Title: Pharmacology of GABA Receptors in *Ascaris suum* Muscle: an Electrophysiological Study.

Name: DUITTOZ, Anne, Hélène

Dissertation submitted to the University of Edinburgh for the degree of Doctor of Philosophy.

1990

Preface:

The experimental work described herein was performed under the supervision of Dr R.J.Martin, during the tenure of a university studentship in the department of Preclinical Veterinary Sciences.

I, hereby, declare that the experimental work is entirely my own composition and that it has not previously been submitted in whole, or in part, to this or any other University. The substance of Sections 5 and 6 has been accepted for publication. Preprints are included at the end of the thesis.

Acknowledgements:

My thanks are due to Dr. Richard Martin for the facilities placed at my disposal and for the frequent and helpful discussions. I would like also to thank Professor Arthur Duggan for reading a draft of the manuscript and for valuable advice.

I am particulary grateful to Professor Camille Wermuth and his team for their collaboration and helpful suggestions.

My thanks are also to Mrs Judith Merten who provided technical assistance; to Miss Fiona Manson and Mr Colin Warwick, who provided the arty touch.

This work was made possible by the award of the Harriet Thompsom scholarship, and a research project grant by the Wellcome trust to Dr. Richard Martin.

"...La preuve est dans le fait, et le fait n'a de sens qu'autant que lui en donnent ceux qui font la profession d'établir les faits..."

Henry Miller, Tropique du Capricorne.

CONTENTS:

LIST OF TABLES:

LIST OF FIGURES:

SUMMARY

The aim of this work was to describe the pharmacological profile of the *Ascaris* muscle GABA receptor, and to compare its properties to those of GABA receptors in vertebrates. The interest of such study is to show the existence of pharmacological differences between *Ascaris* and vertebrate GABA receptors, which may be exploited in antiparasitic drug research. A short review will be presented first on the anatomy, physiology and pharmacology of the neuro-muscular system of *Ascaris*, and on the pharmacological characteristics of GABA receptors in vertebrates and invertebrates. It will be followed by a simplified presentation of the receptor theory and the subsequent development of drug-receptor interaction theories.

The effects of GABA and various GABA-agonists on the membrane input conductance of *Ascaris* muscle cells were studied using a current-clamp technique. Dose-response curves were obtained and described by a modified Hill equation for each agonist tested. The relative potencies of GABA-agonists in *Ascaris* are compared to those obtained at vertebrate GABA-A receptors. Despite some differences: inactivity of sulphonic acid derivatives and the loss of relative potency of rigid analogues of GABA, such as muscimol, THIP and isoguvacine, the agonist profile of *Ascaris* muscle GABA receptor appears correlated with the agonist profile of vertebrate GABA-A receptors.

In contrast, the antagonist profile of *Ascaris* GABA receptors differs greatly from the antagonist profile of the vertebrate GABA-A receptor. The two classical GABA-A antagonists, picrotoxin and bicuculline, were found very weak or inactive at antagonizing GABA responses in *Ascaris*. The steroid derivative, RU5135, a very potent competitive antagonist in vertebrate (IC50=5nM, rat cuneate nucleus, Simmonds and Turner, 1985), was found to hold back some activity in *Ascaris* , but in a non competitive manner and with an IC50 of 117μM.

The effects of the arylaminopyridazine GABA derivatives SR95103 and SR95531 on GABA responses were investigated on GABA-induced conductance changes using a current-clamp technique. Arylaminopyridazine GABA derivatives are specific and competitive antagonists at the vertebrate GABA-A receptor, SR95531 being the most potent analogue. In *Ascaris* SR95103 was found more potent than SR95531 at antagonizing GABA responses. This potency order contrasts with that at vertebrate GABA-A receptors. The antagonism of SR95103 was associated with a parallel shift to the right in the GABA dose-response relationship. A modified Schild plot was used to describe the action of SR95103, the data is consistent with a competitive mechanism

involving two molecules of GABA but one molecule of antagonist interacting with the receptor. The estimated K_B for SR95103 is 64±13μM (mean±SE, n=14) and the pA_2=4.

The actions of SR95103 were further examined on GABA-activated single-channel currents using an outside-out patch-clamp technique. The presence of SR95103 (30-100μM) did not alter the GABA-activated channel main conductance, but reduced the open-probability of the channels. No effect on the mean burst duration, corrected mean open-time and the distribution of burst-durations, was observed with 30μM SR95103. However, when a higher concentration was used, 100μM, mean open-time and mean burst duration were reduced, and the proportion of short bursts (mainly represented by single openings) was increased. The mode of action of SR95103 is discussed, and it is suggested that most of the antagonism is competitive and produced by a single molecule of SR95103 combining with the receptor-channel complex, as suggested by the current-clamp data. However, an additional non-competitive component, possibly a channel block, is detected at high concentrations. This non-competitive component accounts only for a small proportion of the antagonism, since no reduction in the maximal response was detected by the current-clamp technique, even when high antagonist concentrations were used (1mM).

Other arylaminopyridazine derivatives were tested on GABA response in *Ascaris* using a current-clamp technique. The order of potency of these compounds on *Ascaris* muscle GABA receptor, was found different from the order of potency at the vertebrate GABA-A receptor. One analogue, SR95132, virtually inactive in vertebrate preparations was found equipotent to SR95103 in *Ascaris*. When tested on GABA dose-response curves, SR95132 (K_B=65μM) and other potent analogues, displaced GABA dose-response curves to the right without decrease in the maximal response. The modified Schild plots for these compounds, were also consistent with a competitive mechanism involving two molecules of GABA and only one molecule of antagonist interacting with the receptor. Structure-activity relationships for this series of compounds are examined in *Ascaris* and compared to the vertebrates. Substitution on the pyridazine ring in 4-position, while detrimental for the antagonist potency at the vertebrate GABA-A receptor, appears to be a prerequisite for antagonistic activity on *Ascaris* muscle GABA receptor. Newly synthesized arylaminopyridazine derivatives confirmed that the presence of a substituent in the 4-position is very important for the antagonistic activity at the *Ascaris* GABA receptor. The ethyl-4-substituted derivative, NCS247-90 with K_B=55μM, was found to be more potent than SR95103 and SR95132. Benzyl- (NCS251-90) and the isopropyl-(NCS252-90) 4-substituted derivatives also appeared to be potent antagonists. Besides the role of the 4-position,

the length of the side chain and the acidic function were also investigated. A longer side-chain (5C), as in NCS194-83, decreases the potency but does not produce a complete loss of potency. Replacement of the carboxylic acid group by a sulphonic acid group produces a total inactivation of the compound, as seen with NCS249-90 and NCS250-90. This is in agreement with the lack of potency of sulphonic acid derivatives as agonists. These results are discussed in terms of the accessory binding sites theory of Ariëns, and suggest the existence of different accessory sites responsible for the binding of antagonists on *Ascaris* GABA receptor.

In conclusion, the *Ascaris* muscle GABA receptor differs from vertebrate GABA-A receptors in terms of antagonist properties, whereas its agonist properties are very similar to those of vertebrate GABA-A receptors. Among GABA antagonists, the exploration of the arylaminopyridazine GABA derivatives family may lead to the discovery of a new generation of anthelminthics. Already, one analogue, SR95132, shows an antagonistic activity in *Ascaris* but not in vertebrates.

GENERAL INTRODUCTION

Ascaris suum is a large intestinal nematode parasite of the pig. Nematode infestation is the most common cause of parasitism in the world, in both humans and animals. *Ascaris suum* is so similar to its close relative, *Ascaris lumbricoides*, parasite of humans, that they have been previously classified as a same species: *Ascaris lumbricoides* (Soulsby, 1965). *Ascaris lumbricoides* is the most abundant and prolific of all helminths in humans, it is said to infest more than one billion people (Lancet editorial, 1989). There is no equivalent parasite in apes and monkeys, suggesting that man has acquired *Ascaris*, not phylogenetically, but from close contact with pigs. Although, *Ascaris suum* can sometimes infect humans, and *Ascaris lumbricoides*, pigs, the cross transmission is normally very rare. Because of its resemblance to the human parasite, *Ascaris suum* is a good experimental model and has been used in pathological studies (Crompton, Nesheim and Pawlowsky, 1989), and pharmacological studies (Toscano-Rico, 1926; Baldwin and Moyle, 1949; Norton and De Beer, 1957; Del Castillo, De Mello and Morales, 1964c; Ash and Tucker, 1965; Martin, 1982, 1985; Harrow and Gration, 1985; Holden-Dye, Hewitt, Wann et al., 1988; Holden-Dye, Walker, Nielsen et al., 1989).

Ascaris lumbricoides was described by the ancient Romans as an earth-worm, *Lumbricus teres*, able to live in man (Lancet, 1989). To combat *Ascaris* infection, the ancient Greeks used santonica, an extract of the Levant wormseed, *Artemica maritima* (Bowman and Rand, 1980). Santonin, in conjunction with kainic acid which potentiates its action (Bowman and Rand, 1980), has been for a long time the main treatment for ascariasis. The chenopod oil, extracted from *Chenopodium ambrosioides*, was also used widely as a "vermifuge". *Chenopodium ambrosioides*, is mentioned also in the Sahágun's Florentine codex, written by Aztec botanists and physicians, in the XVI century (Kliks, 1990).

Besides these classical treatments, the major advance in the development of anthelminthic therapeutics was made by Boismaré, a French pharmacist, who observed that people treated for gout with piperazine, eliminated worms as well. The discovery of Boismaré led to a more elaborate investigation of anthelmintic properties of piperazine by Fayard in his medical Thesis (1949) (quoted by Goodman and Gilman, 1985).

The mode of action of piperazine was then, the object of numerous studies. Piperazine was first shown to induce a reversible paralysis of *Ascaris suum* in vitro (Standen, 1955) and Norton and DeBeer (1957) suggested that it acted as an acetylcholine antagonist. Later, Del Castillo, De Mello and Morales (1964b, 1964c), measuring the changes in membrane potential of *Ascaris suum* muscle cells, proposed that piperazine acted like the endogenous inhibitory transmitter, γ-aminobutyric acid (GABA). Using voltage-clamp and patch-clamp techniques, Martin (1982, 1985) confirmed that piperazine acts at the *Ascaris suum* muscle GABA receptor and activates the same Cl channels. Other classical anthelminthics such as pyrantel, morantel and levamisole have been studied in *Ascaris suum* and were shown to act at the acetylcholine receptor (Harrow and Gration, 1985). Recently ivermectin (IVOMEC®) has also been studied using *Ascaris suum* as an experimental model (Kass, Wang, Walrond et al., 1980; Kass, Larsen, Wang et al., 1982; Holden-Dye et al., 1988; Martin and Pennington, 1989), and shown to act as a GABA antagonist at micromolar concentrations (Holden-Dye et al., 1988; Martin and Pennington, 1989). The GABA receptor in helminths emerges thus as a possible target for anthelminthic drugs. When this present study started, little was known on the GABA receptor in *Ascaris suum*. In this thesis on the pharmacology of the *Ascaris* GABA receptor, a review of *Ascaris suum* neuromuscular anatomy, physiology and pharmacology, of GABA receptors in vertebrates and invertebrates and of the basis of drug-receptor interactions theories, will be presented in Section 1. The experimental work was carried out on the *Ascaris suum* muscle preparation, using current-, voltage- and patch-clamp techniques. General methods are described in Section 2, but each specific technique will be described in more details in the appropriate sections. The agonist profile of the GABA receptor is presented and compared to the vertebrate GABA-A receptor in Section 3. Sections 4 describes the action of classical GABA-A antagonists, at the *Ascaris* GABA receptor using current-clamp; Section 5 describes the action of the two arylaminopyridazine GABA derivatives, SR95531 and SR95103. The antagonist effects of SR95103 are investigated at the single-channel current level in Section 6. Finally, Section 7 examines the structure-activity relationships of a series of pyridazine derivatives and compares their antagonist properties seen in *Ascaris* with those seen in vertebrate studies. The work presented in the last section was realized in collaboration with Professor

C.G.Wermuth, Dr M.Schmitt and J.M.Sitamze from the "Laboratoire de Neurochimie du CNRS", Strasbourg, France.

SECTION I: LITERATURE REVIEW

A. *ASCARIS SUUM*

I *ASCARIS* MUSCLE CELLS

For reviews, see Cappe de Baillon (1911) and DeBell (1965). The anatomy of *Ascaris suum* was studied as early as 1866 (Schneider, quoted by DeBell, 1965). Schneider was the first to describe the muscle cell as composed of two histologically separate regions: striated and vesicular. The striated region is the contractile part of the cell, and the vesicular region is the non contractile part. At that time, few people could conceive of the existence of a non contractile part in a muscular fibre. Schneider was therefore the first to claim that the transverse strands, described by others as circular muscles (Cloquet, 1824, quoted by Cappe de Baillon, 1911, and DeBell, 1965) or nerves (Meissner, 1853, quoted by Cappe de Baillon, 1911, and DeBell, 1965), were in fact part of the muscle cell itself:

> *"...In the nematodes, the nerves do not branch out to the muscles, instead branches of the muscle reach the nerves..."*

After Schneider, numerous scientists found an interest in the anatomy of *Ascaris* muscle and its nervous system, leading to various theories and harsh discussions concerning the organization of the muscle cell and the neuromuscular junction (Apáthy, 1893, 1894; Bilek, 1909, 1910; Bütschli, 1874, 1892; Deineka, 1908; Glane, 1910; Goldschmidt, 1908, 1909, 1910; Hesse, 1892; Rohde, 1885; quoted by Cappe de Baillon, (1911) and DeBell, (1965)). The anatomical terms of arm ("bras"), belly ("panse") and spindle ("fuseau") were first introduced by Cappe de Baillon (1911), who gave a good account of the histology of *Ascaris* muscle cells. However the anatomy of the neuromuscular junction was not described before the use of the electron microscope (Rosenbluth, 1963, 1965b).

1. Morphological studies of the muscle cells

1.1. Belly or bag region

The belly is a large round bag shaped part of the somatic muscle cell, which lies in the pseudocoelomic space. The cytoplasm contains the nucleus and is almost entirely filled with particulate glycogen (Rosenbluth, 1963, 1965b). Harris and Crofton (1957) suggested that the muscle cells bellies played the role of an endoskeleton. See Figures 1 and 2.

1.2. Arm and syncytium

The arm is a thin process leaving the base of the belly of the muscle cell in the direction of the nerve cord, where it makes synaptic contacts (see Figures 1 and 2). Several arms can leave the same cell (Cappe de Baillon,1911). The arms were originally believed to form a syncytium surrounding the nerve cord (Del Castillo, De Mello and Morales, 1963a). This was also suggested by Cappe de Baillon (1911),

> "*...il nous parait absolument impossible de nier qu'il existe une liaison très intime pour ne pas dire de continuité entre les fibrilles de soutien qui sortent des bras et celles qui forment manchon autour des fibres nerveuses et des autres formations de la ligne médiane..*".

But in fact, the processes are separated from each other with numerous tight junctions present in the distal part near the synaptic region, increasing the electrical coupling of the muscle cells (Rosenbluth, 1965b). Recent scanning electron microscopy studies show that the muscle arms approach the nerve cord in bundles separated by wide gaps and that the arms extend longitudinally, overlapping with those from other arms to form the so called "syncytium" (Del Castillo, Riviera, Solórzano et al., 1989). Synapses are formed between the "syncytium" and the nerve cord.

1.3. Spindle

The spindle is the contractile part of the muscle cell forming a long tube lying against the hypodermis (see Figures 1 and 2). The apparent lack of cross-striations gave rise to the suggestion that the *Ascaris* muscle cells were a smooth muscle cell type (Rosenbluth, 1973). Rosenbluth (1965a) has reconstructed the fibrillar structure of the *Ascaris* muscle cells using electron microscopy, and shown that the H, A and I bands are present as in the striated skeletal muscle; the Z band is replaced by a less organized

Figure 1: Diagram of a muscle cell from the body wall of *Ascaris suum* (from Rosenbluth, 1973)

The cell consists of three parts: the belly (b) which contains the nucleus and glycogen stocks; the spindle (s) or contractile part; the arm (a) which stretches to the nerve cord (n) to make neuromuscular junctions. (h) is the hypodermis and (c) the cuticule.

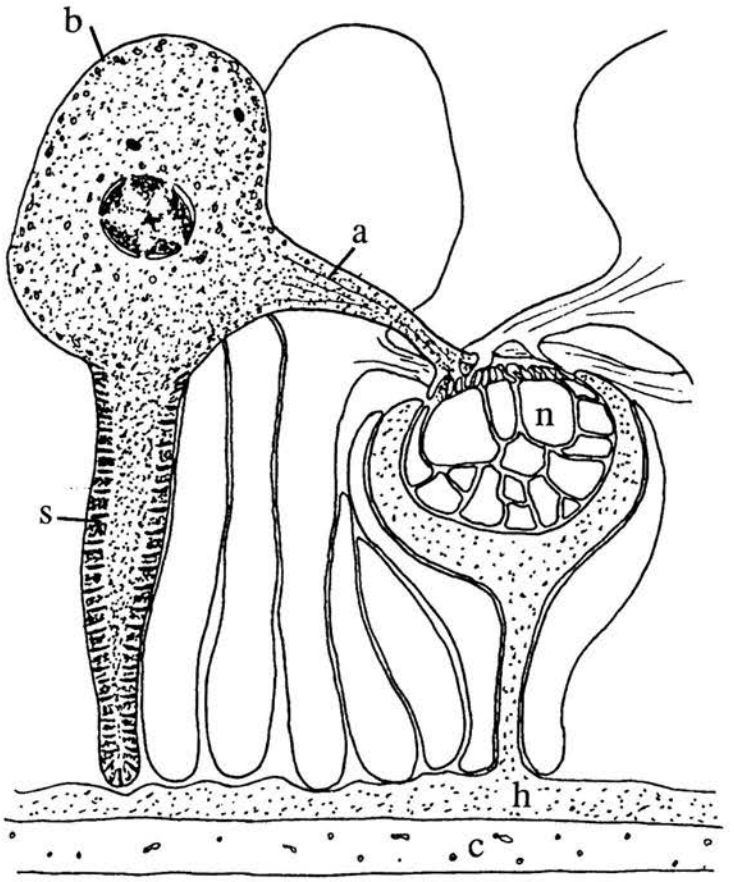
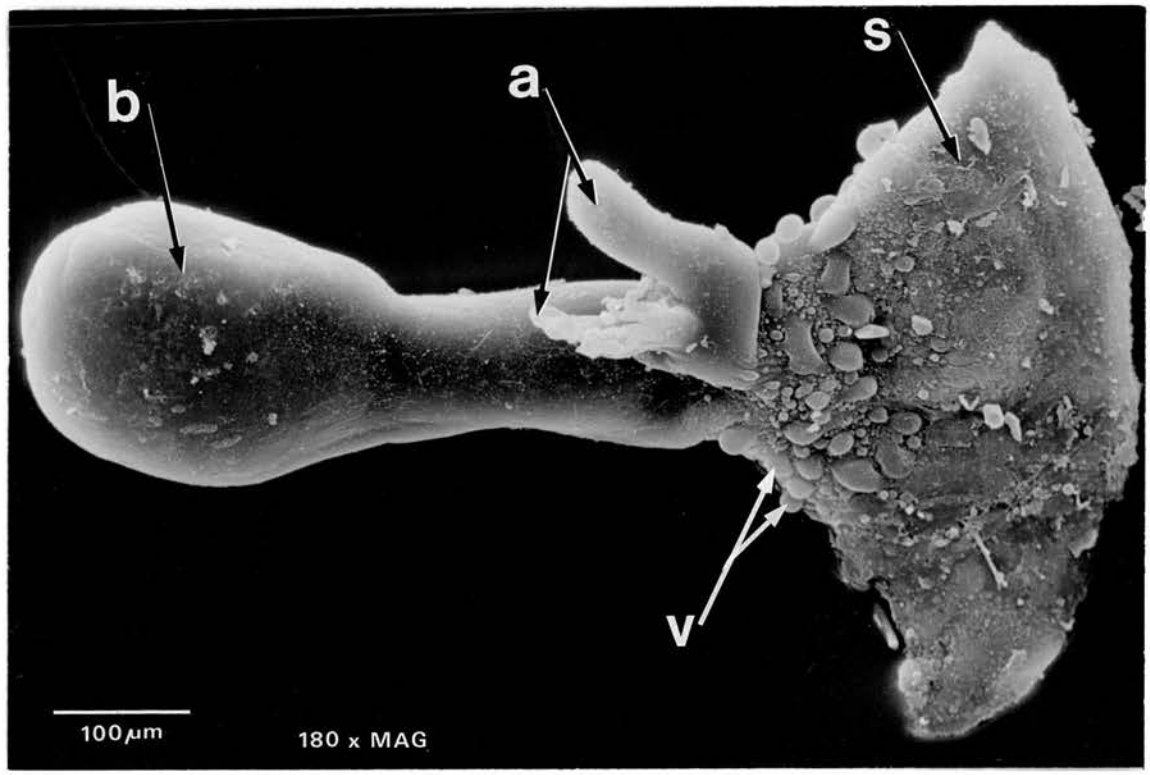

b
a
n
s
h
c

Figure 2: *Ascaris* muscle cells: scanning electron micrograph

To obtain free muscle cells, *Ascaris* muscle was treated with collagenase and kept at 35ºC, until free muscle cells appeared in the medium.
(a) arm; (b) belly; (s) spindle; (v) vesicles
Note the presence of two arms leaving the base of the belly, one is damaged and appears torn.


b
a
s
v
100 µm
180 x MAG

structure of dense bodies. However the striation does not form a 90º angle as in the skeletal muscle, but only a 6º angle (Figure 3). This explains why the striations had previously remained unnoticed, although Apàthy (1894, quoted by Cappe de Baillon (1911)), detected some striation:

> "*...les bandes ne sont pas toutes parallèles, mais sont groupées en bande parallèles entre elles. Ces groupements se confrontent très obliquement dans une grande variété de plans...*" .

Rosenbluth (1973) classified the *Ascaris* muscle as a new type, the "Obliquely striated muscle". It turned out later that the obliquely striated muscle is widely distributed among soft bodied invertebrates but is not known to occur in any vertebrate (Rosenbluth, 1965a; and see Rosenbluth, 1973 for review).

Conclusion: this particular arrangement allows sliding of the thin filaments with respect to the thick ones and shearing of the thick filaments. The shearing phenomenon confers great extensibility and the sliding phenomenon preserves the velocity in length change. Obliquely striated muscle has therefore the extensibility of smooth muscle and the velocity of contraction of skeletal muscle.

2. Biochemistry of contraction

2.1. Actin and myosin

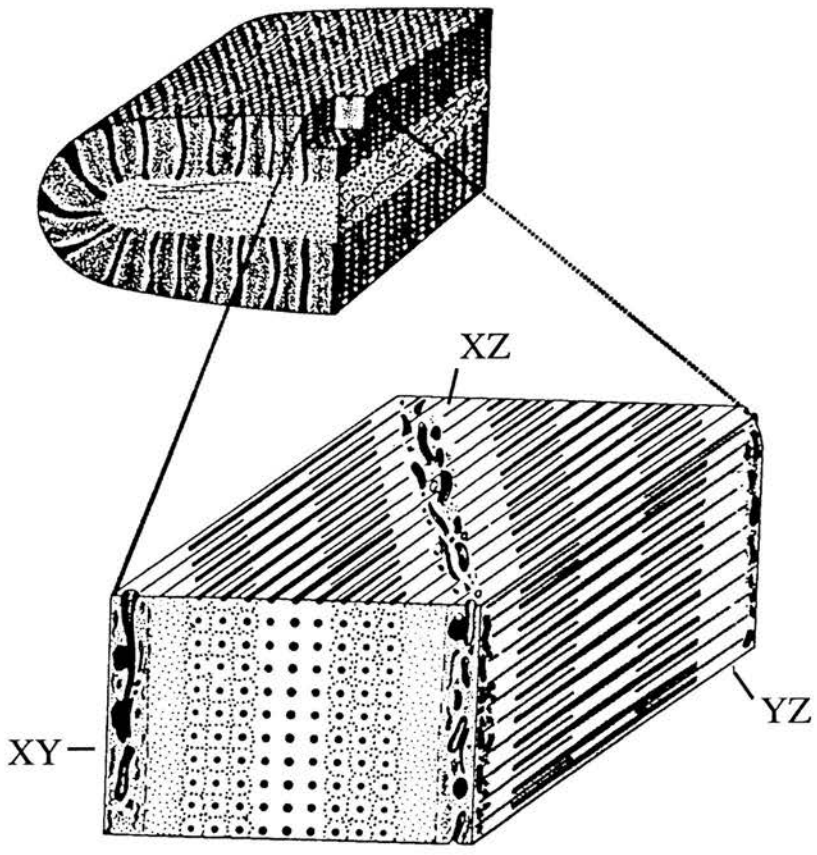
It is commonly reported that Ca mediated actin fixation is the principal mechanism of contraction in the skeletal muscle, whereas the Ca-dependent myosin phosphorylation is the principal mechanism in the vertebrate smooth muscle (Rosenbluth, 1973). In *Ascaris* muscle, the two mechanisms are present. The fast twitch is apparently regulated by a Ca-mediated actin fixation and muscle relaxation involves the myosin phosphorylation mechanism (Martin and Donahue, 1987). The fibrillar composition of obliquely striated muscle is very close to vertebrate skeletal muscle: troponin and tropomyosin are present in a similar stoichiometry and the myosin light chain is present in three isoforms, similar to those present in the rabbit skeletal muscle (Donahue, Michnoff and Masaracchia, 1985). It appears therefore that the mechanisms underlying muscle contraction in *Ascaris* are very similar to those involved in the skeletal muscle.

Figure 3: Diagram of the striation of a muscle fibre from *Ascaris suum* (from Rosenbluth, 1973)

Rosenbluth (1965a) has reconstructed the fibrillar structure of the *Ascaris* muscle cells using electronic microscopy (E.M.).
XY is the transversal plan (transverse section of the body)
XZ is the lateral plan (tangential section of the body)
YZ is the horizontal plan (longitudinal section of the body)

A. Part of the spindle region of a muscle cell

B. The tangential section XZ shows the oblique striation
The H, A and I bands are present as in the striated skeletal muscle. The Z band is replaced by a less organized structure of dense bodies. However the striation does not form a 90º angle as in the skeletal muscle.


XZ
XY
YZ

2.2. Energy metabolism

Histological observations (Rosenbluth, 1963) suggest that, glycogen plays an important role in the supply of energy for muscle contraction. Glycogenolysis provides the principal source of substrates for ATP synthesis during non feeding intervals (Donahue, Yacoub, Kaeine et al., 1981c, Donahue, Yacoub and Harris, 1982). Two pathways are present as in the skeletal muscle: a cAMP dependent mechanism, analogous to the mammalian pathway, where serotonin is believed to be the primary messenger which induces the rise in cAMP (Donahue, Yacoub, Michnoff et al., 1981a; Donahue, Yacoub, Kaiene, 1981b; Donahue et al., 1981c); a cAMP independent pathway, less characterized but which appears to be Ca-dependent and activated by acetylcholine (Donahue et al., 1981c, 1985).

Conclusion: histologically the *Ascaris* somatic muscle is different from the vertebrate skeletal and vertebrate smooth muscle, and has been classified as a special type: "Obliquely striated muscle" (Rosenbluth 1965a; see Rosenbluth, 1973, for review). Biochemically however, the mechanisms and enzymes involved are very similar to those in the vertebrate skeletal muscle (Donahue et al., 1981b, 1981c, 1985).

3. Electrical activity of the muscle cells

3.1. Resting membrane potential

The main characteristic of the resting membrane potential of *Ascaris* muscle cells is its insensitivity to changes in ionic composition of the extracellular solution (Del Castillo, De Mello and Morales, 1964a). The muscle bags are normally bathed with perienteric fluid (mM: Na 129; K 24; Ca 6; Mg 5; Cl 53; organic anions 120, mainly volatile fatty acid), (Hobson, Stephenson and Beadle, 1952). Most electrophysiological studies have been performed using artificial perienteric fluids where organic anions were never considered. However they may play an important role. Several mechanisms have been proposed to explain how the cell maintains its resting potential, but none of them is fully satisfactory.

a. The Na shunt conductance

Del Castillo et al. (1964a) were the first to study the influence of ion substitution on the resting membrane potential. The membrane potential was little affected by changes

in the K concentration, but was affected by changes in external Cl concentration. Their conclusion was that a Cl battery with a low internal resistance seemed to be largely responsible for the maintenance of the membrane potential. The role of a K battery was limited by a high internal resistance. Both Cl and K batteries were shunted by a high Na conductance. In the vertebrate smooth muscle the membrane potential is due to a Cl battery as well, and is shunted by a high Na conductance. The ionic permeability sequence in vertebrate smooth muscle is: $Cl>SO_4>Br>NO_3>I$; and in *Ascaris* muscle: $NO_3>I>Br>Cl>SO_4$, NO_3 is the most permeant anion.

b. Compensatory changes in membrane conductance

Brading and Caldwell (1964) first suggested that compensatory changes in membrane permeability counteracted changes in ion concentration. But after using labelled ion flux experiments, Caldwell and Ellory (1968) refuted this hypothesis.

c. Operation of an electrogenic pump

Assuming that the constant field theory is applicable to *Ascaris* muscle, Brading and Caldwell (1971) studied the effect of ion replacement using the Goldman-Hodgkin-Katz equation (Hille, 1984a):

$$E = \frac{RT}{F} \cdot \log_n \frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i + x}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o + y}$$

Their results suggested that there was a major factor determining the *Ascaris* muscle membrane potential other than the contribution of the identified K, Na and Cl ions. Brading and Caldwell (1971) proposed the existence of a carboxylic acid transport system associated with Na transport. However, when they looked at the effect of several transmitters, they concluded that GABA did not affect P_{Cl} or P_K, but inhibited the putative carboxylic acid transport system and consequently decreased the Na secretion. GABA does not act this way, but activates a Cl conductance in *Ascaris* muscle (Martin, 1980), suggesting either that the mechanism proposed by Brading and Caldwell (1971) is unlikely to occur, or that their experimental conditions were not satisfactory, making their results difficult to interpret.

Conclusion: a satisfactory explanation for the resting membrane potential in *Ascaris* muscle remains to be found, although it seems possible that an active transport of organic anions is involved.

3.2. Spontaneous electrical activity of the muscle cells

Jarman (1959) was the first to report the spontaneous depolarizing potentials in *Ascaris* muscle. Later, DeBell, Del Castillo and Sanchez (1963), showed that these depolarizations were myogenic, arising from what they described as a syncytial region of the muscle cells. DeBell et al. (1963) reported two functional classes of depolarization: spikes of variable amplitude and of duration 5-50ms, and slow waves up to 20mV amplitude and 100-1000ms duration. Weisblat, Byerly and Russel (1976) reported a third class: long lasting modulations of 5mV amplitude and 3-20s duration which corresponded to rhythmic contractions of the whole animal. The pattern of each type of electrical activity is considered in more details in the next paragraphs.

a. Spike potentials

Spike potentials are graded in their amplitude (Jarman, 1959) and originate from the syncytial region near the nerve cord (DeBell et al., 1963). Ion replacement studies have shown that they are mediated by Ca (Weisblat et al., 1976). Variations in K concentration and application of TEA do not affect the spike potentials, suggesting that K is not involved in terminating the spike potentials in *Ascaris* muscle.

b. Slow waves

Ion replacement experiments showed that slow waves can be carried by Na or Ca (Weisblat et al., 1976). TEA, externally applied had no effect upon the amplitude or the duration of individual slow waves (Weisblat et al., 1976), suggesting that a K conductance is not involved. The mechanism of generation and termination of slow waves is not known.

c. Modulation and square waves

Modulation is sometimes seen in spontaneously active preparations and coincides generally with the contraction of the whole animal. Modulation is a rhythmic depolarization (lasting 3-20s), inducing a burst of electrical activity such as slow waves and spike potentials. Experimentally, TEA, and Ca replacement by Sr or Ba, induced

long lasting depolarizations similar to the modulation waves, called square waves by Weisblat et al. (1976).

Low-Ca and high-Mg solutions also induced square waves, but the replacement of Na by TRIS inhibits the generation of square waves. These facts suggest that Na but not Mg can carry currents generating the square waves. Weisblat et al. (1976) suggested that square waves corresponded to the modulation activity seen under normal conditions in spontaneously active preparation, and could be carried by Ca, Na, Ba and St as for the slow waves. The rapid rise and fall of square waves seems to indicate that voltage-dependent Ca channels are involved. Weisblat et al. (1976) proposed a mechanism in which the internal accumulation of Ca itself regulates the Ca conductance. The accumulated Ca would need to be pumped out before the next square wave.

Conclusion: three different types of spontaneous electrical activity have been demonstrated in *Ascaris* muscle. Although their ionic mechanisms remain imperfectly understood, Ca appeared to play an important role. These three activities are myogenic and are probably involved in coordinating contraction.

Conclusion on *Ascaris* muscle cells:

The histology of the muscle cell of *Ascaris suum* differs greatly from the skeletal and smooth muscle cell types of vertebrates. Firstly the cell itself is specialized in three parts: spindle (contractile), belly (energy and metabolism), arm (connection with the nervous system). Secondly, the contractile part is obliquely striated and forms a new type of muscle cell besides the classical skeletal and smooth muscle cells. Obliquely striated muscle cells combine the elasticity of a smooth muscle and the velocity of a skeletal muscle. The mechanisms and enzymes involved in the contraction are very similar to those present in skeletal muscle, but in terms of spontaneous electrical activity, the *Ascaris* muscle resembles the vertebrate smooth muscle type.

II *ASCARIS* NERVOUS SYSTEM

The nervous system of *Ascaris* was studied as early as 1892 (Hesse). Goldschmidt (1908,1909, quoted by DeBell, 1965) reconstructed the anatomy of the neurones in the head and the tail, showing that they are completely reproducible from animal to animal and that only a small number of neurones are present (250). These two features have made the nematode *Ascaris* attractive for the analysis of nervous system function.

1. Morphological studies

Near the head, a series of ganglia is associated with a nerve ring from which the ventral and dorsal nerve cords originate. In the posterior region, there is a second set of ganglia. However the motorneurone cell bodies, located in the ventral nerve cord, are not arranged in ganglia, but are scattered (Goldschmidt, 1908, 1909, quoted by DeBell, 1965). Two pairs of lateral nerve cords are also present, a dorsal lateral cord running along the anterior and posterior parts of the body, a ventral lateral cord situated only in the posterior part of the worm. In the anterior part of the parasite, the dorsal lateral nerve cord is accompanied by two sublateral nerve cords: dorsal and ventral (Figure 4). Stretton, Fishpool, Southgate et al. (1978) have studied the structure of the motorneurones in the ventral nerve cord. Based on anatomical features, they have been divided in seven classes: V-1, V-2, DE1, DE2, DE3, DI, VI. They are named after their targets and their effects. D cells innervate the dorsal musculature; V cells innervate the ventral musculature; E cells are excitatory and I cells are inhibitory. V-1 and V-2 appeared to be excitatory, although there is no direct physiological evidence yet. Each motorneurone is composed of a cell body and a process in the ventral nerve cord. Five types also have a dorsal process in the dorsal nerve cord, linked to the ventral process by a single transverse fibre called a commissure (Hesse, 1892). The commissures are arranged in a highly reproducible pattern along the body of the worm, forming five segments each containing eleven motorneurones (Figure 5). The segments are linked by six non segmental interneurones (Stretton et al., 1978). Dorsal excitatory neurones, besides making neuromuscular junctions, excite ventral inhibitory neurones. Dorsal excitatory neurones receive their input from interneurones in the ventral nerve cord. V-1 and V-2 ventral excitatory motorneurones also receive their input from interneurones but excite dorsal inhibitory motorneurones (Walrond, Kass, Stretton et al., 1985).

Besides the well documented ventro-dorsal commissures, Johnson and Stretton (1987), described latero-ventral commissures. They are processes of neurones whose cell bodies are located in the lateral nerve cords or in the tail. In the male, there is a large number of lateral to ventral commissures associated with sensory papillae in the tail. There are also latero-ventral commissures associated with the nerve ring in the head (Johnston and Stretton, 1987).

Figure 4: Diagram of the major and minor nerve cords in *Ascaris suum* (from Johnson and Stretton, 1987)

The largest section is made in the anterior part of the parasite; the smallest section is made in the posterior part. (D) dorsal nerve cord; (V) ventral nerve cord; (DL) dorsal lateral nerve cord; (VL) ventral lateral cord; (DsubL) dorsal sublateral nerve cord; (VsubL) ventral sublateral nerve cord; (c) cuticule; (h) hypodermis; (LL) lateral lines.

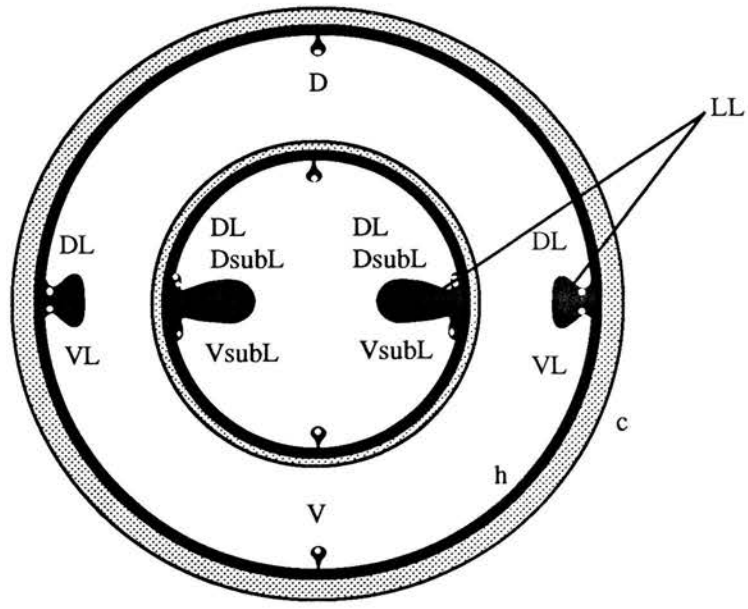
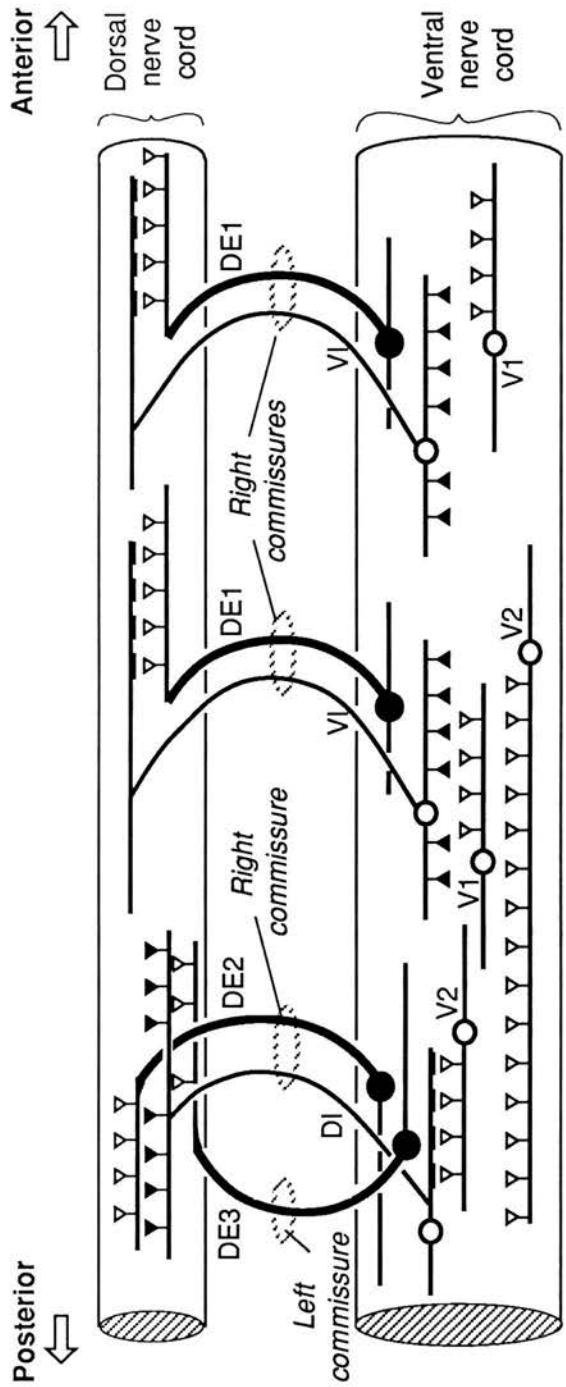

D
LL
DL
DL
DsubL
DL
DsubL
DL
VL
VsubL
VsubL
VL
c
h
V

Figure 5: Schematic diagram of the organization of the dorsal and ventral nerve cord in *Ascaris suum*: one typical repetitive segment

The diagram represents one of the seven repetitive segments found in the *Ascaris suum* body:

○ Inhibitory motorneurone
● Excitatory motorneurone
△ Inhibitory output
▲ Excitatory output
——— Input

Each neurone is classified according the shape of its cell body:
dorsal excitatory motorneurones: DE1, DE2, DE3
ventral excitatory motorneurones: V-1, V-2
dorsal inhibitory motorneurones: DI
ventral inhibitory motorneurone: VI
V-1 and V-2 do not have commissures. All commissures are right handed except DE3.


Anterior
Posterior
Dorsal nerve cord
Ventral nerve cord
DE1
DE1
DE2
DE3
Right commissures
Right commissure
Left commissure
DI
VI
VI
V1
V1
V2
V2

2. Physiological studies

Electrical stimulation of the nervous system evokes either excitatory or inhibitory potentials in muscle cells (DeBell et al., 1963; Del Castillo, De Mello and Morales, 1967; Weisblat et al., 1976). Walrond et al. (1985) have developed a technique allowing the stimulation of an identified neurone and the recording of the muscle response in the innervated area. Davis and Stretton (1989a) have recorded intracellularly from the commissure of identified motorneurones. These studies have allowed a model for the role of the nervous system in locomotion to be developed (Walrond and Stretton, 1985a, 1985b).

2.1. Graded responses

a. Graded neuromuscular response

Electrical stimulation of the ventral nerve cord produced graded muscle cell responses, no all or none threshold response was recorded (Walrond et al., 1985). Similarly, direct intracellular recordings from commissural neurones have shown that the neurones do not propagate action potentials, but show graded responses (Davis and Stretton, 1989b).

b. Passive membrane properties

Since the muscular response is graded with the neuronal stimulus, signalling appears to rely on passive properties of neuronal membranes. This was confirmed by the unusual membrane properties of *Ascaris* motorneurones: high membrane resistance (61-251 $k\Omega cm^2$) and large space constant (4-10mm); that make them well designed for conducting passive voltages over long distances (Davis and Stretton, 1989a). Cells with such membrane properties and long processes, have been described among sensory cells conveying graded responses (Prasztor and Bush, 1982).

c. Graded active responses

Although motorneurones can function with passive signalling, small voltage activated currents are recorded in the commissure. These currents underlie small active responses obtained at the offset of the depolarizing or hyperpolarizing stimulus. These offset responses are graded with the stimulus intensity (Davis and Stretton, 1989b). In

inhibitory motorneurones, strong depolarization induces oscillations of the membrane potential. Such oscillations can sometimes be found spontaneously and may be related to the presence of these voltage activated channels (Davis and Stretton, 1989b).

2.2. Tonic release of neurotransmitter

The presence of voltage-activated channels at the normal resting membrane potential, may suggest a tonic release of neurotransmitter from the commissure (Davis and Stretton, 1989b). A tonic release of acetylcholine had been shown earlier by Del Castillo et al. (1963a) since curare, an acetylcholine antagonist, hyperpolarized and neostigmine, an inhibitor of acetylcholinesterase, depolarized the muscle cells.

2.3. Complex neuromuscular responses

Besides the unusual signalling properties of the *Ascaris* motorneurones, several important types of neuromuscular responses have been described. Complex intracellular potentials are often obtained after stimulation of a single motorneurone. For example, the stimulation of an excitatory motorneurone often produced a depolarization followed by a small hyperpolarization (Stretton et al., 1978). These complex responses may result from neurone-neurone interaction in the dorsal nerve cord or membrane properties of muscle cells.

2.4. Reciprocal inhibition

Dorsal excitatory motorneurones interact synaptically with ventral inhibitory neurones. A neuronally evoked dorsal contraction is complemented by a neuronally evoked ventral inhibition. A similar circuit seems to exist within the ventral nerve cord, involving V-1, V-2 and DI (Walrond and Stretton, 1985b).

2.5. Excitatory and inhibitory muscle activity evoked by stimulation of single dorsal excitatory motorneurones.

Stimulation of a dorsal excitatory motorneurone leads to depolarization in the corresponding musculature and to an hyperpolarization in the surrounding dorsal region. This hyperpolarization seems to be due to synaptic activation of DI by the DE neurones (Walrond and Stretton., 1985b). Stimulation of a dorsal excitatory motorneurone leads to muscle contraction in the corresponding innervated dorsal area,

and to relaxation of the corresponding ventral muscle. The surrounding dorsal musculature is also inhibited, giving an omega shape (Ω) to the body. The mechanism of wave propagation remains to be determined, but the interneurones may play an important role (Walrond and Stretton, 1985b).

III NEUROMUSCULAR PHARMACOLOGY

Toscano-Rico (1926) was the first to develop a technique for testing effects of drugs on *Ascaris*; he used a portion of worm linked to a kymograph, and observed the contracting effect of nicotine. The technique was improved in order to bring the drugs in direct contact with the musculature and to short-circuit the protective effect of the cuticule (Baldwin and Moyle, 1947, 1949). The two main effects reported were: contraction and relaxation.

1. Cholinergic neuromuscular pharmacology

1.1. Excitatory effects of acetylcholine

Acetylcholine at micromolar concentrations, produces muscle contraction (Baldwin and Moyle, 1949; Del Castillo et al., 1963a; Natoff, 1969; Rozhkova, Malyutina and Shishov, 1980), muscle cell depolarization and increase in cation conductance (Martin, 1982; Harrow and Gration, 1985; Colqhoun, Holden-Dye and Walker, 1990). Stimulation of the ventral nerve cord often produces muscle contraction (Walrond and Stretton, 1985a) suggesting that the neurotransmitter for muscle contraction is acetylcholine. Choline acetyl transferase activity was found in the commissure, and cell bodies of identified excitatory motorneurones (Johnson and Stretton, 1987) and was in agreement with acetylcholine being an excitatory transmitter in *Ascaris*. At first, acetylcholine receptors were thought to be exclusively located in the nervous system and at neuromuscular junctions (Norton and DeBeer, 1957). However, Brading and Caldwell (1971) recorded acetylcholine responses from the bag region of muscle cells after section of the arms: they suggested that receptors were also present extrasynaptically on the bellies. This was confirmed later by ionophoresis experiments (Martin, 1982).

1.2. Pharmacology of the *Ascaris* muscle acetylcholine receptor

Contraction is also produced by nicotine at low concentrations (Toscano-Rico, 1926; Baldwin and Moyle, 1949; Natoff, 1969). Acetylcholine induced contractions are blocked by tubocurarine but not by atropine (Baldwin and Moyle, 1949; Natoff 1969; Rozhkova et al., 1980). Natoff (1969), Rozhkova et al. (1980) and Colquhoun et al. (1990), tried to further classify this receptor into ganglionic or muscular nicotinic types. The selective and potent ganglionic agonist, dimethylphenylpiperazinium (DMPP), has been shown to be the most potent agonist on *Ascaris* acetylcholine receptor (Natoff, 1969; Colqhoun et al., 1990). Moreover, mecamylamine, a relatively specific ganglionic antagonist is the most potent cholinergic antagonist in *Ascaris* (Natoff, 1969) after N-methylaconitine (Colquhoun et al., 1990). However there is some doubt about the potency of nicotine; Baldwin and Moyle (1949) and Natoff (1969), reported nicotine to be more potent than acetylcholine, whereas Rozhkova et al. (1980) and Colqhoun et al. (1990) found it was less potent. The low potency of the ganglionic blocker hexamethonium casts doubt on the proposed ganglionic nicotinic type for the *Ascaris* acetylcholine receptor (Rozhkova et al., 1980; Colquhoun et al,. 1990). The two anthelmintics; morantel and pyrantel have been shown to be potent agonists on *Ascaris* muscle acetylcholine receptor, and were nearly as potent as DMPP (Harrow and Gration, 1985). Levamisole has also been shown to act as an acetylcholine agonist but with a much lower potency (Harrow and Gration, 1985). The Tables 1 and 2 summarize the pharmacological characteristics of this receptor.

1.3. Acetylcholine-gated channels in *Ascaris* muscle

The acetylcholine-activated single currents in *Ascaris* muscle have been shown to have two conductances. The larger being 30-50pS, and the smaller being 15-25pS (Pennington and Martin, 1990). The channel kinetics for the larger conductance are described by two exponentials for the open durations, and three exponentials for the closed durations (Pennington and Martin, 1990). Similar results are found in vertebrate preparations.

Conclusion: acetylcholine appears to be the excitatory neurotransmitter in *Ascaris*, synaptic and extrasynaptic acetylcholine receptors are present on the muscle cells. Pharmacological studies show that the *Ascaris* acetylcholine receptor is closely related to a vertebrate ganglionic type, but has some specific characteristics which may be exploited therapeutically.

Table 1: Summary of the properties of various agonists on *Ascaris* cholinoreceptor

	Baldwin and Moyle 1957 (1)	Natoff 1969 (2)	Rozhkova et al., 1980 (3)	Harrow and Gration 1985 (4)	Colquhoun et al., 1990 (5)
DMPP	——	0.2-0.6	0.7	——	2
Morantel	——	——	——	0.4	——
Pyrantel	——	——	——	0.4	——
Propionylcholine	——	2	——	——	——
Nicotine	1	2-38	3.5	——	0.3
Carbachol	——	5-55	4.1	——	0.5
Suberyldicholine	——	——	4.7	——	——
Acetylcholine	1	6-55	6.1	110	1
PTA	——	——	1.1	——	——
Levamisole	——	——	——	20	——
TMA	——	——	2.9	——	0.05
Succinyldicholine	——	——	3.1	——	——
Decamethonium	——	73-366	——	——	——
Arecoline	——	——	——	——	0.002
Furtrethonium	——	——	——	——	0.007

DMPP=dimethylphenylpiperazinium

PTA=pentyltrimethylammonium

TMA=trimethylammonium

(1) ribbon like stripe of dorsal musculature, lowest concentration inducing contraction (μM)

(2) strip of dorsal musculature, range of concentrations inducing contraction (μM)

(3) strip of dorsal musculature, contraction EC50 (μM)

(4) muscle flap preparation, measure of the change in membrane input conductance EC50 (μM)

(5) muscle flap preparation, measure of the change in membrane input conductance, relative potency

Table 2: Summary of the properties of various antagonists on *Ascaris* cholinoreceptor

	Baldwin and Moyle 1949 (1)	Natoff 1969 (2)	Rozhkova 1980 (3)	Colquhoun et al., 1990 (4)
N-methylaconitine	——	——	——	0.23
Bungarotoxin	——	——	——	1.4
Mecamylamine		6.6 (0.25)	——	0.33
Strychnine	100	——	——	1.29
d-tubocurarine	100	5.63-2.34	6.3 (0.5)	3.1
TEA	——	——	5.2 (6)	——
Hexamethonium	——	——	4.4 (40)	43
Pancuronium	——	——	——	3.2
Atropine	——	4.1 (80)	4.1 (80)	6.7

(1) ribbon like stripe of dorsal musculature, lowest concentration inducing contraction (μM)

(2) strip of dorsal musculature, pA_2 (calculated IC50 μM)

(3) strip of dorsal musculature, pA_2 (calculated IC50 μM)

(4) muscle flap preparation, IC50 (μM)

2. GABA-ergic neuromuscular pharmacology

2.1. Inhibitory effects of GABA

GABA applied at micromolar concentrations produces muscle relaxation, cell membrane hyperpolarization (Del Castillo et al., 1964b) and an increase in membrane conductance (Martin, 1980; Holden-Dye et al., 1988). GABA has been suggested to be the inhibitory transmitter in *Ascaris*, and GABA immunoreactivity was found in the commissures and cell bodies of identified inhibitory motorneurones (Johnson and Stretton, 1987). As for acetylcholine, it was originally claimed that GABA receptors were exclusively present at the neuromuscular junction (Norton and DeBeer, 1957), although later they were found on the extrasynaptic muscle bag region (Brading and Caldwell, 1971; Martin, 1980).

2.2. Pharmacology of the *Ascaris* muscle GABA receptor

The anthelmintic piperazine has been shown to induce a reversible paralysis of *Ascaris suum* in vitro (Standen, 1955), and was suggested to act as an acetylcholine antagonist (Norton and De Beer, 1957). However, using a denervated preparation, Del Castillo et al. (1963a, 1964b), Del Castillo, Morales and Sanchez (1963a), showed that spontaneous contractions of the muscle were of myogenic origin. Acetylcholine increased the rhythm of contraction while tubocurarine antagonized acetylcholine induced contractions but not the spontaneous ones (Del Castillo et al., 1964c). They then proposed that piperazine acted like the endogenous inhibitory transmitter, since piperazine paralysis could be mimicked by stimulation of the nerve cord. Finally, Del Castillo et al. (1964c) showed that GABA has similar effects to piperazine, but was 100 times more potent. Ash and Tucker (1966), investigated the pharmacology of the GABA receptor using measurements of the length of the dorsal musculature, and confirmed the paralysing actions of GABA (0.5μg/ml) and piperazine (50μg/ml). Although the GABA receptor appeared to be a suitable target for anthelmintic drugs, its pharmacological profile was not investigated seriously until recently (Wann, 1987; Hewitt, 1988; Holden-Dye et al., 1988,1989). The purpose of this work presented here is to study the pharmacological profile of *Ascaris* muscle GABA receptor. A detailed description of the pharmacology is given in the Section 3 for the agonists and in the Sections 4, 5, 6 and 7, for the antagonists.

2.3. GABA-gated channel

GABA activates a Cl conductance in *Ascaris* muscle, as at the vertebrate GABA-A receptor (Del Castillo et al., 1964b; Martin, 1982, 1985; Holden-Dye et al., 1988). The characteristics of the *Ascaris* GABA-activated channel are similar to that of the vertebrate GABA-A-activated channels (Martin, 1985). In outside-out patches, GABA produces channels with a main-state conductance of 22.0pS, and two rare subconductances of 30% and 70% of the full level (Martin, 1985). In the presence of GABA 3μM, the effective mean open time is 30.5ms; the channel open times kinetic is described by two exponentials: 2.1 and 46.0ms; the closed times kinetic is described by three exponentials: 1.6, 40.0 and 1320ms (Martin, 1985). At concentration greater than 5μM, GABA-activated channels show desensitization (Martin, 1985).

3. Serotonin: muscular effects

3.1. Serotonin is an endogenous modulator

There is evidence for serotonin (5-hydrotryptamine; 5-HT) receptors in *Ascaris* muscle based on radiolabelled ligand binding techniques (Chaudhuri and Donahue, 1989). Serotonin can be absorbed from the host or synthesized by the parasite (Chaudhuri , Martin and Donahue, 1988).

3.2. Serotonin regulates the glycogen metabolism

Serotonin does not induce contraction or relaxation, but appears to regulate glycogen metabolism in *Ascaris* muscle (Donahue et al., 1981a). Glycogen metabolism regulation relies mainly on the activity of two enzymes: glycogen synthetase, responsible for the synthesis of glycogen from glucose-6-phosphate; and glycogen phosphorylase, responsible for the breakdown of glycogen into glucose-6-phosphate. These enzymes can exist in several states of activation depending on their level of phosphorylation. Serotonin induces inactivation of glycogen synthetase but activates the glycogen phosphorylase and produce a three fold increase in cAMP (Donahue et al., 1981b).

Hence it appears that the glycogen metabolism can be regulated by two mechanisms which are either cAMP dependent or cAMP independent. The excitatory neuromuscular-transmitter acetylcholine, besides producing muscle contraction,

activates the glycogen synthetase, and prevents inactivation of glycogen phosphorylase; there is no increase in cAMP. GABA induces hyperpolarization and relaxation which require energy; GABA prevents inactivation of glycogen synthetase and the conversion of glycogen phosphorylase to an inactivated form, there is no increase in cAMP (Donahue et al., 1981b). Serotonin has been identified as the primary messenger which promotes the increase in cAMP. Serotonin in *Ascaris* muscle appears to play a role similar to adrenaline in skeletal muscle. Adrenaline and noradrenaline have no effect on muscle contraction or glycogen metabolism in *Ascaris* (Donahue et al., 1981a).

3.3. Pharmacology of the serotonin receptor

Binding studies have shown that the *Ascaris* serotonin binding site has greater affinity for the vertebrate 5-HT2 agonists and antagonists than for the vertebrate 5-HT1 agonists and antagonists. Specific binding is found in the muscle and in the intestine (Chaudhuri and Donahue, 1989).

4. FMRF-amide: neuro-muscular effects

FMRF-amide is a tetrapeptide which was first isolated from the cardiorespiratory system of a mollusc (Price and Greenberg, 1977). FMRF-amide-like peptides (FLPs), have since been isolated in a great variety of species (Cowden, Stretton and Davis, 1989). FLPs have a wide variety of actions mediated by different types of receptor: activation of sodium, potassium and chloride currents, suppression of calcium currents and depression of voltage-dependent potassium and calcium currents (Cowden et al., 1989).

4.1. FMRF-amide immunoreactivity in *Ascaris*

FLP immunoreactivity appears widespread in *Ascaris*; it is found in the anterior region of the parasite, concentrated near the pharyngeal nerves, as well as in the four major nerve cords along the length of the body (Davenport, Lee and Isaac, 1988).

4.2. Physiological activity of a purified FLP: AF1

FLPs extracted from *Ascaris* heads are heterogeneous; whether or not they belong to an intragenic family is not known. One purified and sequenced form, called AF1, is an heptapeptide (A for *Ascaris*, F for FMRFamide, 1 for the first purified form), and has been shown to be biologically active in *Ascaris* (Cowden et al., 1989). AF1 reduces the input resistance in inhibitory motorneurones (recorded from the commissures), but has no effect on excitatory motorneurones. The decrease in input resistance produces changes in the membrane space constant that may impair inhibitory transmission (Cowden et al., 1989). One putative target for AF1 is the slow voltage-sensitive conductance activated by strong depolarization: AF1 could activate this conductance.

It has been proposed that AF1 suppresses the inhibitory circuit leading to paralysis of the body, and this during the feeding period. However there is no direct evidence yet for the role of FLPs in *Ascaris*, and it seems likely that the variety of FLPs and receptors will make the picture even more complex in the future.

B. γ-AMINOBUTYRIC RECEPTORS

I VERTEBRATE

The first report of γ-aminobutyric acid (GABA) as a neuro-inhibitory substance was made by Florey (1954). He described the inhibitory activity of a substance extracted from the mammalian nervous system, Factor I, on the crayfish stretch receptor. Later, Factor I was shown to be mostly GABA (Bazemore, Elliot and Florey, 1957). The recognition of GABA as an inhibitory transmitter in the vertebrate nervous system came with the demonstration of its wide distribution in association with the related enzymes required for its synthesis and degradation, and the discovery of its physiological role (Curtis and Johnston, 1974; Krnjevíc, 1974). GABA has an inhibitory action at the cellular level, but it may produce disinhibition and leads to a general excitatory effect at a more integrated level. GABA-ergic neurotransmission is involved in the control of more specific physiological mechanisms (see for a review Matsumoto, 1989; Sieghart, 1989) such as: hormone secretion of prolactin and growth hormone, control of cardiovascular function, mechanisms underlying pain, anxiety and feeding and aggressive behaviour. This list is certainly not exhaustive, but gives an idea of the important role played by GABA neuro-transmission in the vertebrate systems. GABA receptors subtypes were discovered using new techniques such as radiolabelled-ligand binding and molecular cloning. Two GABA receptor types are commonly recognized, A and B (Bowery and Hudson, 1979), which have different physiological roles (see Matsumoto, 1989 for a review). There is also indirect evidence for a third type, non-A, non-B, tentatively named a GABA-C receptor by Johnston (1986), on which baclofen and bicuculline are inactive (Ault and Nadler, 1983). Different pharmacological subtypes have also been described, within GABA-A and GABA-B types. Whether or not these subtypes have different physiological functions is not known. In this chapter, a short review of the GABA receptors in vertebrates is presented; it concentrates mostly on the GABA-A type, since the *Ascaris* GABA receptor is more closely related to this type than to the B type.

1. GABA-A receptor complex

1.1. Association of the GABA receptor with a Cl channel and regulatory sites

Rather than "GABA-A receptor", the term of "GABA-A receptor-channel complex" is more appropriate. Despite early evidence for the coupling between the GABA receptor and a Cl channel (Bowery and Brown, 1974), the use of the term "GABA receptor-channel complex" really emerged with the discovery of the association of benzodiazepine receptors and GABA receptors (Tallman, Thomas and Gallager, 1978). Now several others regulatory binding sites have been identified: the picrotoxin binding site; the benzodiazepine binding site; and the steroid binding site.

These sites were shown to interfere with the binding of GABA and to interact with each other. The description of these interactions and of their physiological relevance is discussed below.

a. Benzodiazepine binding sites

Benzodiazepines were first shown to potentiate presynaptic inhibition in the spinal cord (Polc, Möhler and Haefely, 1974), in cultured mammalian neurones (MacDonald and Barker, 1978), and pre- and post-synaptic inhibition in cat cuneate nucleus (Polc and Haefely, 1976). The interaction between GABA-A and benzodiazepine receptors was confirmed using binding techniques (radiolabelled-ligand binding techniques), where GABA was shown to enhance benzodiazepine binding (Tallman et al., 1978; Karobath and Sperk, 1979). This enhancement is antagonized by GABA-A competitive antagonists, such as bicuculline (Tallman et al., 1978), suggesting an action mediated by the GABA receptor itself. However, GABA does not increase the binding of benzodiazepine antagonists, such as Ro15-1788, and even decreases the binding of benzodiazepine inverse agonists, such as methyl-β-carboline-3-carboxylate (Braestrup and Nielsen, 1981; Ehlert, Roeske, Braestrup et al., 1981).

The benzodiazepine site interferes reciprocally with [^{3}H]GABA binding; agonists at the benzodiazepine receptor increase the affinity of the GABA receptor (Guidotti, Toffano and Costa 1978). It was proposed that benzodiazepines act by competing or removing an inhibitory protein at the GABA receptor: GABA modulin (Guidotti et al., 1978; Guidotti, Konkel, Ebstein et al., 1982). An increase in GABA binding affinity is also obtained by pretreatment of the membranes with triton X-100 or by freezing-thawing cycles (Enna and Snyder, 1977). These treatments were thought to denature an

inhibitory protein which might be similar to the one regulated by the benzodiazepines. More recently the patch-clamp technique has shown that benzodiazepines increase the probability of channel opening without affecting the mean open time; but at high concentrations agonists like diazepam appear to accelerate desensitization of GABA receptors (bovine chromaffin cells, Mathers, 1987). An attractive model for the interaction benzodiazepine-GABA receptors has been proposed by Mathers (1987): GABA-activated channels opening is regulated by a putative protein preventing repetitive opening and subsequent desensitization. In the presence of benzodiazepines, this regulatory protein is inhibited and the channel open probability is increased until a desensitization threshold is reached.

b. Picrotoxin binding site

The recording of GABA activated single channel currents in patch-clamp studies (Bormann, Sakman and Seifert, 1983) and the measurement of changes in the flux of ^{36}Cl in brain slices (Wong, Leeb-lundberg, Teichberg et al., 1984) or brain microsacs (Harris and Allan, 1985), has confirmed the widespread assumption that GABA-A receptors are directly coupled to Cl channels. The well known non competitive GABA-A antagonist, picrotoxin, its close derivative picrotoxinin and the "cage convulsant" TBPS, are believed to bind a site located on the channel itself (Squires, Casida, Richardson et al., 1983; Supavilai and Karobath, 1984). These compounds also interfere with non GABA-gated Cl channels in invertebrates, again suggesting that their site of action is on the channel itself (Eldefrawi and Eldefrawi, 1987).

Barbiturates are believed to act on the same site as well. Depressant barbiturates such as pentobarbitone potentiate GABA by increasing the receptor affinity (Ticku and Olsen, 1978). At high concentrations they have a direct GABA mimetic effect (Leeb-Lundberg, Snowman and Olsen, 1980; see Johnston and Willow, 1982 for review). In patch-clamp studies, barbiturates promote a burst-like pattern of channel openings (Mathers, 1987) by increasing the burst duration (Twyman, Rogers and MacDonald, 1989), and may well be responsible for desensitization at higher concentrations (Mathers, 1987). Whether the barbiturate-GABA interaction is relevant is not known since the concentrations of barbiturates required to potentiate GABA responses are not compatible with therapeutic concentrations. The channel binding site is certainly of great pharmacological importance; the recent discovery of the action of some naturally occurring steroids brings a new interesting development to this field. Because of its importance, the action of steroids on the GABA-A receptor-channel complex will be treated in a separate paragraph.

c. Benzodiazepine and picrotoxin binding sites interactions

Barbiturates can increase, indirectly, benzodiazepine binding by increasing the GABA-receptor affinity; but in the presence of an excess of GABA, barbiturates still increase benzodiazepine binding (Leeb-Lundberg et al., 1980). A similar type of interaction is described with the anxiolytics etazolate and cartazolate: they are also supposed to bind to the Cl channel, and they increase benzodiazepine binding, but in contrast to barbiturates they do not interfere with GABA binding (Beer, Klepner, Lippa et al., 1978; William and Risley, 1979; Supavilai and Karobath, 1979). The antiparasitic drug, avermectin B1 has been shown to have similar properties as well (Williams and Yarbrough, 1979). In view of the difficulties in interpreting binding studies, only rational physiological experiments are likely lead to a better understanding of the interactions between the different regulatory sites at the GABA-A receptor-channel complex.

d. Steroid binding site

Recently, the GABA receptor complex has been shown to be modulated by steroids in a stereospecific manner (Simmonds, Turner and Harrison, 1984; Majewska, Harrison, Schwartz et al., 1986). Naturally occurring steroid hormone metabolites are effective at very low concentrations (Majewska, Bisserbe and Eskay, 1985; Majewska et al., 1986; Turner, 1986; Callahan, Cottrell, Hather et al., 1986; Barker, Harrison, Lange et al., 1986; Morrow, Suzdack and Paul, 1987; Ong, Kerr and Johnston, 1987; see Gee, 1988 for a review) and so is the steroid anaesthetic alphaxalone (Harrison and Simmonds, 1984; Barker et al., 1986; Cottrell, Lambert and Peters, 1987). The receptor involved is not the classical cytoplasmic steroid receptor since the stereospecificity is different, and steroids displace [^{35}S]TBPS from its binding site. The steroid binding site seems identical to the previously described TBPS, picrotoxin, barbiturate binding site. However, the stimulatory effects of secobarbital and pregnenolone on the GABA receptor can be blocked selectively and the stimulation of [^{3}H]muscimol binding by secobarbital and pregnenolone is additive (Kirkness and Turner, 1988). These two facts are in favour of a separate site of action, but here as well, it would be interesting to know if this difference can be demonstrated under more physiological conditions. Steroids are an interesting area of research and may be considered as natural modulators at the Cl channel level.

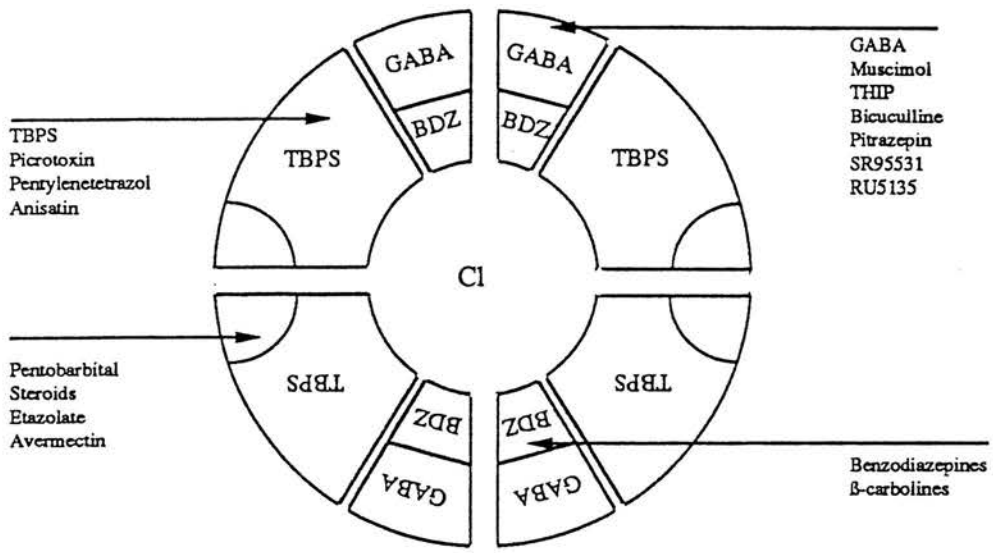


Figure 6: Schematic diagram of the GABA-A receptor-channel complex (from Braestrup et al., 1986).

Conclusion: all these pharmacological and biochemical approaches have revealed different sites on the GABA-A receptor, which can be pharmacologically manipulated. Figure 6 is a schematic representation of these different sites of the GABA-A receptor (from Braestrup and Nielsen, 1986).

1.2. Pharmacology of the GABA-A receptor

The main aspects of the pharmacology of the vertebrate GABA-A receptors are summarized in Table 3, and compared with the GABA-B receptor. The rigid analogues of GABA, muscimol, isoguvacine and THIP are usually potent and rather specific agonists at GABA-A receptors. Bicuculline and picrotoxin are potent, specific antagonists at GABA-A receptors. Bicuculline is a competitive antagonist, whereas picrotoxin acts non-competitively at the channel level. However, there are some variations in the relative potencies of agonists and antagonists within the population of the GABA-A receptors, indicating of the heterogeneity of the GABA-A receptors (Krogsgaard-Larsen, 1988).

1.3. Heterogeneity of GABA-A receptors

a. Pharmacological evidence

If GABA-A receptors are defined as bicuculline sensitive and baclofen insensitive receptors (Bowery, Hill and Hudson, 1983), different subtypes can be described. Their classification is made on the basis of either their localization (central or peripheral, synaptic or extrasynaptic), affinity (high, medium or low affinity) or putative function (uptake sites or autoreceptors) (see Krogsgaard-Larsen, 1988, for a review).

- High, medium and low affinity sites

Two GABA-A binding sites: high and low affinity (Guidotti, Gale, Suria et al., 1979) have been described. The relative density of the high affinity site varies within the brain; a high density is found in the striatum and the substantia nigra. After lesion of the striato-nigral GABA-ergic system, there is an augmentation in the number of high affinity sites but not in the number of low affinity sites. A similar increase in the proportion of high affinity GABA sites is seen in brains from humans suffering from Huntington's Chorea. This was interpreted as up-regulation of the high affinity receptor, suggesting that the high affinity site was physiologically relevant in the

Table 3

Summary of the main differences between GABA-A and GABA-B receptor types (from Bowery et al., 1983).

	GABA-A	GABA-B
muscimol	potent agonist	weak agonist
isoguvacine	potent agonist	inactive
baclofen	inactive	agonist
bicuculline	competitive antagonist	inactive
picrotoxin	non competitive antagonist	inactive
Cl	increase conductance	no effect
Ca or K	no effect	decrease Ca conductance or increase K conductance
G protein	not linked	second messenger ?
benzodiazepines	potentiate	no effect
barbiturates	potentiate	no effect

striato-nigral system (Guidotti et al., 1979). There are as well some pharmacological differences between these two binding sites: dihydromuscimol, muscimol and thiomuscimol are more potent on the low affinity site, and are the most potent activators of benzodiazepine binding. In contrast THIP is a potent [^{3}H]GABA displacer on the high affinity site, but is weak at enhancing benzodiazepine binding. The extreme case is the sulphonic derivative P4S, which is a potent agonist on the high affinity site but which has a deactiving effect on benzodiazepine binding (Krogsgaard-Larsen, 1988). These differences lead to the hypothesis of two separate GABA receptors, only one being associated with the benzodiazepine binding sites. Pharmacological similarities between the low affinity binding site and the benzodiazepine binding stimulation, and the fact that micromolar concentrations of GABA are required to get physiological responses, suggest that the low affinity binding site is the physiological GABA-A receptor.

Falch and Krogsgaard-Larsen (1982), using THIP as a ligand, described a third, medium affinity, binding site in the CNS. They proposed that this medium affinity site was physiologically relevant since its pharmacological profile was similar to the pharmacological properties observed in electrophysiological studies in the CNS.

Conclusion: the distinction between high, medium and low affinity binding sites is based on binding studies and may not reflect the real actions of GABA and GABA agonists observed under more physiological conditions. The difference in affinity detected may correspond to different states of the same receptor; these states may have different pharmacological properties (see Mathers, 1987).

- Synaptic and extrasynaptic GABA-A receptor subtypes

With hippocampal neurones, GABA applied ionophoretically on the soma induced hyperpolarization, but GABA applied on the dendrites induces depolarization (Alger and Nicoll, 1982). The different response is explained by differences in the Cl potential between the two regions. However the two responses present a different pharmacological sensitivity: the depolarizing response is 10-100 times more sensitive to picrotoxin and bicuculline than the hyperpolarizing response; THIP elicits only hyperpolarizing responses (Alger and Nicoll, 1982); and ethylenediamine on the contrary elicits depolarizing responses (Blaxter and Cottrell, 1987). Since, on hippocampal neurones, synaptic receptors are concentrated on the soma, the hyperpolarizing response was said to be the result of synaptic receptors activation, whereas the depolarizing response was due to the activation of extrasynaptic receptors (Alger and Nicoll, 1982). Extrasynaptic receptors are also found on peripheral nerves

and their activation produces depolarization of the unmyelinated (Brown and Marsh, 1978) and myelinated (Morris, Di Costanzo, Fox et al., 1983) axons. In electrophysiological studies, the pharmacology of extrasynaptic myelinated nerve GABA-A receptors is similar to the central GABA-A receptors. However in binding studies, there is a marked disparity: neither bicuculline or baclofen displace [^{3}H]GABA from this binding site (Barolet, Kish and Morris, 1985). There is therefore biochemical and physiological evidence for different subtypes of GABA-A receptors corresponding to synaptic and extrasynaptic locations.

- Central and peripheral GABA-A receptors

GABA receptors are present centrally (Curtis and Watkins, 1965; Curtis and Johnston, 1974) and peripherally on: sympathetic ganglia (Adams and Brown, 1974; Bowery and Brown, 1974), sensory dorsal root ganglia (Feltz and Raminsky, 1974), peripheral nerve trunks (Brown and Marsh, 1978; Morris et al., 1983; Barolet et al., 1985), primary afferent terminals (Barker and Nicoll, 1973), and adrenal chromaffin cells (Katoaka, Gutman, Guidotti et al., 1984). Nistri and Constanti (1979) proposed four key compounds to distinguish between two groups of GABA-A receptors: guanidoacetic acid, β-guanidinopropionic acid, γ-aminohydroxybutyric acid, and trans-1,3-aminocyclopentane carboxylic acid. GABA receptors found in sympathetic ganglia, spinal motorneurones, and spinal interneurones are rather insensitive to these compounds whereas GABA-A receptors found in primary afferents terminals, dorsal root ganglia, cortical neurones are rather sensitive. Another classification based more simply on the potency of bicuculline and the relative potency of 3-APS, distinguishes three main types: ganglionic, spinal and supraspinal GABA-A receptors (Krogsgaard-Larsen, 1988). Ganglionic GABA receptors (sensory or autonomic ganglia) produce depolarization of the neurone membrane and 3-APS is equipotent to GABA. Spinal receptors, in spite of small differences between primary afferent terminals, interneurones and motorneurones, are fairly homogenous: 3-APS is more potent than GABA itself. Bicuculline acts as a potent competitive antagonist at both ganglionic and spinal receptors, in contrast to the central GABA receptors where bicuculline acts as a weak non competitive antagonist. These results illustrate the pharmacological variety of GABA-A receptors. Since these differences are associated with regional variation, they may well reflect differences in the physiological role of GABA-A receptors.

- Uptake sites

Three different sites are commonly distinguished: neuronal high affinity uptake, neuronal low affinity uptake and glial uptake; they ~~are all sensitive to bicuculline, but~~

present pharmacological properties different from the classical GABA-A receptor (Iversen and Kelly, 1974; Hitzemann and Loh, 1978; Krogsgaard-Larsen and Johnston, 1975; Allan, Dickenson and Fong, 1986b). For example nipecotic acid, 2,4-diaminobutyric acid and β-alanine are inhibitors of GABA uptake; β-alanine is a potent blocker of glial uptake but not of neuronal uptake and 2,4-diaminobutyric acid is an inhibitor at the neural uptake sites. These uptake sites are interesting targets since their specific pharmacological manipulation can lead to a potentiation of GABA neurotransmission.

- Autoreceptors

The release of GABA from an inhibitory nerve may be regulated by GABA itself (Mitchell and Martin, 1978). These sites are bicuculline sensitive but present different pharmacological properties. For example δ-aminolaevulinic acid is a specific agonist at GABA-A autoreceptors (Brennan and Cantrill, 1979). GABA autoreceptors can also be manipulated pharmacologically to modulate GABA neurotransmission.

b. Molecular biology

See Stephenson, (1988); Schofield, (1989); Olsen and Tobin, (1990), for reviews. In purified and solubilized preparations of central GABA receptors, only two protein bands were originally detected: 50-53 kDa (α) and 55-57kDa (β) (Schofield, Darlison, Fujita et al., 1987). The β protein chain was photolabelled with [^{3}H]muscimol and the α protein chain with [^{3}H]flunitrazepam (Schofield et al., 1987). Cloning and expression of these subunits suggested that the organization of GABA-A receptor was of the type 2αchains+2βchains (2α2β) (Schofield et al., 1987). However, the cloned 2α2β GABA-A receptors expressed in xenopus eggs, although functional, were only weakly potentiated by benzodiazepines (Schofield et al., 1987). Recent preparations have revealed the existence of novel subunits: γ, δ and ε, and new subtypes of subunits: α_1, α_2, α_3, α_4; β_1, β_2, β_3; γ_1, and γ_2 (see Schofield, 1989, for a review). Molecular cloning techniques confirmed that each subunit and each subtype were originating from distinct genes. Reconstitution with the cloned subunits αβ2γ yielded to functional receptors that were modulated by benzodiazepines. In contrast to the previous findings, the γ but none of the α subunits, are photolabelled by [^{3}H]flunitrazepam. The combination and expression of these various subtypes of subunits gives functional receptors with different sensitivities and pharmacological properties. The molecular biology techniques have therefore confirmed the multiplicity of GABA-A receptors. Recent in situ hybridization studies have shown that the γ_2 subunit is encoded in various neuronal subsets known to contain high affinity

benzodiazepine binding sites (Olsen and Tobin, 1990); localization of mRNA for the δ subunit also shows a good correlation with the distribution of the high affinity muscimol binding site. These new results provide strong evidence for the existence of GABA-A receptor heterogeneity and are based on genic differences. These molecular differences explain the pharmacological heterogeneity of the GABA-A receptors described earlier.

Conclusion: GABA-A receptors are complex molecules, some can present binding sites for benzodiazepines, barbiturates and steroids, and are pharmacologically and geographically heterogeneous. GABA-A receptor heterogeneity is also seen with molecular biology techniques. These GABA-A receptors are the product of multiple genes, and the regulation of their expression in various biological situations may explain differences in location found with the pharmacological GABA-A subtypes.

2. GABA-B receptor

See Bowery, (1989) for a review. GABA-B receptors also inhibit neuronal activity but mainly by pre-synaptic inhibition of neurotransmitter release. GABA-B receptors inhibit the release of neurotransmitter in the peripheral and in the central nervous system (Bowery and Hudson, 1979; Bowery, Doble, Hill et al., 1980a; Bowery, Hill, Hudson et al., 1980b). However a post-synaptic physiological role for GABA-B receptors has been proposed in hippocampal pyramidal cells: activation of post-synaptic GABA-B receptors results in a slow inhibitory post-synaptic potential mediated by an increase in K conductance (Dutar and Nicoll, 1988). This contrasts with the fast post-synaptic inhibitory potential induced by GABA-A activation in the same cells (Dutar and Nicoll, 1988). The physiological role of GABA-B receptors is less well-known than is the role of GABA-A receptors, but they seem to be specifically associated with depression, memory and analgesia (Bowery, 1989).

2.1. GABA-B receptors are coupled with Ca or K conductances

GABA-B receptors, in contrast to GABA-A receptors, are not linked to a chloride channel but are related to Ca or K conductances, and they have a distinct pharmacological profile. GABA reduces a Ca conductance via GABA-B receptors in rat dorsal root ganglion (Desarmenien, Feltz, Loeffler et al., 1982) and in rat embryonic sensory neurones (Dunlap, 1981); but GABA increases a K conductance via GABA-B

receptors in rat hippocampus (Dutar and Nicoll, 1988). It would seem that the Ca-coupling is predominant at presynaptic terminals whereas the K-coupling is more important post-synaptically. In contrast to the GABA-A site, the coupling of GABA-B receptors with Ca or K conductances, is not direct and involves a second messenger: a G protein appears to be the best candidate. The fine mechanism is still unknown. One problem is the existence of contradictory effects on the production of cAMP: GABA-B receptor activation can either depress the accumulation of cAMP induced by forskolin, or enhance the formation of cAMP induced by the stimulation of β-adrenoreceptors (Scherer, Ferkany and Enna, 1988). These two actions may involve different receptors (Scherer et al., 1988), but whether or not these two mechanisms are related to the coupling with two different conductances, is not known yet.

2.2. Pharmacological profile of the GABA-B receptor

The pharmacological properties of the GABA-B receptors are very different from those of the GABA-A receptor complex; the main points are;

- Baclofen is a specific GABA-B agonist which has no action on the GABA-A receptor (Bowery et al., 1980b). [^{3}H]baclofen binds specifically GABA-B receptors (Hill and Bowery, 1981; Bowery and Hudson, 1983).

- Bicuculline does not antagonize GABA responses at the GABA-B receptors (Bowery and Hudson, 1979; Bowery et al., 1980a) and does not interfere with [^{3}H]baclofen binding (Hill and Bowery, 1981, Bowery and Hudson, 1983).

- The rigid analogues of GABA such as, isoguvacine, THIP, and the sulphonic acid derivatives, 3-APS and P4S, are inactive (Bowery, Price, Hudson et al., 1984). The partially rigid analogues such as muscimol are less potent than on the GABA-A receptor (Bowery and Hudson, 1983).

On the basis of the activity of flexible and rigid GABA analogues, Johnston and Allan (1984) proposed that GABA-A receptors accept the GABA molecule in an extended conformation, whereas GABA-B receptors accept the GABA molecule in a folded conformation. Table 3 shows a summary of the main differences between GABA-A and GABA-B receptors. Baclofen was the first specific GABA-B agonist discovered (Bowery et al., 1980b). Now phosphinic derivatives of GABA are known to be potent and selective GABA-B agonists: 3-aminopropane phosphinic acid, is 10-100 times more potent than baclofen. Surprisingly the phosphonic derivative of baclofen, phaclofen, does not act as an agonist but as an antagonist (Dutar and Nicoll, 1988). Similarly, the GABA-A agonist DAVA acts as an antagonist on GABA-B

receptors in rat anococcygeus muscle (Muhyaddin, Roberts and Woodruff, 1982), and its restricted analogue Z-5-aminopent-2-enoic acid is five times more potent than phaclofen as a GABA-B antagonist (Dickenson, Allan, Ong et al., 1988).

2.3. Heterogeneity of GABA-B receptors

As mentioned before, there is evidence for heterogeneity of GABA-B receptors; the agonist 3-aminopropyl phosphinic acid acts as a full agonist in inhibiting the accumulation of cAMP stimulated by forskolin, but acts as a partial agonist on the potentiation of cAMP formation by β-adrenergic agonists. A close analogue: 3-aminopropyl phosphonic acid mimics the action of baclofen on the accumulation of cAMP stimulated by forskolin, but is inactive on the potentiation of cAMP formation by isoprenaline (Scherer et al., 1988). There is also some pharmacological evidence for the existence of GABA-B autoreceptors (Pittaluga, Asaro, Pelligrini at al., 1987).

Conclusion: GABA-B receptors also appear to be heterogeneous; they have a great physiological importance and seem to be more involved in analgesia and depression than GABA-A receptors (Bowery, 1989).

II INVERTEBRATES

Evidence for GABA receptors in invertebrates was discovered very early, since Florey (1954) detected the inhibitory effects of Factor I on a crustacean stretch receptor preparation. The study of GABA receptors in invertebrates has mainly been performed in arthropods, and to a certain extend in molluscs. One characteristic of the GABA receptors in invertebrate, is the variability of their pharmacological properties, between and within species. However their common features are (see, Nistri and Constanti, 1979; Simmonds 1983; Walker and Holden-Dye, 1989, for review):

- Invertebrate GABA receptors are directly linked to a Cl channel (except in molluscs, where different ionic conductances can be associated with GABA receptors).
- Invertebrate GABA receptors are insensitive to baclofen
- Invertebrate GABA receptors are insensitive to sulphonic derivatives such as the GABA-A agonists P4S and 3-APS.
- Bicuculline has inconsistent effects, and is weak or inactive.
- Picrotoxin is a good antagonist.

- The presence of modulatory sites, such as benzodiazepine receptor, associated with the GABA receptor, is inconsistent.

1. Insensitivity to baclofen

Baclofen has been shown to be inactive in virtually all invertebrate preparations, suggesting that invertebrate GABA receptors are not GABA-B receptors. However, to reject the existence of a GABA-B type of receptor, one would have to test several compounds, rather than to rely on only one agonist. Invertebrates may possess GABA-B-like receptors with slightly different pharmacological properties such as weak activity of baclofen. A baclofen sensitive GABA receptor has been reported in only one invertebrate study, on the larvae of a sea gastropod (*Haliotis rufescens*), where it is involved in the trigger of metamorphosis (Trapido-Rosenthal and Morse, 1986). These larvae are induced to settle and metamorphose in response to exogenous GABA and a number of GABA agonists. Binding studies have shown that [^{3}H]baclofen specifically labelled the chemoreceptors involved (Trapido-Rosenthal and Morse, 1986). More recently, Benson (1989) described a GABA receptor in the heart of *Limulus* linked to a chloride channel on which the specific GABA-B agonist, 3-aminopropyl phosphonous acid is the most potent agonist; it was also insensitive to bicuculline and baclofen. These results indicate that receptor classification should not rely on a single compound. In conclusion, most invertebrate preparations are baclofen insensitive but this does not exclude the existence of a baclofen insensitive GABA receptors which is physiologically related to the vertebrate GABA-B receptor.

2. Insensitivity to sulphonic acid derivatives

In contrast to the GABA-A receptor, all invertebrate GABA receptors have shown an insensitivity towards the sulphonic acid derivatives such as P4S and 3-APS (Simmonds, 1983; Walker and Holden-Dye, 1989). Obviously, the structural and electron differences between the tetrahedrally oriented sulphonate group and the planar carboxylate group (Krogsgaard-Larsen, 1988) play an important role accounting for the differences of properties between GABA and P4S at invertebrate GABA receptors.

3. Bicuculline sensitivity

Tables 4,5, and 6, summarizes the sensitivity of various preparations to bicuculline and GABA antagonists. One can distinguish two classes of invertebrate GABA

receptor: those that are insensitive to bicuculline and those on which bicuculline has a weak antagonist action.

3.1. Insensitivity of GABA responses to bicuculline

GABA receptors in the central nervous system and at neuromuscular junctions in insects are insensitive to bicuculline (Scott and Duce, 1987; Lees, Beadle, Neumann et al., 1987; Satelle, Pinnock, Wafford et al., 1988; Benson, 1988; Wafford, Satelle, Abalis et al., 1988). In crustaceans, bicuculline is ineffective on the peripheral stretch receptor neurones (Swagel, Ikeda and Roberts, 1973), but has a weak antagonist effect at neuromuscular junctions in the crayfish, the lobster (Takeuchi and Onodera, 1972; Shank, Pong, Freeman et al., 1975; Constanti, 1978) but not in the crab muscle (Earl and Large, 1974).

3.2. Weak GABA antagonism induced by bicuculline

Crustacean neuromuscular junctions are sensitive to bicuculline (10-250μM), but in contrast to the vertebrate GABA-A receptor, bicuculline acts as a non competitive antagonist (Takeuchi and Onodera, 1972; Shank et al., 1975; Constanti, 1978). In molluscs, bicuculline antagonizes fast hyperpolarizing responses to GABA (Piggott, Kerkut and Walker, 1977; Yarowsky and Carpenter, 1978; Vehovszky, Bokisch, Krogsgaard-Larsen et al., 1989). These responses involve Cl channel activation. However, bicuculline has been reported also to antagonize some depolarizing responses to GABA, which are due to Na conductance activation or K conductance inactivation (Pigott et al., 1977). Apart from its weak GABA antagonist properties in some species, bicuculline has been reported to antagonize nicotinic Na-mediated responses in the locust (Benson, 1988), and acetylcholine hyperpolarizing Cl-mediated responses in the snail (Piggott et al.,1977).

4. Picrotoxin sensitivity

Picrotoxin antagonizes most of the GABA responses in invertebrates. The main difference with the GABA-A receptor lies in the mechanism of action. Several reports suggest that picrotoxin acts in a competitive manner in crustaceans (Grundfest, Reuben and Rickles, 1959, Shank et al.,1974, Hori, Ikeda and Roberts,1978), but it seems more likely that "mixed" antagonism is involved (Constanti, 1978). At the vertebrate GABA-A receptor complex, picrotoxin is believed to bind a site located at the chloride

channel. It is therefore not surprising, that picrotoxin is able to antagonize acetylcholine induced hyperpolarizing (Cl-mediated) responses in molluscs (Piggott et al., 1977). However, picrotoxin has been shown to block GABA depolarizing (Na-mediated) responses in the snail (Vehovszky et al., 1989) and acetylcholine (Na-, K-mediated) depolarization in the cockroach (Satelle et al., 1988). From these results, it is clear that, if picrotoxin really interacts with the channel part of the receptor, it is not specific to Cl channels, in invertebrates, specially in molluscs.

5. Modulatory sites

Although benzodiazepine receptors were originally thought to occur only in the vertebrates (Nielsen, Braestrup and Squires, 1978), they have been described in insects (Table 7) (Abalis, Eldefrawi and Eldefrawi, 1983; Lummis and Satelle, 1986). They seem to be associated with GABA receptors since benzodiazepine potentiate GABA induced currents (Lees et al., 1987) and GABA binding (Satelle et al., 1988). Barbiturate potentiation of GABA effects has also been demonstrated in insects (Table 7) (Scott and Duce, 1987; Lees et al., 1987; Satelle et al., 1988).

Conclusion: invertebrate GABA receptors are similar to vertebrate GABA-A receptors: most of the GABA-A agonists such as muscimol, act as agonists, and the receptors are usually directly linked to a Cl channel. The main differences are that bicuculline is virtually inactive at invertebrate GABA receptors, and that sulphonic acid derivatives are not potent agonists. There are exceptions to this generalization: mollusc neurones respond in five different ways to GABA, three responses are excitatory mediated by Na or K conductance changes, and two responses are inhibitory mediated by Cl conductance changes (Vehovszky et al., 1989). It has been pointed out that most of the invertebrate GABA receptors are insensitive to baclofen, and this is interpreted as invertebrate GABA receptors being not GABA-B receptors. However in some cases, this classification criteria loøses its meaning. For example, the GABA-B agonist 3-aminopropyl phosphonous acid, is the most potent agonist at the GABA receptor in *Limulus* heart (Benson, 1989); this does not mean that this receptor is a GABA-B receptor, since the GABA-A agonists muscimol, isoguvacine and THIP act as agonists, and baclofen is inactive.

More generally, this short review on GABA receptors shows that receptor classification is not simple. Classifying a GABA receptor cannot be achieved solely by using a single drug or technique, but receptor classification, although an oversimplification, is still required for convenience of communication. In the next

Sections, the terms of GABA-A and GABA-B receptors will be used, bearing in mind that GABA-receptor classification is more complex and involve numerous pharmacological subtypes.

Table 4:Summary of the effects of GABA antagonists on Insects GABA receptors

		INSECTS	
Bicuculline	Locust muscle	1mM, no effect	Scott and Duce, 1987
	Locust neurones	100μM no effect on GABA responses but 0.1-10μM antagonized acetylcholine responses	Lees et al., 1987 Benson, 1988
	Cockroach neurones	100μM no effect on GABA evoked currents on [^{36}Cl] influx stimulation by GABA	Satelle et al., 1988 Wafford et al., 1987
	Limulus *heart	inactive at 100μM	Benson, 1989
Picrotoxin	Locust muscle	1-5mM blocked GABA responses	Scott and Duce, 1987
	Locust neurones	1μM, antagonized GABA responses	Lees et al., 1987 Benson, 1988
	Cockroach neurones	0.1-1μM antagonized GABA and acetylcholine responses (Na,K currents) 100μM antagonized [^{36}Cl] influx stimulation by GABA	Satelle et al., 1988 Wafford et al., 1987
	*Limulus** neurones	5μM, antagonizes GABA responses	James et al., 1982
	Limulus *heart	inactive at 100μM	Benson, 1989
Pitrazepin	Locust neurones	100μM no effect	Lees et al., 1987
	Cockroach neurones	1-10μM no effect 100μM, small voltage sensitive antagonism of GABA currents	Satelle et al., 1988
	*Limulus**heart	inactive at 10μM	Benson, 1989
RU5135	Cockroach neurones	1-10μM no effect 100μM, small voltage independent antagonism of GABA currents	Satelle et al., 1988
SR95103	Limulus* heart	inactive at 100μM	Benson, 1989

* *Limulus* is not an insect but belongs to the class of Chelicerata, which is close to the class of Arachneidea.

Table 5: Summary of the effects of some antagonists on crustaceans GABA receptors

	CRUSTACEANS		
Bicuculline	Lobster muscle	10-250μM, true non competitive antagonist	Shank et al., 1975
	Crayfish muscle		Constanti, 1978 Takeuchi and Onodera, 1972
	Crayfish muscle	Ki=350μM	Olsen et al., 1975
	Crab muscle	1mM no effect	Earl and Large, 1974
	Crayfish stretch receptor	10-100μM, no effect	Swage et al., 1973
Picrotoxin	Lobster muscle	0.1-10μM mixed antagonism	Constanti, 1978
		2μM, true competitive antagonism	Shank et al., 1974 Grundfest et al., 1959
	Crayfish muscle	1-30μM, true non competitive antagonism * inactive at 300μM	Takeuchi and Takeuchi 1969 Olsen et al 1975
	Crab muscle	1-50μM, true non competitive 10-500μM, blocks acetylcholine Na, K currents	Earl and Large, 1974 Marder and Paupardin-Tritsh, 1980
	Crayfish stretch receptor	2μM, competitive antagonism	Hori et al., 1978

Table 6: Effects of some antagonists on molluscs GABA receptors

MOLLUSCS			
Bicuculline	Snail neurones	0.1-1000µM antagonizes H but not D responses to GABA 80µM antagonizes, H and D responses to GABA and H response to acetylcholine	Vehovszky et al., 1989 Piggott et al., 1977
	Aplysia neurones	10-1000µM, antagonizes the fast hyperpolarization (Cl) response to GABA	Yarowsky and Carpenter, 1978
Picrotoxin	Snail neurones	0.1-10µM blocks H and D GABA responses 8µM blocks H responses to GABA and acetylcholine	Vehovszky et al., 1989 Piggott et al., 1977
	Aplysia neurones	1-100µM blocks the fast hyperpolarization (Cl) response to GABA	Yarowsky and Carpenter, 1978
Pitrazepin	Snail neurones	1-100µM, no effect	Vehovszky et al., 1989
SR95531	Snail neurones	1-10µM, blocks H and D responses to GABA	Vehovszky et al., 1989
Curare	*Aplysia* neurones	1mM decrease GABA response	Yarowsky and Carpenter, 1978
Strychnine	*Aplysia* neurones	100µM no effect	Yarowsky and Carpenter, 1978
	Snail neurones	8µM, blocks H and D responses to GABA and H responses to acetylcholine	Piggott et al., 1977

Table 7: Effects of benzodiazepines and barbiturates on insects GABA receptors

		INSECTS	
Benzo-diazepines	Locust neurones	1-10μM potentiation	Lees et al., 1987
	House Fly muscle	specific binding [3H] flunitrazepam	Abalis et al., 1983 Lummis and Satelle, 1986
Barbiturates	Locust neurones	100μM potentiation	Lees et al., 1987
	Locust muscle	100μM-2.5mM potentiation	Scott and Duce, 1987
	Cockroach neurones	1μM potentiation	Satelle et al., 1988

C. RECEPTOR THEORY

I AN HISTORY OF RECEPTOR THEORY

1. The concept of receptor

See Dean, (1987) for a review. The notion of specific receptors for drug molecules or neurotransmitters started with Langley. In his early studies of salivary secretion, Langley (1873) speculated on the association of drugs with some part of the tissue:

> "*...we may, I think, without much rashness, assume that there is some substance or substances in the nerve endings or gland cells with which both atropin and pilocarpin are capable of forming compounds...*".

Later, Ehrlich, working on the immune response, formulated the concept of specific surface groups on cells, that could interact with drug molecules showing specific binding properties. The term, receptor, was then used (Ehrlich and Morgenroth, 1900):

> "*...For the sake of brevity, that combining group of the protoplasmic molecule will hereafter be termed receptor...*" .

Langley (1905), then referred in his subsequent work on toxins and drugs to Ehrlich's theory of immunity, and mentioned the existence of receptive substances:

> "*...I conclude then that in all cells two constituents at least must be distinguished, (1) substances concerned with carrying out the chief functions of the cell such as, contraction, secretion, the formation of special metabolic products, and (2) receptive substances especially liable to change and capable of setting the chief substance in action...*".

2. Classical models for drug-receptor interaction

Langley (1873) himself suggested that the action of a drug or toxin could follow:

> "*...some law of which their relative mass and chemical affinity are some factors...*".

A student of Langley, Hill (1909), applied the law of mass action to describe the relation between the concentration of an agonist and the amount of compound formed between this agonist and the "receptive substance". However, it was only 50 years later, that the first model for drug-receptor interaction was proposed by Clark (1926).

2.1. Clark-Gaddum theory of occupancy

Clark's quantitative study of the action of acetylcholine on isolated frog muscle showed that the effects of acetylcholine could be expressed as a function of the drug concentration:

$$K.x = \frac{y}{(100-y)} \qquad (1)$$

where x is the concentration of acetylcholine, y is the response as a percentage of the maximum response and K is a constant. He related this equation to the Langmuir's adsorption isotherm, describing the adsorption of a gas at the surface of a solid. By analogy, Clark interpreted y as the percentage of receptor occupied, and K_A as the affinity constant of the drug for the receptor:

$$A + R \rightleftharpoons AR$$

The effect of a drug is therefore related to the proportion of receptors occupied by this drug; Clark's theory is also called *occupancy theory*. Authors refer either to Langmuir's isotherm or to the law of mass action (Hill, 1909). Both equations are the same. Although there is no evidence that the drug-receptor interaction can be described by the law of mass action or by Langmuir's isotherm, it is assumed to give a good approximation.

Gaddum (1936), extended Clark's theory to the antagonist-receptor interaction. He assumed that antagonist molecules occupy the receptors without any effect. If z is the percentage of receptors occupied by the antagonist, y is the percentage of receptors occupied by the agonist, C_1 and C_2 are the concentrations of agonist and antagonist, K_1 and K_2 the dissociation constants for agonist and antagonist, then,

$$A + R \rightleftharpoons AR$$

$$B + R \rightleftharpoons BR$$

with $y = K_1.C_1.(100 - y - z)$, $z = K_2.C_2.(100 - y - z)$ and the relation between agonist and antagonist is,

$$K_1.C_1 = \frac{(1 + K_2.C_2).y}{(100 - y)} \quad (2)$$

However, although the response is expected to be proportional to occupancy, the relation between them is not known in most preparations. The work on antagonism was then mainly carried on by Schild, who first used the notion of null hypothesis: the same tissue response should correspond to the same receptor occupancy (see 2.1.).

2.2. Ariëns and the concept of "intrinsic activity"

Ariëns (1954) introduced the notion of intrinsic activity. Working on the acetylcholine receptor, he noticed that some compounds showed a dual action: agonist or antagonist, depending on the tissue to which they were applied. The response appeared not only dependent upon the fraction of receptor occupied as in the Clark-Gaddum theory, but also on an intrinsic property of the agonist on a given tissue: the intrinsic activity,

$$E_A = \alpha.[RA]$$

where E_A is the response, [RA] the fraction of receptors occupied, and α the intrinsic activity of the compound. A full agonist has an intrinsic activity of 1, whereas a full antagonist has an intrinsic activity of 0. Compounds with values in between correspond to partial agonists. α is a property specific to the agonist and the tissue on which it is applied, whereas the proportion of receptors occupied depends on the receptor affinity.

2.3. Stephenson and the concept of "efficacy"

Stephenson (1956), introduced the idea that the response may not be linearly proportional to the fraction of receptors occupied, and that drugs may have different capacities to induce a response. He then defined, e, the efficacy and S, the stimulus. The stimulus is proportional to the fraction of receptors occupied: $S=e.y$, where e is the efficacy and y the proportion of receptors occupied. The response R is an unknown function of S: $R=f(S)$. This contrasts with Ariëns theory, where the response is directly proportional to the fraction of receptor occupied. Although efficacy and intrinsic activity

are both characteristics of the agonist and bear some similarities, they are not equivalent.

Conclusion: Clark-Gaddum, Ariëns, and Stephenson theories, are occupancy theories. The basic assumption is that the drug-receptor interaction is approximated by the law of mass action (or Langmuir's isotherm). Whereas early theories suggested that the response was directly related to the receptor occupancy, Stephenson introduced the notion of stimulus and stated that the response is an unknown function of the stimulus. However, even if the relationship between response and receptor occupancy is not known, the use of null methods allows the estimation of equilibrium constants and efficacy.

2.4. Paton and the "rate theory"

Paton (1961), suggested that the stimulus may depend upon the rate of combination of the drug with the receptor rather than on the receptor occupancy. A compound would have to form a complex which is rapidly broken up, and the rate constant for the break-up would be analogous to the efficacy described by Stephenson (1956). In the occupancy theories, it is assumed that the response will be produced as long as the receptor is occupied, whereas Paton suggested that the response will be produced only when the molecule leaves its binding site. Because, most of the experiments are carried out at the equilibrium and under the null hypothesis, there is no difference between the rate theory and the occupancy theory,

$$y = \frac{A}{A + k_2/k_1} \qquad (3)$$

where A is the agonist concentration, y is the fraction of receptors occupied, k_1 is the rate of association and k_2 the rate of dissociation.

This theory suggested for the first time and before the two-state models and the allosteric theory, the existence of some structural modifications of the receptor after the binding of the agonist.

2.5. Hill and the "cooperative model"

The use of more direct approaches and parameters more related to the primary events following receptor activation, showed that the dose-response curve was not always hyperbolic, but in some cases distinctly sigmoid (Jenkinson, 1960). It was then

suggested that the model for cooperative interaction between haemoglobin and oxygen developed by Hill (1913), could be applied to the drug-receptor theory. Hill (1913), explained the sigmoidicity of the binding kinetic curve by the possibility of having several binding sites on the molecule of haemoglobin; the binding of one molecule will help the binding of the subsequent molecules.

Two cooperative models for drug-receptor interaction are distinguished. The first one, is equivalent to Hill's model: the binding of the first molecule of agonist helps the binding of the subsequent molecules, and the receptor is activated (Nistri and Constanti, 1979),

$$nA + R \rightleftharpoons A_nR$$

$$y = \frac{R}{Rmax} = \frac{x_A{}^N}{x_A{}^N + K_A} \quad (4)$$

where y is the fraction of receptor occupied, R is the response, Rmax is the maximal response, x_A is the agonist concentration, K_A is the apparent dissociation constant for the complex A_nR, and N is the number of sites.

In the second model, the activation of the receptor requires the binding of N molecules of agonist, but the binding sites are independent (Nistri and Constanti, 1979),

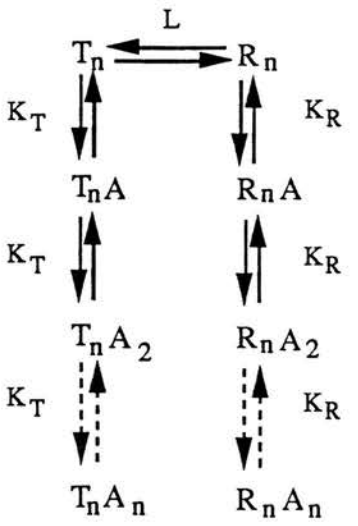
$$y = \frac{R}{Rmax} = \left(\frac{x_A}{x_A + K_A}\right)^N \quad (5)$$

where y, R, Rmax, x_A, and N are the same as before, but here K_A is the apparent dissociation constant for one of any of the n sites. In the first model there is a "binding cooperativity", whereas in the second model, there is a "physiological cooperativity". In radiolabelled ligand binding studies, cooperativity will be detected only for the first model.

2.6.Two-state models and the Monod-Wyman-Changeux model

In their experiment on eel electroplax, Changeux and Podleski (1968), suggested that the cooperativity detected between agonist and the acetylcholine receptor could not be explained by the Hill cooperative model described above. They related their observations to the allosteric model developed for enzymes (Monod, Wyman and Changeux, 1965) This led to the formulation of two-state models (see Colquhoun,

1973). The receptor is then defined as one or several protomers or subunits. Each subunit bears a binding site and can exist in two conformations for which the affinity for the drug molecule can be different.



T_n and R_n are the two states of the receptor. L is the equilibrium constant $L=\frac{[T_n]}{[R_n]}$. K_T and K_R are the microscopic constants for the interaction with binding sites on protomers in the T and R conformations.

In the dependent subunit model or Monod-Wyman-Changeux model, the n subunits are linked in such a way that all the subunits are constrained to adopt the same conformation: "concerted transition" (Monod et al., 1965), so that,

$$P_{open}= \frac{1}{1 + L\left(\frac{1 + \sum Mc}{1 + \sum c}\right)^n} \qquad (6)$$

where c is the normalized concentration of agonist: $c = \frac{x}{K_R}$, and $M = \frac{K_R}{K_T}$.

Under simplifying conditions, this model can be described by the Hill equation (5) (Colquhoun, 1973). In the independent subunit model, the observed cooperativity need not to involve any interaction between the binding sites. The channel will open only when all the protomers are in the right conformation.

$$P_{open}= \left(\frac{1}{1 + L.\frac{1 + \sum Mc}{1 + \sum c}}\right)^n \qquad (7)$$

All symbols are as before. Under simplifying conditions this model can also be described by equation (4) (Colquhoun, 1973).

Conclusion: the occupancy theories adequately described the drug-receptor interactions until the emergence of new techniques, which allowed the measurement of more fundamental events following receptor activation. With the discovery of the allosteric properties of proteins (Monod et al., 1965), the two-state model was proposed for drug-receptor interaction (Karlin, 1967; Changeux and Podleski, 1968; Colquhoun, 1973). The consequences of these theories are discussed subsequently in terms of experimental design and analysis of the results.

II MEASUREMENT OF DRUG-RECEPTOR INTERACTION

This chapter examines the practical application of drug-receptor interaction theories for the design of experimental procedures. If one wants a correct measure of drug-receptor interaction, two points are critical: the drug concentration and the response measured.

1. Drug concentration

In the original occupancy theory, the intensity of a pharmacological effect is considered to be directly proportional to the number of occupied receptors and this, in turn, is dependent upon the concentration of the drug in proximity of the receptor. It is therefore critical to know exactly the concentration in proximity of the receptor. Bath application of the drugs to a preparation, has the advantage of a known and more even concentration around the receptor when compared to local administration. However, uptake, metabolizing systems and diffusion barriers, can locally reduce the drug concentration around the cell, so that the bath concentration is not equivalent to the concentration around the receptor.

2. Measurement of the response

To describe qualitatively and quantitatively the interaction between a drug and its receptor, the choice of the measured response is of crucial importance; too often the chosen response is not linearly related to the primary event following receptor occupation. In the case of a receptor directly coupled to an ion channel, such as the GABA receptor, the primary event following the agonist-receptor interaction is the

opening of the Cl channels, leading to an increase in membrane conductance. The measurement of the change in membrane conductance is therefore directly related to the number of receptors activated. The change in membrane potential is a secondary event following receptor activation and is less related to the number of receptors activated. The change in membrane potential is also limited by the equilibrium potential for Cl. After several applications of GABA or agonist, Cl ions tend to accumulate intracellularly (if the resting membrane potential of the cell is less negative than the equilibrium potential for Cl), and the membrane potential is shifted to more negative values. This shift in resting membrane potential is illustrated in the Figure 34, Section 5, where GABA 30μM has been applied several times to the same cell and shows that the responses are similar in terms of membrane conductance but not in terms of membrane potential. (There is also a shift in equilibrium potential for Cl to a less negative value, since the intracellular concentration is increased.)

III ANALYSIS OF AGONIST AND ANTAGONIST ACTION

In this chapter, for more clarity, it is assumed that the response measured is the change in membrane conductance following the activation of a receptor linked to an ion channel such as the GABA-A receptor.

1. Agonists

The dose-response relationship is more direct when changes in membrane conductance are measured. All the equations proposed are based on the Hill equation (4) or (5), and therefore on the assumption of the Langmuir's isotherm. One useful way to describe quantitatively the action of an agonist is to define the EC50: concentration which produces 50% of the maximal response. The EC50 is equal to K_A, the dissociation constant, when there is no cooperativity (N=1), but is equal to $\sqrt[N]{K_A}$ when there is binding cooperativity (KA is the apparent dissociation constant of the complex A_nR, see equation 4).

The relation between drug-response and EC50 is illustrated by the modified Hill equation described by Nistri and Constanti (1979),

$$\Delta g = \frac{\Delta gmax}{1+(EC50 / [X_A])^N}$$

where Δg is the change in input conductance, $\Delta gmax$ is the maximum response, EC50 is the concentration of agonist producing 50% of the maximum response, $[X_A]$ is the agonist concentration and N is the Hill coefficient. Agonist activity is therefore quantified by three parameters: EC50, $\Delta gmax$ and N.

2. Antagonists

Antagonism has been widely studied and various models have been used (see Gaddum, 1936; Schild, 1947a, 1947b, 1949, 1957; Arunlakshana and Schild, 1959). This chapter presents the different approaches to drug antagonism and discusses their various implications.

2.1. Schild and modified Schild analysis

Schild (1947a) carried on the work started by Gaddum (1936) on drug antagonism. Its main achievement was to develop a method allowing estimation of the dissociation constant for an antagonist, in the absence of reliable information on the relationship between occupancy and response. For this purpose, he applied the notion of dose-ratio that Clark and Gaddum had already suggested. He defined also the pA_x, as the negative logarithm of the molar concentration of antagonist which reduces the effect of a multiple dose (x) of an agonist, to that of a single dose of agonist in absence of antagonist. x is the dose ratio. Schild modified Gaddum's expression to,

$$\log(x-1) = \log K_2 - pA_x \quad (8)$$

where by definition $pA_x = -\log B$, B is the antagonist concentration, K_2 is the dissociation constant for the antagonist. The plot of log(x-1) versus pA_x gives a straight line of slope: -1. He used the pA_x values to classify drugs and receptors (Schild, 1947b). The difference between pA_2 and pA_{10} can also be used as a test for competitive antagonism (Schild, 1949),

$$pA_2 - pA_{10} = \log 9$$

Arunlakshana and Schild (1959), considered the possibility of having n molecules of antagonist interacting with the receptor. Therefore the slope for the Schild plot: log(x-1) versus $\log(X_B)$, could differ from unity :

$$\log (x-1) = \log K_2 - npA_x \qquad (9)$$

where x, pA_x,and K_2 are as before, and n is the number of antagonist molecules required. They even considered the possibility of having different affinity constants for each antagonist molecule binding the receptor, and showed that log dose-effect curves would still be parallel. It is interesting to note that in this case, the plot log(x-1) against logB, would still be linear and with a slope different from 1. But the intercept would represent a mixture of the affinity constants for the antagonist. In all these cases described, the log dose-response curves are parallel, with same maximal response. Their main conclusion was that (Arunlakshana and Schild, 1959):

> "*...It is always possible to fit an equation for competitive antagonism to a set of parallel dose-effect curves, although the equation may be complicated, but on the other hand, parallel dose-effect curves are no proof of a competitive antagonism...*" .

The interaction of more than one molecule of agonist and antagonist, was first considered by Rang (1971):

> "*...the logarithmic plot of the dose-ratio against the antagonist concentration would not be linear, having a slope of M at low dose-ratios and M/N at high dose-ratios...*",

$$r_A{}^N - 1 = \frac{x_B{}^M}{K_B} \qquad (10)$$

where r_A is the dose-ratio (equivalent to x in the Arunlakshana and Schild 1959 formulation), x_B is the antagonist concentration, K_B the dissociation constant for the antagonist, N the Hill coefficient or the number of agonist molecules required to activate the receptor and M the number of antagonist molecules required to block the receptor activation (equivalent to n in the Arunlakshana and Schild, 1959, formulation). However he used this argument to dismiss the modified Langmuir equation which accounts for cooperative interaction between drug and receptor, since the classical Schild plot has proved to be linear and with a slope of one for a great variety of competitive antagonists (Rang 1971):

> "*...The results are in favour of the simple Langmuir equation...*" .

But Rang did not explain the regular occurrence of a Hill number higher than 1 found with some neurotransmitters, like GABA.

It is only recently that Williams, Smith, Burton et al., (1988) gave an example of bimolecular interaction of agonist and antagonist with the receptor, and formulated the modified Schild equation :

$$\log(DR^N-1) = M\log X_B + pK_B$$

where N is the number of agonist molecules required to activate the receptor and: M is the equivalent of n in the Arunlakshana and Schild formulation (1959); DR is the dose-ratio; pK_B is the negative log of the dissociation constant. In this case the plot; log(DR-1) versus log X_B, is not any more linear, but the log dose-response curves are still parallel with the same maximal response. Considering all these small variations of the original Schild equation, it appears that a slope of unity for the Schild plot; log(DR-1) versus logX_B, and even the linearity of the plot, are not necessary requirements for competitive antagonism. A more fundamental examination of the analysis of competitive antagonism has followed the realization that some receptors possess binding sites that may not be equivalent (Jenkinson, 1987). The implications can extend both to a departure from parallelism in the log dose-response curves and to non-linear Schild plot (Jenkinson, 1987; and see Appendix). In conclusion these models are classically used to describe agonist- and antagonist-receptor interactions, although they may not be representative of the real mechanisms, they nonetheless, provide a reasonable approximation.

2.2. Determination of the IC50

The estimation of the dissociation constant, is based upon a theoretical model of drug-receptor interaction. To compare antagonist properties of different compounds involving different mechanisms of action, a parameter estimated independently of any theoretical model, such as IC50, is more appropriate. The IC50 is the antagonist concentration which reduces the response to a given agonist concentration by 50%. In the case of non competitive antagonism, the IC50 is the only parameter available. The IC50 can be estimated independently of any theoretical assumption and is widely used to compare antagonists between each others or between different preparations. One problem encountered is that IC50 must be estimated graphically from the antagonist dose-response curve using only one agonist concentration. IC50 may appear better than K_B since it does not rely on any theoretical model of interaction between drug and receptor. However if the mechanism of action is competitive, K_B estimation is more

appropriate. The IC50 is only a snapshot of the antagonist-agonist interaction. The choice between IC50 or K_B depends on use: the IC50 is more appropriate for comparing antagonists that have different mechanisms of action, or for comparison of antagonists between different preparations. K_B is more appropriate for comparing antagonists that have the same mechanisms of action. This is especially true if they belong to a same chemical family and are tested on the same preparation.

Conclusion: theories have evolved with the advance of new experimental techniques. The theory of occupancy, in particular, was dramatically shaken by the emergence of single channel current recording and molecular biology techniques. Theoretical models are always based on a simplified interpretation. However, even imperfect models are of value; they provide useful information even if they are only approximations, and they allow the testing of hypotheses. In contrast to mathematics, biology is a versatile science, and to quote Jenkinson in his tribute to Heinz Schild (1987):

> "*...It is sobering that receptor pharmacology can already provide several examples of fortious agreement between experimental observations and the predictions of hypotheses we know now to be quite incorrect...*".

SECTION 2:
GENERAL METHODS

I COLLECTION AND MAINTENANCE OF THE PARASITES

Specimens of *Ascaris suum* were collected on a weekly basis from the local slaughter house. During transport, they were kept in a thermos flask filled with Locke's solution (solution A: see composition in V) at 37ºC. In the laboratory, they were kept in glass tanks filled with Locke's solution placed in a water bath set at 37ºC. The average concentration was ten parasites for 100-200ml. The solution was changed every day, and parasites were usually kept up to four days. Parasites, smaller than 10cm, were discarded because they were too small to be dissected without damaging the muscle cells. Very large parasites, longer than 30cm, were also rejected. As a matter of fact, large parasites did not produce good recordings. One possible reason is that the connective layer situated at the surface of the musculature was too thick: electrode penetration could not be achieved easily without damaging the cell, and diffusion of the drugs was impaired. Parasites showing no swimming behaviour when disturbed, were also discarded.

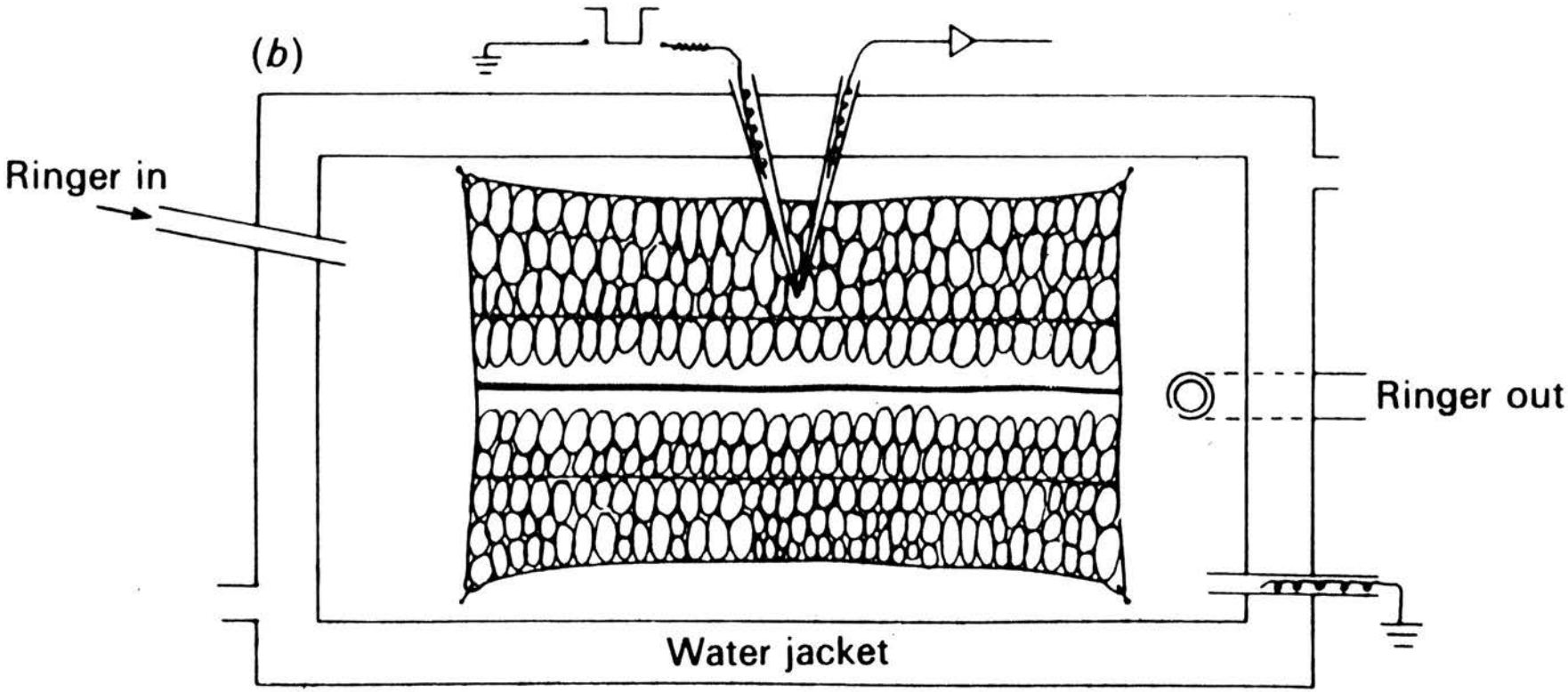
II DISSECTION

A cylindrical section of the worm about 2cm long was obtained from the anterior part of the parasite, approximatively 1-2cm anteriorly to the genital organs. A longitudinal incision was made under eye control, along one of the lateral line, and the intestine was gently removed (Martin, 1980). The flap preparation was then mounted in a Perpex™ experimental chamber situated in a Faraday cage placed on an antivibration table. The muscle flap was pinned cuticule down onto Sylgard™. The volume of the experimental chamber in the presence of the flap preparation, was approximatively 2.5ml.

For current- and voltage-clamp experiments: the chamber was continuously perfused with *Ascaris* ringer (solution B: see composition in V), at a rate of 10-15ml/mn. The chamber was maintained at 22ºC by a surrounding water jacket (Figure 7) connected to a water bath (Grant, BS15 bypass), situated outside the Faraday cage to eliminate electrical interference. The perfusion system was gravity fed. Polythene

Figure 7: Schematic diagram of a muscle flap preparation

A section of *Ascaris* is pinned down onto Sylgard™ in an experimental chamber and perfused by a Ringer solution. The chamber is fitted with a water-jacket to maintained the preparation at 22°C. Two microelectrodes are also illustrated. They are placed intracellularly under the control of a microscope. One was used for current injection, the other was used to record voltage responses.


(b)
Ringer in
Ringer out
Water jacket

tubing connected the reservoirs to the bath, and were enclosed with the tubing containing the circulating water of the water jacket, within a larger isolant tubing. Temperature was adjusted and checked at the water bath reservoir and resistance by a thermocouple. The muscle flap was then left for an hour to equilibrate with the ringer solution. During this equilibration phase, due to the low Ca concentration, the muscle usually relaxed, and had to be pinned down again before starting the experiment.

For patch-clamp experiments: the chamber was filled with solution D (see composition in V), in the presence of 350UI/ml collagenase. The temperature of the water bath was set at 35ºC. The enzyme treatment was stopped when vesicules started to form at the surface of the muscle cells membranes, which usually took 20-30mn. The muscle preparation was then washed several times with extracellular solution (solution E: see below).

III EXPERIMENTAL SET-UP

The experimental chamber was located on an antivibration table. The table consisted of a metallic frame bearing a concrete tray. The whole table was itself resting on a concrete base isolated from the rest of the floor. Electrical interference from external AC sources was prevented by a Faraday cage (approximatively 80cm x 70cm x 60cm), placed on the table. The light source was situated underneath the Perpex™ chamber, and the DC power supply (Weir 762-1), outside the cage. Microelectrodes position was achieved with two micromanipulators (Zeiss), and was controled under a dissecting microscope (Olympus, x25). A third micromanipulator (Prior) was installed for microionophoresis in voltage-clamp experiments.

IV MICROELECTRODES

1. Curent- and voltage-clamp microelectrodes

Electrodes were pulled on a vertical glass microelectrode puller (SRI 2001), from filamented glass tubing (Clarke Electromedical Instruments GC 120f-15: internal diameter=0.62mm, external diameter=1.20mm). Once pulled, microelectrodes were heated with a flame, and bent, before being back-filled with solution C: K acetate, 2M. They were then placed into Ag/AgCl pellet electrode holders. For current-clamp experiment, the resistance was typically between 10-30MΩ, for voltage-clamp

experiment, the resistance of the current injecting electrode was 10-15MΩ and the resistance of the recording electrode was 20-50MΩ. Ionophoresis electrodes had a resistance typically of 10-20MΩ.

2. Patch-clamp electrodes

Patch-electrodes were made from microhaematocrit capillary tubes (soft glass Garner glass 7052, internal diameter=1.15mm, external diameter=1.55mm), pulled on a two-stage vertical puller (David Kopf Instruments) and had a resistance of 1-5MΩ. The pipette tip diameter was checked under microscope (Vickers, x400), and was approximatively 0.5μm. Electrode tips were manually coated under eye control, with Sylgard™, to improve the frequency response (typically greater than 2kHz). The pipette tip was then polished, using a heated filament installed under the microscope lens (Vickers, x400), heat polishing was stopped when a physical change in the tip geometry could be detected. The tip was filled by suction with intracellular solution (solution F: see composition below), and then the pipette was backfilled with the same solution.

3. Bath electrodes

For current-clamp and voltage-clamp experiments, a Ag/AgCl bath electrode was used. For patch-clamp experiment, the bath electrode was made with an Agar 150mM KCl gel, set into a polythene tube to limit junction potentials. All connections were made with Ag/AgCl wires.

V SOLUTIONS

Solutions were kept in a cold room and up to one week; except solution C which was highly concentrated in K, and therefore unlikely to be contaminated by bacteria or fungi.

1. Solution A: Locke's solution, (mM): NaCl, 155; KCl, 5; $NaHCO_3$, 1.5; glucose, 5; $CaCl_2$, 2.

2. Solution B: *Ascaris* Ringer, (mM): NaCl, 135; KCl, 3.0; $MgCl_2$, 15.7; glucose, 3.0; TRIS, 5.0; pH adjusted to 7.6 with maleic acid.

3. Solution C: pipette solution, (M): Kacetate, 2.

4. Solution D: low-Ca extracellular solution, (mM): NaCl, 35; Na acetate, 105; $MgCl_2$, 2; KCl, 2; HEPES, 10; glucose,3; ascorbic acid, 2; pH=7.2 adjusted with NaOH.

5. Solution E: extracellular solution, (mM): NaCl, 35; Na acetate, 105; $MgCl_2$, 2; $CaCl_2$, 5; KCl, 2; HEPES, 10; glucose,3; ascorbic acid, 2; pH=7.2 adjusted with NaOH.

6. Solution F: intracellular solution, (mM): CsCl, 145; $MgCl_2$, 3; $CaCl_2$, 1; EGTA, 11; HEPES 10; pH=7.2 adjusted with CsOH.

VI CHEMICALS

Chemicals were kept according the recommendations mentioned on the data sheets.

1. Origin

All chemicals used for standard solutions were obtained from Sigma.

collagenase type IA was obtained from Sigma

γ-aminobutyric acid, glycine, β-alanine, δ-aminovaleric acid, ε-aminocaproic acid, guanidoacetic acid, β-guanidinopropionic acid, γ-guanidinobutyric acid, muscimol, nipecotic acid, imidazole-4-acetic acid, 3-aminopropane sulphonic acid, piperidine-4-sulphonic acid, (+)bicuculline, (-)bicuculline methiodide, picrotoxin, were obtained from Sigma.

Isonipecotic acid was obtained from Aldrich.

Trans-aminocrotonic acid, cis-aminocrotonic acid and ZAPA were obtained from Tocris Neuramine.

Thiomuscimol, isoguvacine, RS(±)dihydromuscimol, Homo-β-proline, THIP, were gifts from P.Krogsgaard-Larsen.

Baclofen was a gift from Pfizer.

d-tubocurarine was obtained from Wellcome

SR95103: 2-[3-carboxypropyl]-3-amino-4-methyl-6-phenyl pyridazinium chloride
SR95531: 2-[3-carboxypropyl]-3-amino-6 -p-methoxy phenyl pyridazinium chloride
SR42666: 2-[3-carboxypropyl]-3-amino-4-methyl-6-p-methoxy phenyl pyridazinium chloride
SR95133: 2-[3-carboxypropyl]-3-amino-4-methyl-6-α-naphtyl pyridazinium chloride
SR95132: 2-[3-carboxypropyl]-3-amino-4-phenyl-6-phenyl pyridazinium chloride
SR42627: 2-[3-carboxypropyl]-3-amino-4-methyl-6-p-methyl phenyl pyridazinium chloride
SR42640: 2-[3-carboxypropyl]-3-amino-5-methyl-6-phenyl pyridazinium chloride
were gifts from SANOFI.

NCS194-83: 2-[4-carboxybutyl]-3-amino-4-methyl-6-phenyl pyridazinium bromide
NCS247-90: 2-[3-carboxypropyl]-3-amino-4-ethyl-6-phenyl pyradizinium bromide
NCS248-90: 2-[3-sulfonylpropyl]-3-amino-(c5,6)-benzocyclohepta-pyridazinium
NCS249-90: 2-[3-sulphonylpropyl] -3-amino-4-methyl-6-phenyl pyridazinium
NCS250-90: 2-[3-sulphonylpropyl] -3-amino-4-ethyl-6-phenyl pyridazinium
NCS251-90: 2-[3-carboxypropyl]-3-amino-4-benzyl-6-phenyl pyridazinium bromide
NCS252-90: 2-[3-carboxypropyl]-3-amino-4-isopropyl-6-phenyl pyridazinium bromide
were obtained from Pr C.G.Wermuth and J.M.Sitamze from the laboratoire de neurochimie, CNRS Strasbourg.

RU5135: 3-α-hydroxy-1,6-imino-5-β-17-aza-androstan-11-one was a gift from Roussel-Uclaf

2. Solubilization

Compounds stable in solution, were solubilized in *Ascaris* Ringer (B) and the stock-solution were kept at -20ºC. Dilutions were made before the experiments and kept at 5ºC no more than a week.

RU5135 was dissolved in DMSO, 2% final concentration in Ringer (solution B).
Diazepam was dissolved in DMSO, 1% final concentration in Ringer (solution B).
NCS251-90 was dissolved in DMSO, 1% final concentration in Ringer (solution B).
(+)bicuculline was dissolved first in 0.1N HCl, and used within two hours.

VII ABBREVIATIONS

Chemicals:

AP-5=2-amino-5-phosphonopentanoic acid

3-APS=3-aminopropane sulphonic acid

CACA=Cis-aminocrotonic acid

DAVA=δ-aminovaleric acid

DHM= RS(±)-dihydromuscimol

DHP4S=dihydroxy piperidine sulphonic acid

DMPP=dimethylphenylpiperazinium

DMSO=dimethylsulphoxyde

EGTA=ethylene-bis (oxyethylenitrilo)-tetraacetic acid

β-GP=β-guanidinopropionic acid

GuAc=Guanidoacetic acid

H-β-P=Homo-β-proline

HEPES=4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid

IAA=Imidazole-4-acetic acid

INA=Isonipecotic acid

isoTHAZ=5,6,7,8-tetrahydro-4,11-isoxazolo [3,4-d] azepin-3-ol

4-me-TACA=4-methyl trans-aminocrotonic acid

5-NPB=5-nitro-2-(3-phenylpropylamino) benzoic acid

OH-GABA=hydroxy GABA

P4S=piperidine sulphonic acid

PTA=pentyltrimethylammonium

TACA=trans-aminocrotonic acid

TBPS=tert-butybicyclophosphothionate

TEA=tetraethylammonium

THIP=4,5,6,7 tetrahydroisoxazolo [5,4-c] pyridin-3-ol

TMA=trimethylammonium

TRIS=2-amino-2-(hydroxymethyl)-1,3-propane diol

ZAPA=Cis-3-[(aminoiminomethyl)thio] propanoic acid

Terms: CNS=central nervous system

Units:					
	A	Ampere			
	Å	Ångström			
	C	Coulomb			
	ºC	degree Celsius			
	Da	Dalton	Unit prefix:	k	kilo
	Hz	Hertz		M	mega
	K	Kelvin		m	milli
	M	molar		μ	micro
	m	metre		n	nano
	mol	mole		p	pico
	mn	minute			
	Ω	Ohm			
	S	Siemens			
	s	second			
	V	Volt			

Mathematical abbreviations:	log	logarithm base 10
	ln	logarithm neperian

Constants:	R	gas constant	8.314 $VCK^{-1}mol^{-1}$
	F	Faraday's constant	9.648 10^4 $Cmol^{-1}$

SECTION 3:

ACTIVITY OF SOME GABA AGONISTS AT THE *ASCARIS* MUSCLE GABA RECEPTOR.

INTRODUCTION

Ascaris suum is a suitable model for pharmacological studies on nematodes. By comparison with other nematodes, *Ascaris suum* is a large parasite, and is easy to collect from pigs slaughtered at the abattoir. Its close resemblance to *Ascaris lumbricoides* of humans, make *A. suum* a particular interesting model. Pharmacological studies of *Ascaris suum* muscle started as early as 1926, when Toscano-Rico recorded the muscle response following the application of santonin, chenopode oil, pilocarpine and nicotine. Later, Baldwin and Moyle (1947), developed a more elaborate method for testing potentials anthelminthics, where the drug was applied directly onto the muscle.

An interesting feature of *Ascaris suum*, is the presence of large muscle cells suitable for intracellular electrophysiological recording. After the first intracellular recordings of electrical activity from an *Ascaris* muscle cell (Jarman, 1959), Del Castillo et al. (1963a, 1963b, 1964b, 1964c) used intracellular recording techniques to study the mode of action of acetylcholine, GABA and piperazine.

From the pharmacological studies on *Ascaris suum* muscle, it was clear that acetylcholine and GABA receptors, were potential targets for the action of anthelminthics. Two well known anthelminthics have been shown to interact with GABA receptor in *Ascaris suum:* piperazine (Del Castillo et al., 1963b, 1964c; Martin, 1982, 1985;Holden-Dye et al., 1988, 1989) and ivermectin (Holden-Dye et al., 1988; Martin and Pennington, 1989). However, when this present study started, little was known of the pharmacology of GABA receptors in nematodes. It was decided that a study of the pharmacology of the *Ascaris* muscle GABA receptor, might facilitate the design of new anthelminthic drugs.

In this section, the action of GABA and various GABA agonists on the *Ascaris* muscle GABA receptor, was examined using a current-clamp technique. A modified Hill equation was used to describe and quantify the agonist dose-response relationships, and to compare the agonist potencies at the *Ascaris* muscle GABA receptor with agonist potencies at vertebrate GABA-A receptors. The modulatory effects of diazepam, pentobarbitone and dihydrocortisone on the GABA response were also investigated.

I METHODS

1. Muscle preparation

The muscle dissection is described in the general methods section. The preparation was bathed in a high-Cl, low-Ca solution (B). The chamber was surrounded by a water jacket which maintained the preparation at 22ºC. The temperature was chosen deliberately low since the electrical activity of the cells is temperature sensitive (Wann, 1987). At 22ºC, *Ascaris* muscle cells shows no spontaneous electrical activity which may interfere with the recording. In contrast to the electrical activity, the resting membrane potential presents little temperature sensitivity between 20 and 37ºC (Wann, 1987); this suggests that the mechanisms underlying the maintenance of the resting potential are not, or only weakly, affected by the temperature, and that changes in membrane potential can be safely interpreted at 22ºC. In the case of this preparation, it is also important that GABA or an agonist, does not produce indirect effects by an action on nearby cells or on the nervous system. A low-Ca and high-Mg Ringer (B) was used to impair synaptic transmission (Katz, 1969), and to avoid any interference from GABA receptors present on the nerve cells. A low-Ca solution has also the advantage of limitating muscle movement and the occurrence of Ca-activated Cl channels (Thorn and Martin, 1987). However electrical coupling between cells was not controlled, and was assumed to have little influence on the cell response.

2. Drug-application

GABA was initially bath-applied using a non-cumulative dose method, with washing of the preparation between applications. GABA produced, as reported previously, a dose-dependent hyperpolarization (Del Castillo et al., 1964b) and an increase in input conductance (Martin, 1980; Wann, 1987; Holden-Dye et al., 1988). However, one limitation of this technique was that recovery of the resting input conductance was often incomplete after application of high concentrations of GABA; this may have been due to slight movement of the muscle flap preparation producing a decrease in the leak resistance around the electrodes. It was found that recovery after cumulative application of GABA concentrations, without intermediate washing, was often more complete. The method involved gentle draining of the bath (volume 2.5ml), and then flushing the bath with 5ml of the next drug solution. The bath was not

perfused continuously because of the limited quantities of agonist available. Whether GABA concentrations were applied this way or by using a continuous perfusion, the results were the same. This method has the advantage of being economic in drug solution.

3. Current-clamp

3.1. Technique

Two microelectrodes were inserted into the bag region of a muscle cell (Figure 7). One pipette (resistance 10-20MΩ) was used to inject hyperpolarizing current pulses (40nA, 2s, 0.2Hz) under the control of a Digitimer 100 and a Digitimer 2533 isolated stimulator, the other pipette (resistance 10-20MΩ) was used to record membrane potential. Signals were initially recorded with a laboratory made amplifier (Martin, 1982) and monitored on a Tectronix 2210 oscilloscope and a Lectromed chart recorder. In later experiments, signals were recorded with an Axoclamp 2A (Axon Instruments) which permit the use of higher resistance current-injection electrodes: typically 20-50MΩ. The change in input conductance was measured and assumed to be directly related to the change in membrane conductance since the bag region of the muscle cell can be approximated by a sphere. In *Ascaris* muscle, the I/V relationship of the bag region of the muscle cell, is linear when hyperpolarized current is injected (Figure 8) (Martin, 1980). Similar results obtained in the presence of GABA (Martin, 1980), indicate that the GABA response is not voltage-sensitive. Therefore in *Ascaris*, input conductance can be determined from the response to hyperpolarizing current pulses and the agonist induced changes in input conductance is a useful practical method for studying the GABA receptor in *Ascaris* muscle.

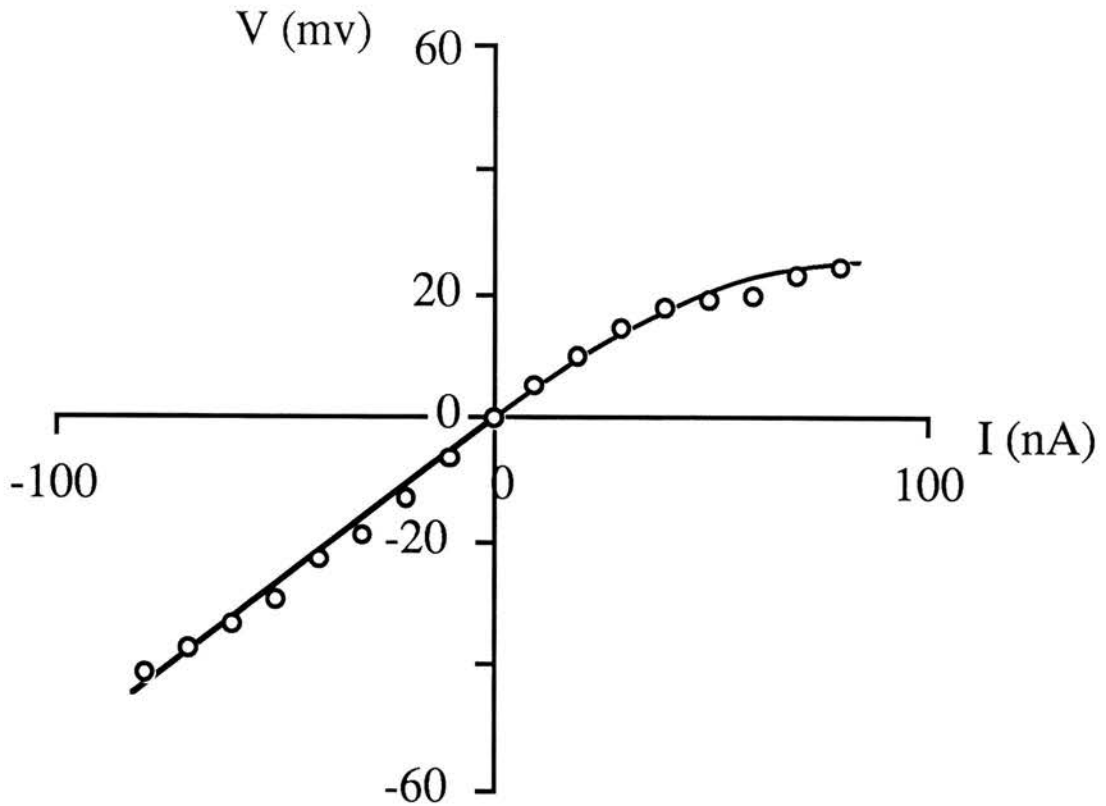
3.2. Data analysis

The input conductance of the bag was calculated directly from the amplitude of the plateau of the voltage response to the injected current pulse. The effects of GABA and agonists were determined by measuring the change in input conductance. Dose-response relationships were described by the modified Hill equation (Nistri and Constanti, 1979):

$$\Delta g = \frac{\Delta gmax}{1+(EC50 / [X_A])^N}$$

Figure 8: I/V relationship

This figure illustrates the typical I/V relationship obtained in *Ascaris* muscle cells using a current-clamp technique.
Abscissa: amplitude of the injected current.
Ordinate: change in steady-state membrane potential recorded during the current injection (pulse length 2s, amplitude 40nA).
The relation is linear at hyperpolarizing currents, but shows a delayed rectification at depolarizing currents. Input conductance (g) was calculated by injecting hyperpolarizing current pulses and using Ohm's law $g = \frac{I}{V}$, where I is the current amplitude and V the change in membrane potential.


V (mv)
60
20
0
-20
-60
-100
0
100
I (nA)

where Δg is the change in input conductance, Δgmax is the maximum response, EC50 is the concentration of GABA producing 50% of the maximum response, $[X_A]$ is the agonist concentration and N is the Hill coefficient. A non linear regression programme (patternsearch Colquhoun, 1971) was used to obtain the least square estimates for Δgmax, EC50 and N. Although there may be theoretical reasons why the Hill equation may not be appropriate, it provides a useful numerical approach for describing dose-response curves.

II RESULTS

The results reported here are based on the analysis of experiments conducted on 124 muscle cells from 80 preparations. Cells selected had resting input conductances between 1.2-4.0μS (2.66± 0.04μS, mean±SE, n=124). The recordings were rejected if the resting input conductance failed to return at least 80% of the control after washing GABA, or GABA-agonist, from the preparation. All the chemical structures illustrated in Figures 13 to 28, are from Krogsgaard-Larsen, Nielsen and Falch (1986), and Krogsgaard-Larsen (1988).

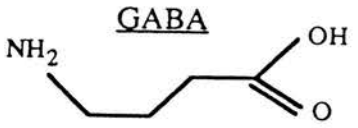
1.Effects of GABA

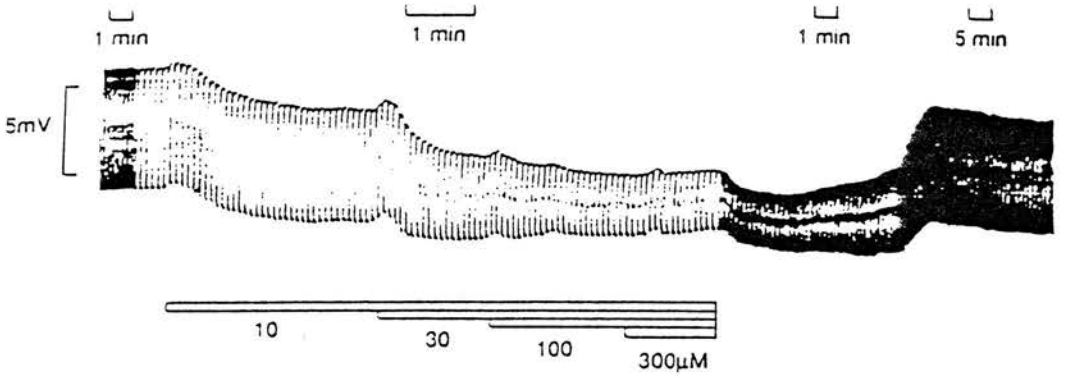
1.1. Dose-dependent hyperpolarization and increase in membrane conductance

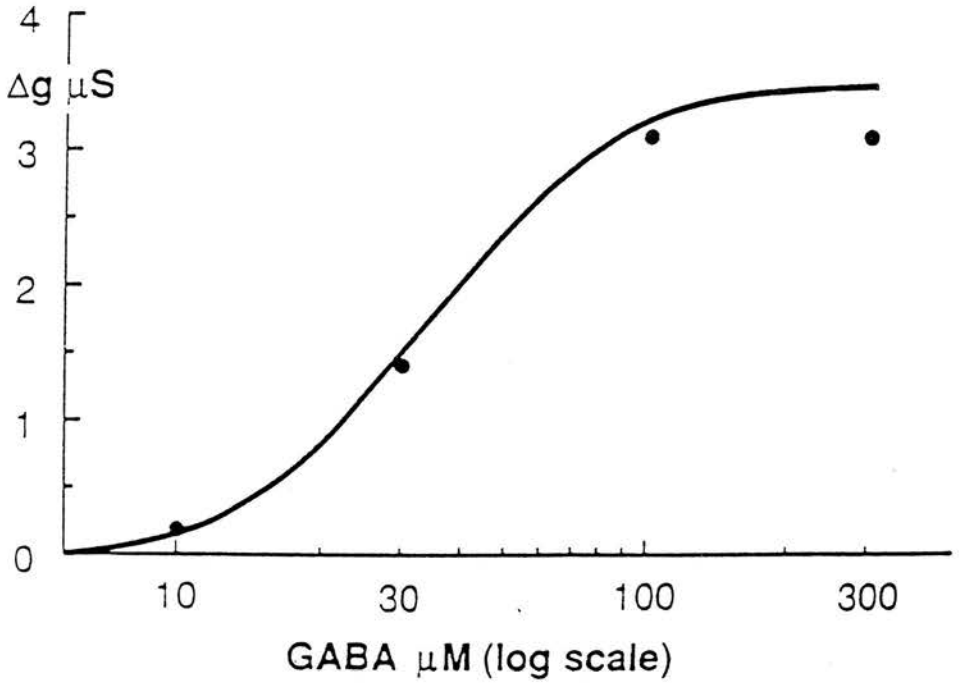
As described before, GABA applied at concentrations greater than 3μM, induced a dose-dependent hyperpolarization of the cell (Del Castillo et al., 1964b), and an increase in membrane input conductance (Martin, 1980; Holden-Dye et al., 1988) (Figure 9). The EC50 for GABA (EC50=30.2±1.9μM, mean±SE, n=38), was similar to the values previously reported (EC50=13μM, Martin, 1980; EC50=20.2±2.5μM, mean±SE, n=6, Holden-Dye et al., 1988). The Hill coefficient (N=2.7±0.2, mean±SE, n=38) varied from 1.5 to 4.9, and was greater than one in all the cells; this indicates positive cooperativity between GABA and its receptor, as previously suggested by Holden-Dye et al. (1988). Δgmax was subject to large variations from cell to cell (1.2 to 10.6μS). The mean value (Δgmax=4.15±0.32μS, mean±SE, n=38), was higher than the values reported by Holden-Dye et al., (1988) (Δgmax=2.58±0.39μS, mean±SE, n=6) and may reflect the difference in drug application techniques (bath application in the present study, and local perfusion, Holden-Dye et al., 1988). The

Figure 9: GABA cumulative dose-response relationship

The upper trace illustrates the change in input conductance during cumulative application of GABA. The width of the trace represents the change in membrane potential during the injection of hyperpolarizing current pulses, I=40nA (see methods). In this experiment, the resting input conductance was 3.0μS. In presence of GABA 10μM, Δg=0.2μS; GABA 30μM, Δg=1.4μS; GABA 100μM, Δg=3.1μS; GABA 300μM, Δg=3.1μS. The conductance returned to the resting level after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented by filled circles (●); the solid line represents the fitted modified Hill equation, Δgmax=3.5μS, EC50=32.6μM, N=2.6.


GABA
NH2
OH
O


1 min
1 min
1 min
5 min
5mV
10
30
100
300μM


4
Δg μS
3
2
1
0
10
30
100
300
GABA μM (log scale)

upper trace in Figure 9, illustrates a typical dose-response relationship obtained from one cell using cumulative concentrations of GABA and the corresponding fitted modified Hill equation.

For the various reasons discussed in the methods, GABA and other agonists, were applied in a cumulative manner, without intermediate washing. Both methods: cumulative and non-cumulative application of GABA, were compared. Values obtained for EC50 (non-cumulative application of GABA, EC50=24.9±3.3μM, mean±SE, n=3; cumulative application of GABA, EC50=30.2±1.8μM, mean±SE, n=38) and N (non-cumulative application of GABA, N=3.2±0.2, mean±SE, n=3; cumulative application of GABA, N=2.7±0.2, mean±SE, n=38), were similar. Δgmax seemed to be higher when non-cumulative concentrations of GABA were used (non-cumulative application of GABA, Δgmax=6.0±0.1μS, mean±SE, n=3; cumulative application of GABA, Δgmax=4.15±0.32μS, mean±SE, n=38), but the difference was not statistically significant. However, non-cumulative dose-response curves for GABA were obtained from only three cells. This contrast with the cumulative dose-response curves obtained from 38 cells, and may explain why the difference is not statistically significant.

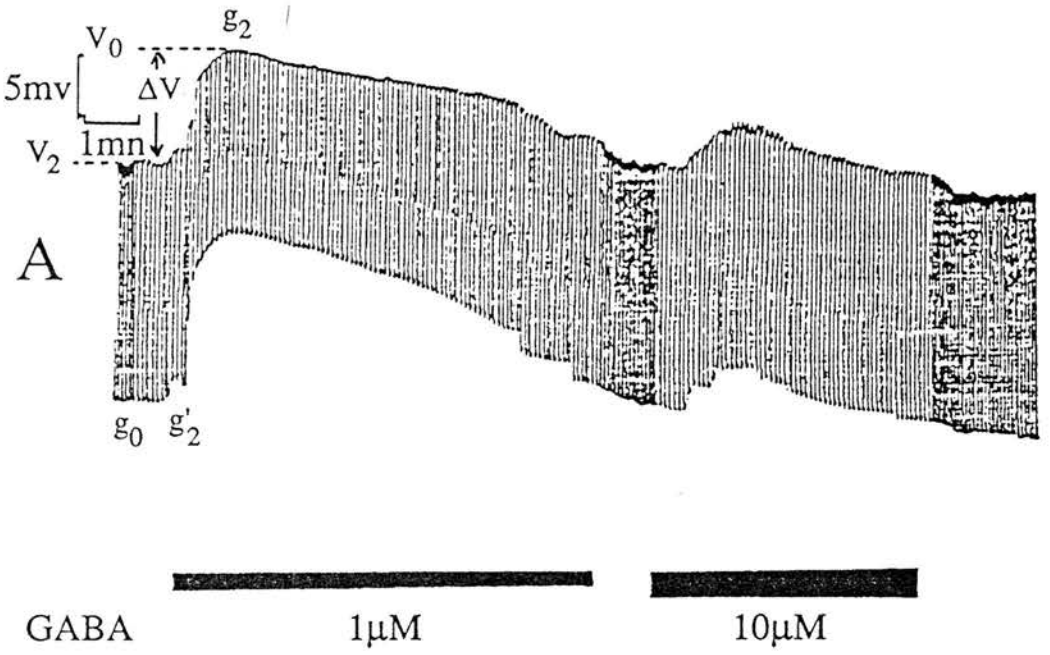
1.2. Transient depolarization following the application of low concentrations

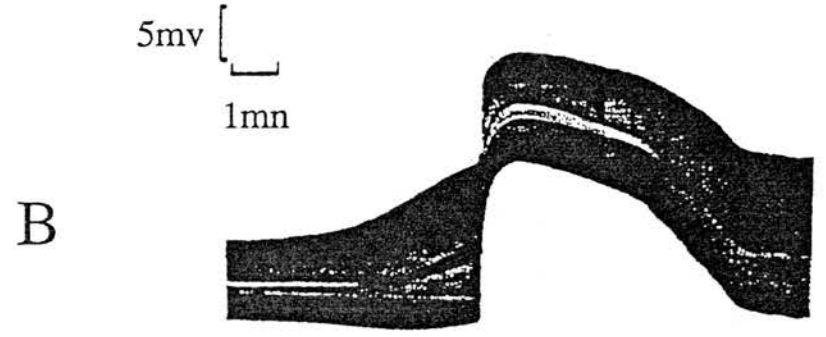
In some cells, low concentrations of GABA (1, 3 or 10μM) produced a transient membrane depolarization (Figure 10). The amplitude of the depolarization was very variable, 1-20mV. In the case of two successive applications of low GABA concentrations, the second depolarization was always smaller, suggesting some sort of desensitization (Figure 10). The depolarization occurred in the presence of low concentrations of GABA (Figure 10A), or at the end of the recovery phases following application of a single concentration or cumulative concentrations of GABA (Figure 10B). The onset of these depolarizations was very rapid and seemed to involve a voltage-sensitive conductance. On recovery the membrane slowly repolarized, and then returned abruptly to its original resting value. This was possibly the consequence of the closing of voltage-sensitive channels. During the membrane depolarization, the membrane input conductance increased to a maximum at the peak of depolarization, and progressively decreased to the original value of the resting conductance. When the depolarization was only a few millivolts, the membrane conductance did not change (Figure 10, A) or even, slightly decreased. In the recorded cell illustrated in Figure 10, the peak depolarization was of 7mV ($\Delta V=V_2-V_0=7mV$). The resting conductance was: $g_0=2.58\mu S$ and the conductance at the peak depolarization was: $g_2=3.40\mu S$. Just

Figure 10: Depolarizations following the application of low GABA concentrations or during the recovery phase after cumulative application of GABA (I=40nA).

A. Low concentration of GABA: On this particular cell GABA 1μM produced a transient depolarization of the membrane of 7mV at its peak. The resting membrane conductance was: go=2.58μS; at the lowest membrane potential value the membrane input conductance increased to: g=3.40μS. The next application of GABA: 10μM produced a smaller depolarization, less than 5mV; the membrane.conductance decreased slightly: Δg=0.1μS, and some spontaneous electrical activity was detected.

B. Depolarization during the recovery phase: In both cases the depolarization occurred at the end of the recovery phase. The measure of the depolarization amplitude was not precise since the response was often out of the range of the pen recorder and had to be manually adjusted.


V_0
g_2
5mv
ΔV
1mn
V_2
A
g_0
g'_2
GABA
1μM
10μM


5mv
1mn
B

before the peak depolarization, the membrane conductance decreased transiently to: g_2'=2.50μS. If the current-voltage relationship is assumed to be linear at depolarizing potentials, the reversal potential of the ion conductance(s) involved in the depolarization can be estimated by extrapolation:

$$E_{rev} = 1/g_0 \cdot \frac{\Delta V}{1/g_2 - 1/g_0}, \qquad \text{here } E_{rev}=+29\text{mV}.$$

However, because its low frequency and the apparent desensitization, this phenomenon was not further investigated.

1.3. Absence of effects of diazepam, pentobarbitone and cortisol on the GABA response

Diazepam (1μM), pentobarbitone (10μM) and cortisol (10μM) were tested on GABA dose-response curves. Control GABA dose-response curves were systematically obtained before or after application of the test drug. In two experiments, diazepam (1μM) had no effect on EC50, Δgmax or N (Table 8, Figure 11). Pentobarbitone (10μM) (n=3 experiments) and cortisol (10μM) (n=2 experiments), had no effect on EC50, N (Table 8, Figure 12). The calculated mean for Δgmax was higher in the presence of cortisol or pentobarbitone, but the difference from controls did not reach statistical significance, partly because of the small number of experiments and partly because of the difficulty in measuring high conductances with accuracy.

2. Inactive compounds

The following compounds had no effects on the membrane conductance at a concentration of 1mM: β-alanine (n=3), glycine (n=2), ε-aminocaproic acid (n=2), nipecotic acid (n=2), 3-APS (n=2), P4S (n=2) and baclofen (n=2) (Figure 13 and 14). These results are in agreement with a previous report (Holden-Dye et al., 1988).

Thiomuscimol (300μM), γ-guanidobutyric acid (300μM) and THIP (1mM), produced only a small increase in membrane conductance and a small hyperpolarization (Figure 15 and 16). To obtain full dose-response curves would have required very high agonist concentrations. They were not determined for this reason. Among these weak agonists THIP had been suggested to act as a partial agonist in *Ascaris* (Holden-Dye et al., 1988), but for the reasons stated above, these findings could not be confirmed or rejected.

Figure 11: Effect of diazepam (1μM) on dose-dependent responses to GABA.

A. Control GABA response: resting membrane conductance, g_0=3.3; GABA 3μM, Δg=0μS; 10μM, Δg=0.3μS; 30μM, Δg=2.8μS; 100μM, Δg=4.7μS; 300μM, Δg=5.4μS; recovery of 97% of the resting membrane conductance. The fitted modified Hill equation gives: Δgmax=5.6μS, EC50=22.4μM, N=1.6. (Injected current: I=40nA)

B. In the presence of 1μM diazepam, g_0=3.4μS; GABA 10μM, Δg=1.3μS; 30μM, Δg=3.3μS; 100μM, Δg=5.4μS; 300μM, Δg=5.4μM. The fitted modified Hill equation gives: Δgmax=5.2μS, EC50=28.8μM, N=2.3. (Injected current: I=40nA). Diazepam (1μM) has no apparent effect on dose-dependent GABA response.

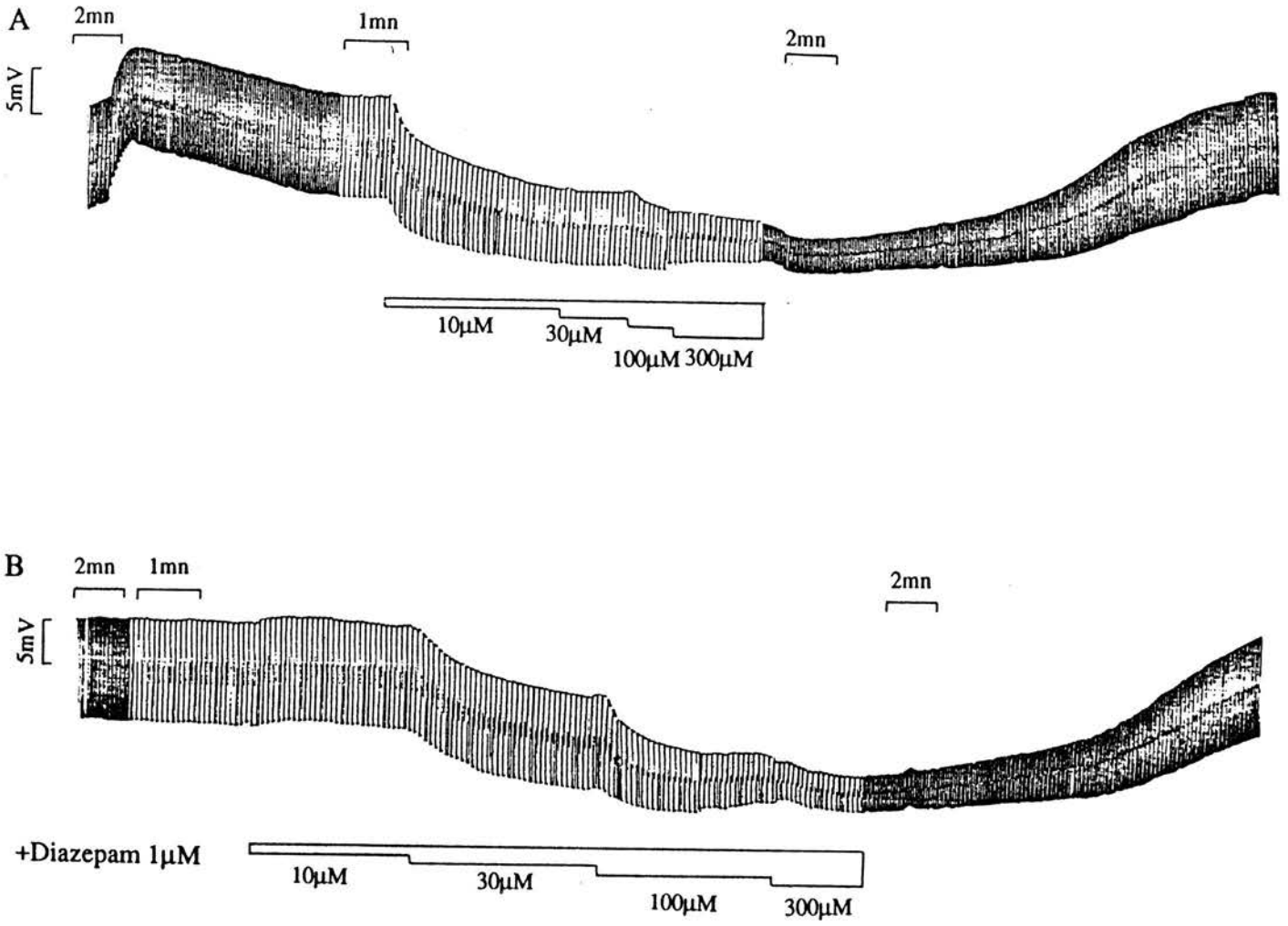

A
2mn
1mn
2mn
5mV
10μM
30μM
100μM
300μM
B
2mn
1mn
2mn
5mV
+Diazepam 1μM
10μM
30μM
100μM
300μM

Figure 12: Effect of pentobarbitone (10μM) and cortisol (10μM) on dose-dependent responses to GABA.

A. Control GABA response:g_0=2.6μS; GABA 3μM, Δg=0.1μS; 10μM, Δg=0.6μS; 30μM, Δg=3.3μS; 100μM, Δg=5.4μS; 300μM, Δg=5.4μS; 97% recovery of the resting membrane conductance. The fitted modified Hill equation gives: Δgmax=5.5μS, EC50=25.0μM, N=2.3. (Injected current: I=40nA)

B. In the presence of 10μM pentobarbitone; g0=2.7μS; GABA 3μM, Δg=0.1μS; 10μM, Δg=0.4μS; 30μM, Δg=3.7μS; 100μM, Δg=6.0μS; 300μM, Δg=6.3μS; 100% recovery of the resting membrane conductance. The fitted modified Hill equation gives: Δgmax=6.2μS, EC50=26.0μM, N=2.7. (Injected current: I=40nA). Pentobarbitone (10μM) has no effect on EC50, but slightly increased Δgmax.

C. In the presence of 10μM cortisol,; g0=2.7μS; GABA 3μM, Δg=0.5μS; 10μM, Δg=0.8μS; 30μM, Δg=3.7μS; 100μM, Δg=6.0μS; 300μM, Δg=6.3μS; 80% recovery of the resting membrane conductance. The fitted modified Hill equation gives: Δgmax=6.4μS, EC50=25.6μM, N=2.7. (Injected current: I=40nA). Cortisol (10μM) has no effect on EC50, but slightly increased Δgmax.

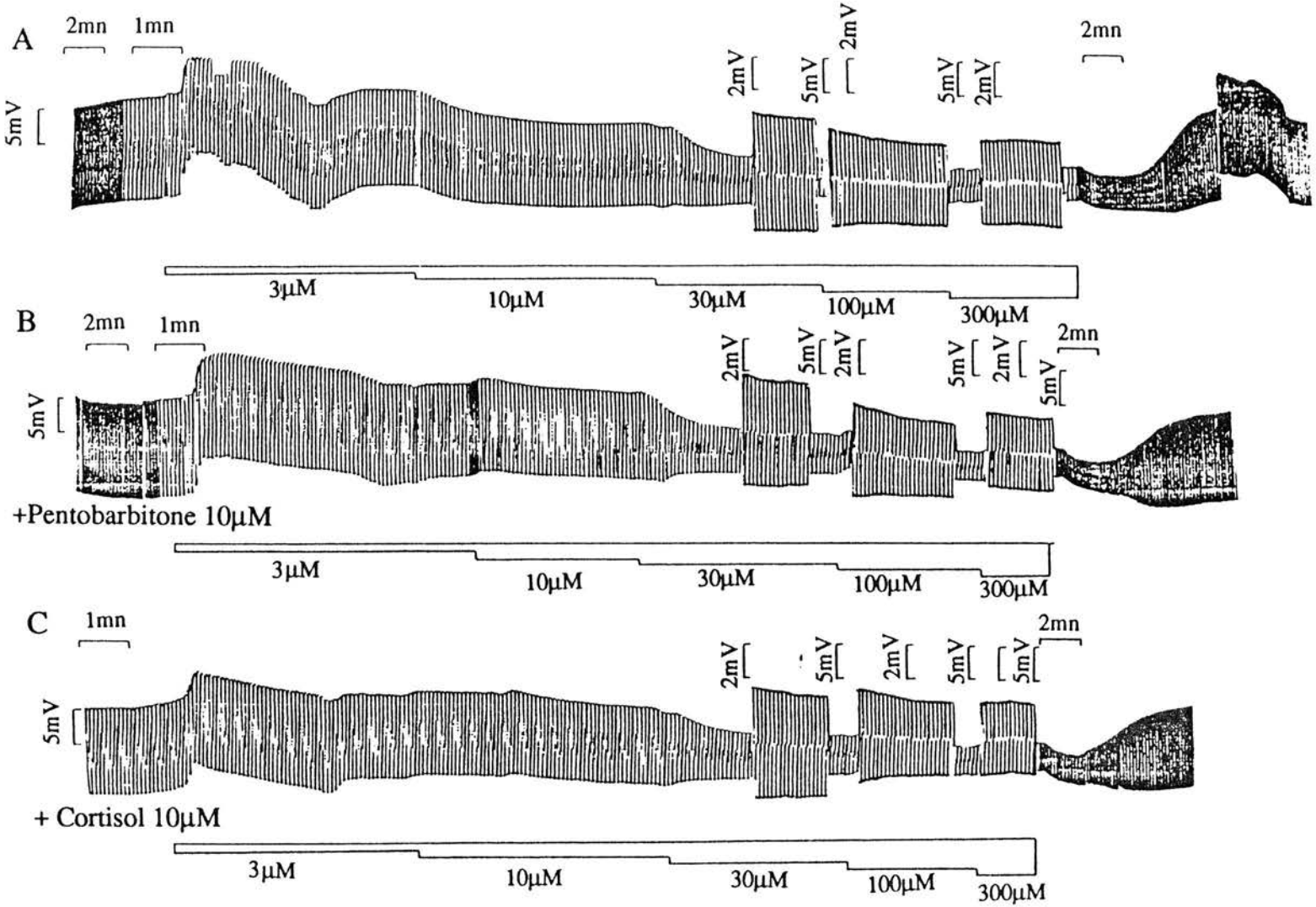

A
2mn
1mn
5mV
2mV
5mV
2mV
5mV
2mV
2mn
3μM
10μM
30μM
100μM
300μM
B
2mn
1mn
5mV
2mV
5mV
2mV
5mV
2mV
5mV
2mn
+Pentobarbitone 10μM
3μM
10μM
30μM
100μM
300μM
C
1mn
5mV
2mV
5mV
2mV
5mV
5mV
2mn
+ Cortisol 10μM
3μM
10μM
30μM
100μM
300μM

Table 8: Effect of diazepam (1μM), pentobarbitone (10μM) and cortisol (10μM) on GABA dose-response curves: EC50, Δmax and N.

	Δgmax (μS)	EC50 (μM)	N
Control (n=2)	6.30±1.10	28.6±0.2	2.2±0.5
+ diazepam (1μM)	6.05±0.45	21.0±1.35	2.1±0.5
Control (n=3)	4.60±0.75	32.4±5.2	2.4±0.5
+ pentobarbitone (10μM)	5.53±0.57	30.2±2.9	2.5±0.1
Control (n=3)	5.70±0.20	24.1±0.8	2.3±0.1
+ cortisol (10μM)	6.35±0.05	24.4±1.1	2.2±0.3

Figure 13: Inactivity of glycine, 3-APS, and β-alanine on membrane conductance

A. The resting membrane conductance was, g_0=2.8μS, in the presence of 1mM glycine, the membrane conductance was not altered: Δg=0μS. (Injected current: I=40nA)

B. Same cell, resting membrane conductance, g_0=2.8μS, in the presence of 1mM 3APS Δg=0μS. (Injected current: I=40nA)

C. Same cell, resting membrane conductance g_0=2.8μS, in the presence of 1mM β-alanine Δg=0. The small depolarization seen after the application of β-alanine was an artefact due to the drug application. (Injected current: I=40nA)

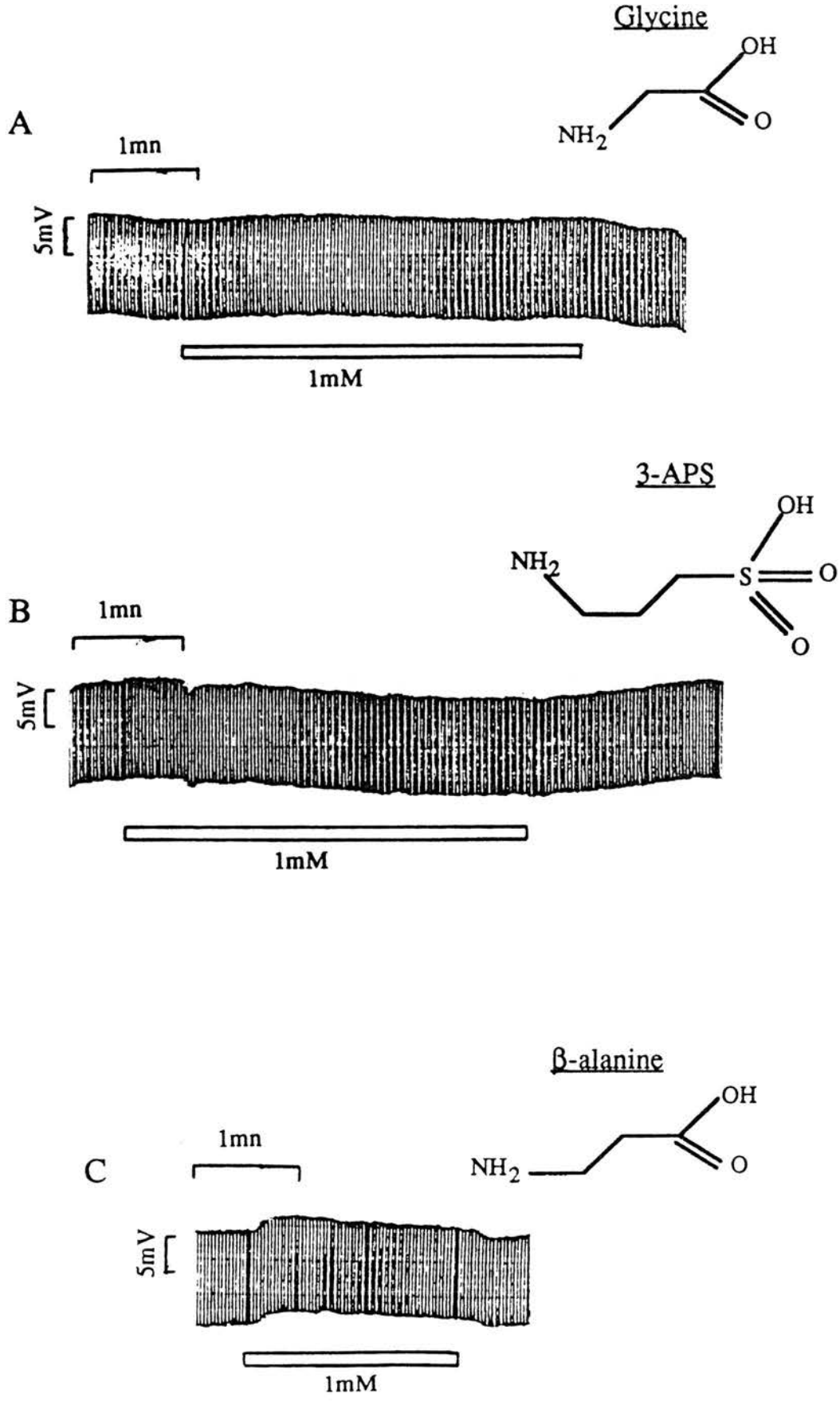
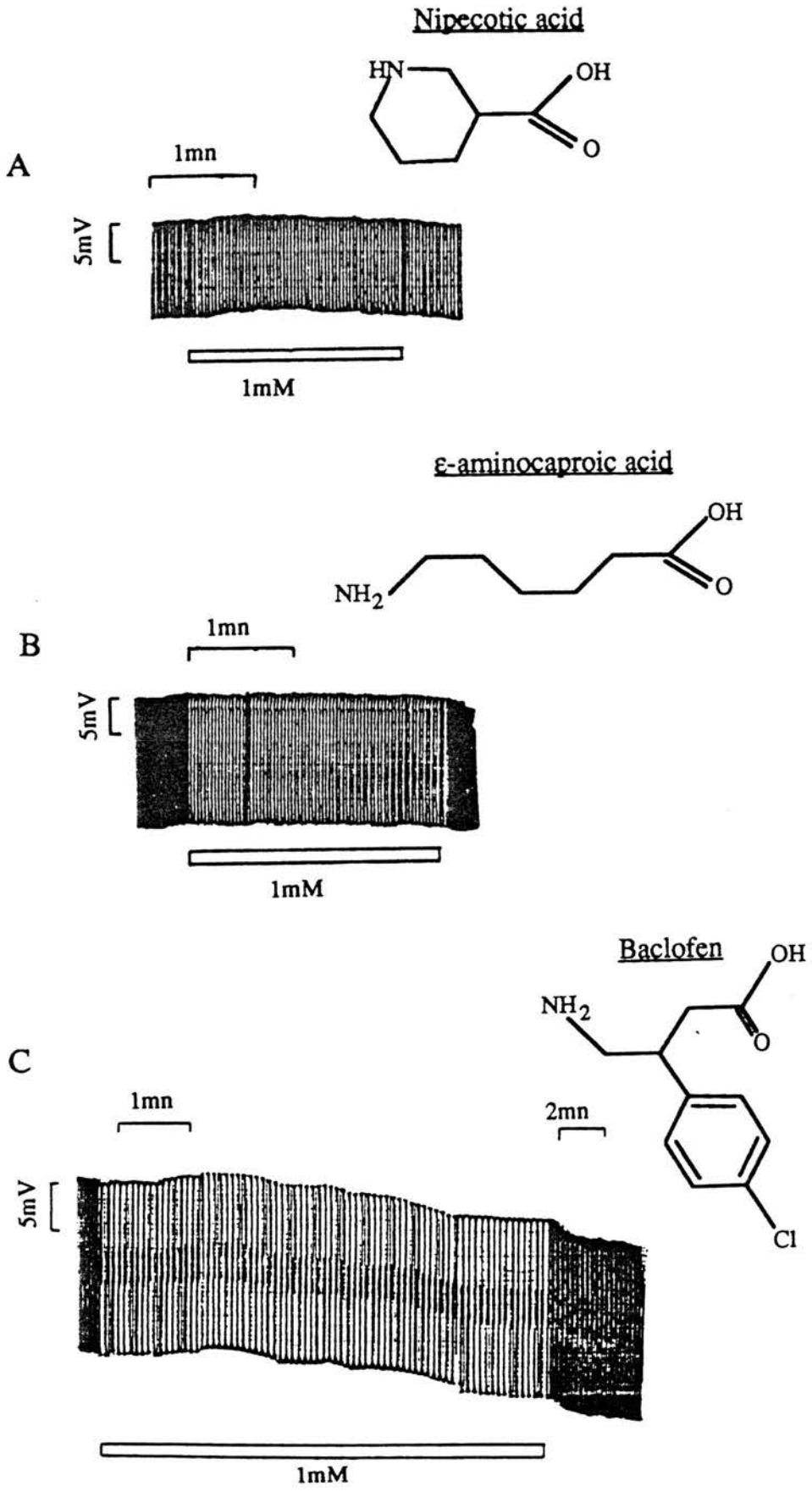

Glycine
OH
O
NH2
A
1mn
5mV
1mM
3-APS
OH
NH2
S
O
O
B
1mn
5mV
1mM
β-alanine
OH
NH2
O
C
1mn
5mV
1mM

Figure 14: Inactivity of nipecotic acid, ε-aminocaproic acid and baclofen on membrane conductance.

A. The resting membrane conductance was, g_0=2.8μS, in the presence of 1mM nipecotic acid, Δg=0μS. (Injected current: I=40nA)

B. Resting membrane conductance, g_0=2.1μS, in the presence of 1mM ε-aminocaproic acid, the membrane conductance remained unchanged, Δg=0μS. (Injected current: I=40nA)

C. Resting membrane conductance, g_0=3.2μS, in the presence of 1mM baclofen, the membrane conductance remained unchanged, Δg=0. (Injected current: I=40nA). The apparent hyperpolarization was not related to the presence of baclofen but rather to an instability of the membrane potential in this particular cell. Instability of the membrane potential has to be related with the spontaneous electrical activity of the muscle cells. It is often seen when the muscle has not been left long enough to equilibrate with the medium before starting recording.


Nipecotic acid
HN
OH
O
A
1mn
5mV
1mM
ε-aminocaproic acid
OH
NH2
O
B
1mn
5mV
1mM
Baclofen
OH
NH2
O
C
1mn
2mn
5mV
Cl
1mM

3. Active compounds

All the results presented here are summarized in Table 9 and illustrated in Figures 13 to 28. For all the agonists tested (except imidazole-4-acetic acid), Δgmax and N are not significantly different from the values obtained with GABA (t-test), suggesting that they act in a similar manner at the *Ascaris* GABA receptor. They appear to be full agonists having the same stoichiometry.

3.1. Hill coefficient

For each agonist, the mean value for the Hill coefficient was greater than one and indicated positive cooperativity between the agonist and the receptor. This result is not surprising, since Hill coefficients greater than one have been obtained with GABA and GABA agonists in various vertebrate and invertebrate preparations (see Nistri and Constanti, 1979, for review). Recently, the purification, cloning and expression, of the vertebrate GABA-A complex have shown the existence of two subunits able to bind the GABA molecule (Schofield et al., 1987), and reinforce the hypothesis that at least two molecules of GABA are required to activate the channel.

3.2. Δgmax

The maximal responses obtained with each agonist were subject to large variation from cell to cell (varying from 0.7 to 22.5μS), and mainly reflected the differences in size of the muscle cells and/or their physiological state. No significant variation was observed between the different agonists tested (except imidazole-4-acetic acid), and GABA. Partial agonists are expected to produce a smaller Δgmax. β-guanidinopropionic acid, δ-aminovaleric acid and cis-aminocrotonic acid, were suggested to act as partial agonists (Holden-Dye et al., 1988), but there is no evidence for this here. Δgmax for: β-guanidinopropionic acid (4.30±0.82μS, mean±SE, n=4), δ-aminovaleric acid (3.77±0.94μS, mean±SE, n=4) and cis-aminocrotonic acid (Δgmax=6.83±2.84μM, mean±SE, n=3), are not significantly different from Δgmax for GABA (4.15±0.32μS. mean±SE, n=38). It is nevertheless interesting to note that, the rigid analogues of GABA: muscimol (Δgmax=2.48±0.77μS, mean±SE, n=5), isoguvacine (Δgmax=3.60±1.00μS, mean±SE, n=4), homo-β-proline (Δgmax=2.96±0.39μS, mean±SE, n=3) and isonipecotic acid (Δgmax=2.50±0.70μS, mean±SE, n=6), had a lower calculated mean for Δgmax than for GABA or other agonists, but if the difference did not reach statistical significance. Only imidazole-4-

acetic acid showed a significantly greater Δgmax than GABA (Δgmax=6.77±1.46μS, mean±SE, n=4, t-test p≤0.05). However, Δgmax should be carefully interpreted, because of the possible desensitization of the GABA receptors and the errors associated with the measurement of high conductance values (see discussion).

3.3. EC50

Table 9 illustrates the various potencies of the different agonists tested, and compares their relative potencies in *Ascaris* to their relative potencies in displacing [^{3}H]GABA from rat brain membranes (Krogsgaard-Larsen, 1988) or in eliciting contractions of guinea-pig ileum (Allan et al., 1986). Dihydromuscimol is the only compound more potent than GABA itself (EC50=8.3±1.5μM, mean±SE, n=5). A racemic mixture of RS(±)dihydromuscimol was used in this present study, but according to Holden-Dye et al., (1989), the (+) isomer is more potent than the (-) isomer; this is similar to the vertebrate central GABA-A receptor (Krogsgaard-Larsen, 1988). The isothiouronium derivative of GABA: ZAPA (EC50=24.1±4.6μM, mean±SE, n=3) was not significantly more potent than GABA. This observation agrees with the previous report of Holden-Dye et al., (1989). All other agonists tested were at least half less potent than GABA; even the potent GABA-A agonists, muscimol (EC50=82.5±20.4μM, mean±SE, n=5) and isoguvacine (EC50=124.2±10.1μM, mean±SE, n=4). Their relative potencies are similar to those reported by Holden-Dye et al., (1989), (See Table 9).

Figure 15: Thiomuscimol

The upper trace illustrates the change in input conductance during cumulative application of thiomuscimol (Injected current: I=40nA). In this experiment, the resting input conductance was 2.1μS. In presence of thiomuscimol, 10μM, Δg=0.0μS; 30μM, Δg=0.0μS; 100μM, Δg=0.0μS; 300μM, Δg=0.3μS; 1000μM, Δg=1.0μS. The conductance returned to 100% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship.

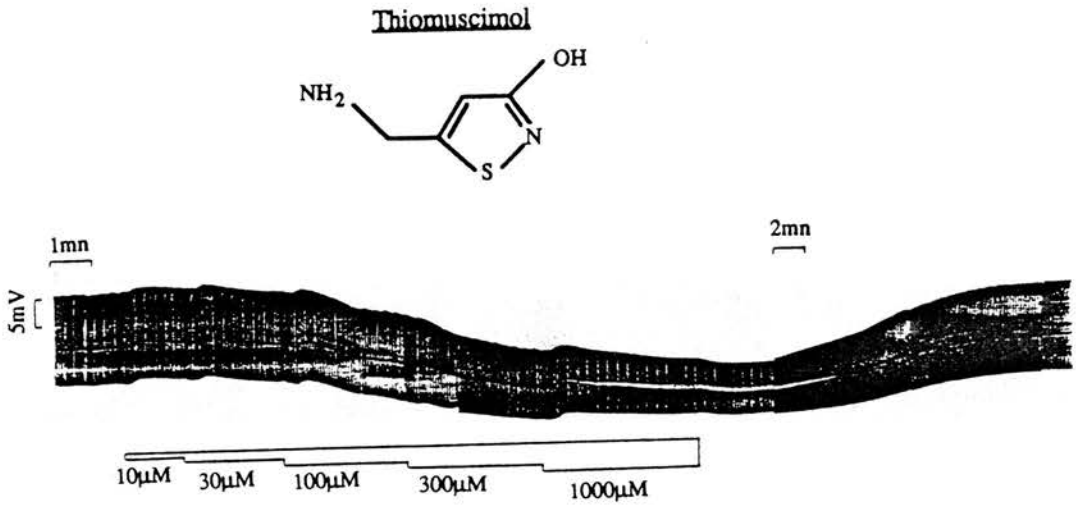

Thiomuscimol
OH
NH2
N
S
1mn
2mn
5mV
10µM
30µM
100µM
300µM
1000µM

Figure 16: γ-guanidinobutyric acid and THIP

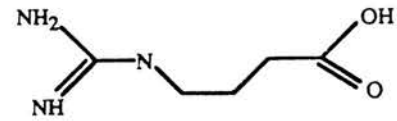
γ-guanidinobutyric acid

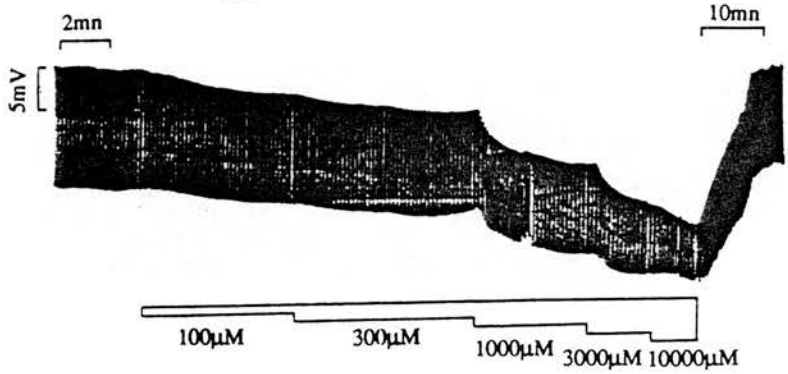
The upper trace illustrates the change in input conductance during cumulative application of γ-guanidinobutyric acid (Injected current: I=40nA). In this experiment, the resting input conductance was 2.8μS. In the presence of γ-guanidinobutyric acid, 100μM, Δg=0.4μS; 300μM, Δg=0.5μS; 1000μM, Δg=1.0μS; 3000μM, Δg=2.2μS; 1000μM, Δg=7.2μS. The conductance returned to 80% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment.

THIP

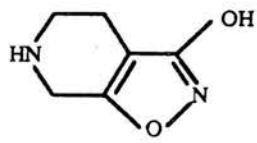
The upper trace illustrates the change in input conductance during cumulative application of THIP (Injected current: I=40nA). In this experiment, the resting input conductance was 2.3μS. In the presence of THIP, 10μM, Δg=0.0μS; 30μM, Δg=0.0μS; 100μM, Δg=0.0μS; 300μM, Δg=0.0μS; 1000μM, Δg=1.0μS. The conductance returned to 100% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment.

γ-guanidinobutyric acid





THIP



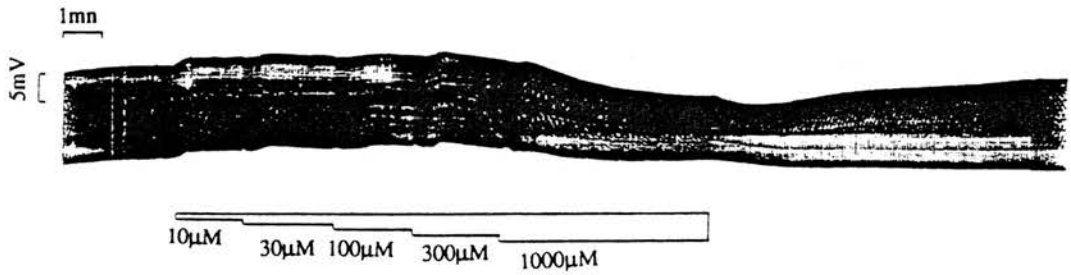
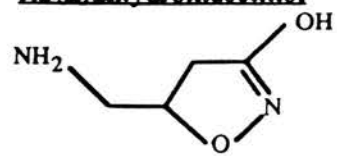
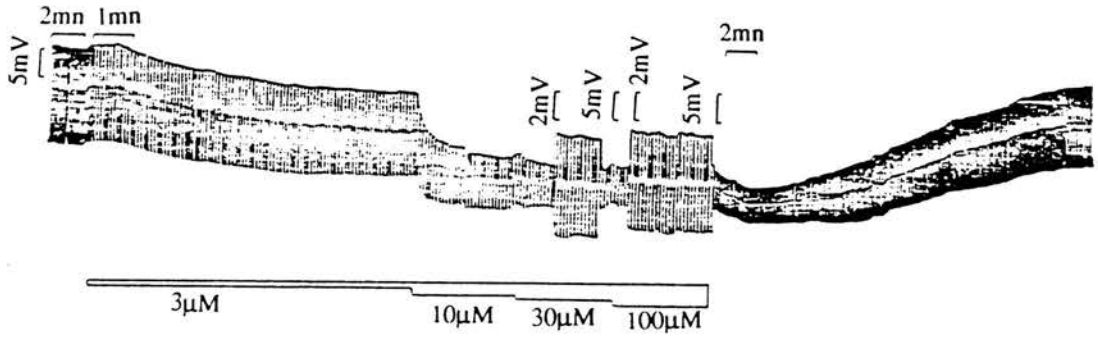


Figure 17: Dihydromuscimol

The upper trace illustrates the change in input conductance during cumulative application of DHM (Injected current: I=40nA). In this experiment, the resting input conductance was 2.3μS. In the presence of DHM: 3μM, Δg=0.4μS; 10μM, Δg=0.9μS; 30μM, Δg=3.2μS; 100μM, Δg=3.2μS. The conductance returned to 85% of the resting value after washing. The chart speed and gain were altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented by (■); the solid line represents the fitted modified Hill equation, Δgmax=3.3μS, EC50=12.9μM, N=3.7.

RS(±)dihydromuscimol





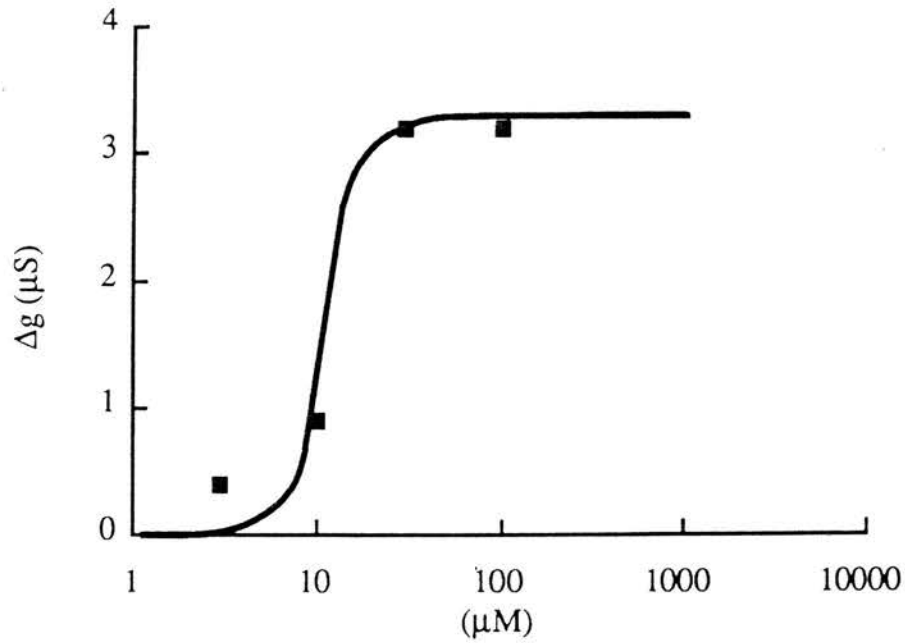
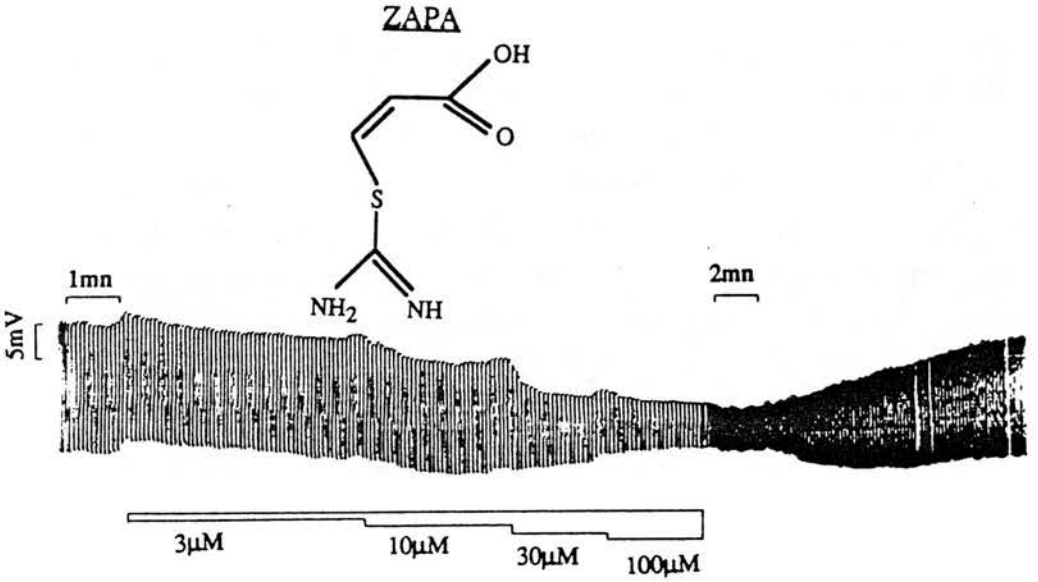


Figure 18: ZAPA

The upper trace illustrates the change in input conductance during cumulative application of ZAPA (Injected current: I=40nA). In this experiment, the resting input conductance was 2.1μS. In the presence of ZAPA 3μM, Δg=0.1μS; 10μM, Δg=0.3μS; 30μM, Δg=1.9μS; 100μM, Δg=3.8μS. The conductance returned to 85% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=4.0μS, EC50=31.0μM, N=2.3.


ZAPA
OH
O
S
NH2
NH
1mn
2mn
5mV
3μM
10μM
30μM
100μM

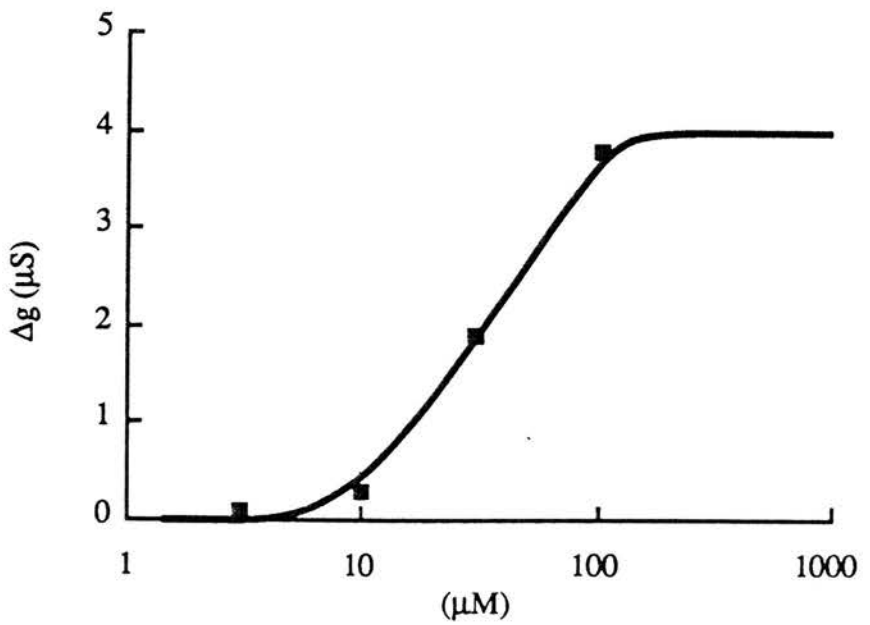
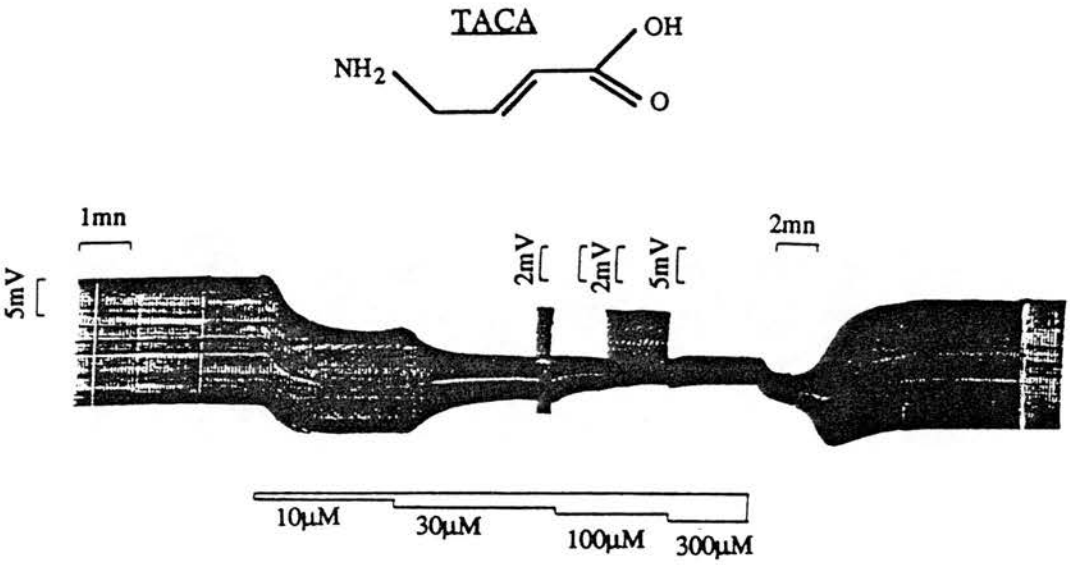

5
4
3
2
1
0
Δg (μS)
1
10
100
1000
(μM)

Figure 19: Trans-aminocrotonic acid

The upper trace illustrates the change in input conductance during cumulative application of TACA (Injected current: I=40nA). In this experiment, the resting input conductance was 2.2μS. In the presence of TACA 10μM, Δg=0.1μS; 30μM, Δg=3.9μS; 100μM, Δg=6.5μS; 300μM, Δg=7.3μS. The conductance returned to 100% of the resting value after washing. The chart speed and gain were altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (●); the solid line represents the fitted modified Hill equation, Δgmax=7.3μS, EC50=28.1μM, N=3.0.


TACA
OH
NH2
O
1mn
2mn
5mV
2mV
2mV
5mV
10μM
30μM
100μM
300μM

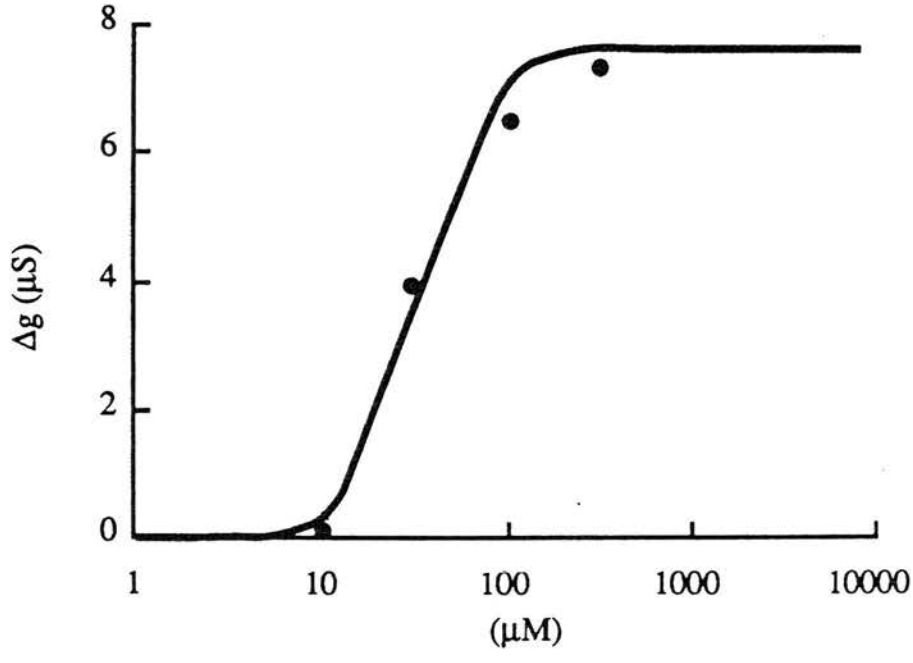
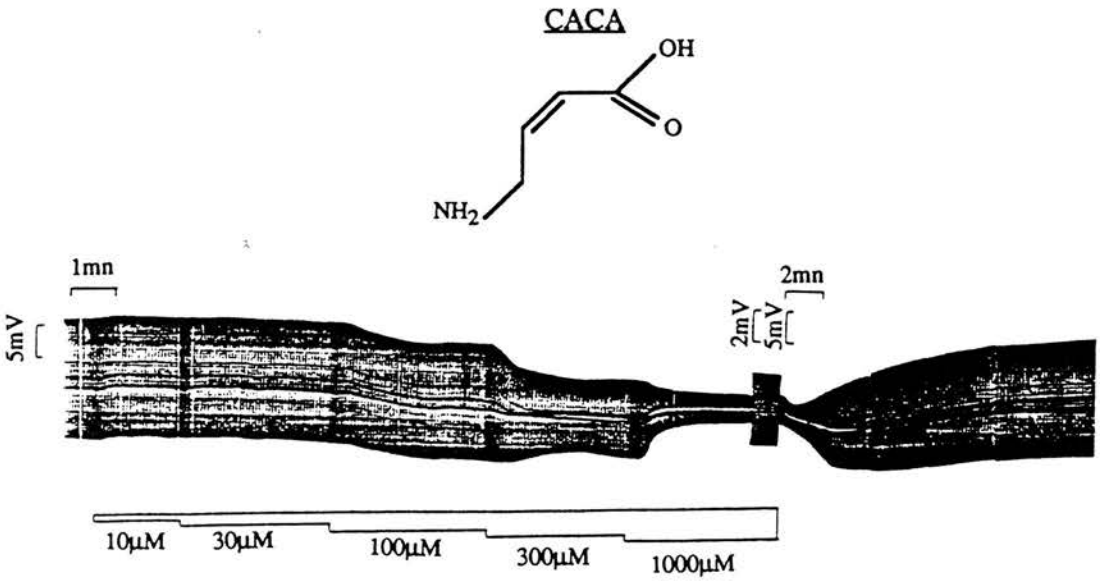

8
6
4
2
0
Δg (μS)
1
10
100
1000
10000
(μM)

Figure 20: Cis-aminocrotonic acid

The upper trace illustrates the change in input conductance during cumulative application of CACA (Injected current: I=40nA). In this experiment, the resting input conductance was 2.1μS (same cell as for TACA). In the presence of CACA, 10μM, Δg=0.0μS; 30μM, Δg=0.0μS; 100μM, Δg=0.1μS; 300μM, Δg=1.2μS; 1000μM, Δg=7.2μS. The conductance returned to 91% of the resting value after washing. The chart speed and gain were altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=7.8μS, EC50=320.6μM, N=3.3.


CACA
OH
O
NH2
1mn
2mn
5mV
2mV
5mV
10µM
30µM
100µM
300µM
1000µM

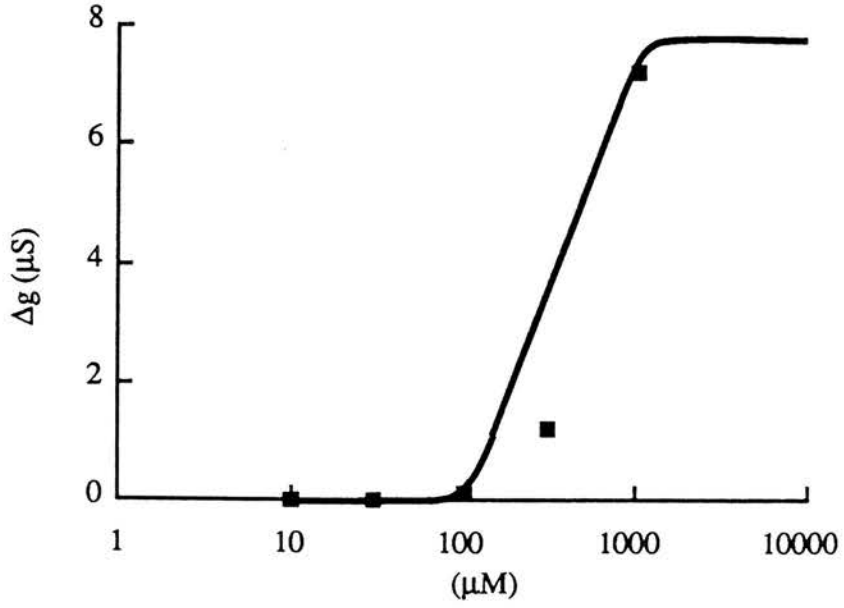
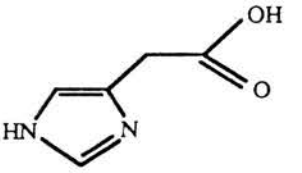
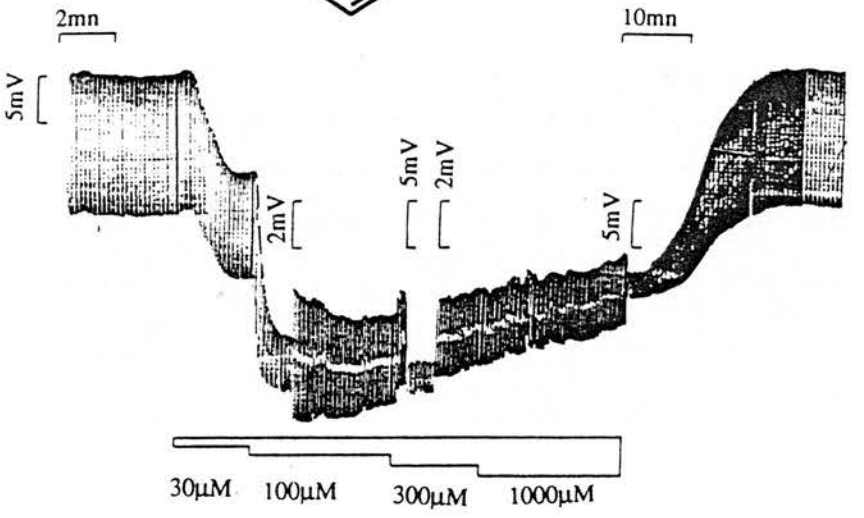

8
6
4
2
0
Δg (µS)
1
10
100
1000
10000
(µM)

Figure 21: Imidazole-4-acetic acid

The upper trace illustrates the change in input conductance during cumulative application of IAA (Injected current: I=40nA). In this experiment, the resting input conductance was 2.7μS. In the presence of IAA 30μM, Δg=0.3μS; 100μM, Δg=7.4μS; 300μM, Δg=9.9μS; 1000μM, Δg=9.9μS. The conductance returned to 100% of the resting value after washing. The chart speed and gain were altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=9.9μS, EC50=75.2μM, N=3.8.

Imidazole-4-acetic acid





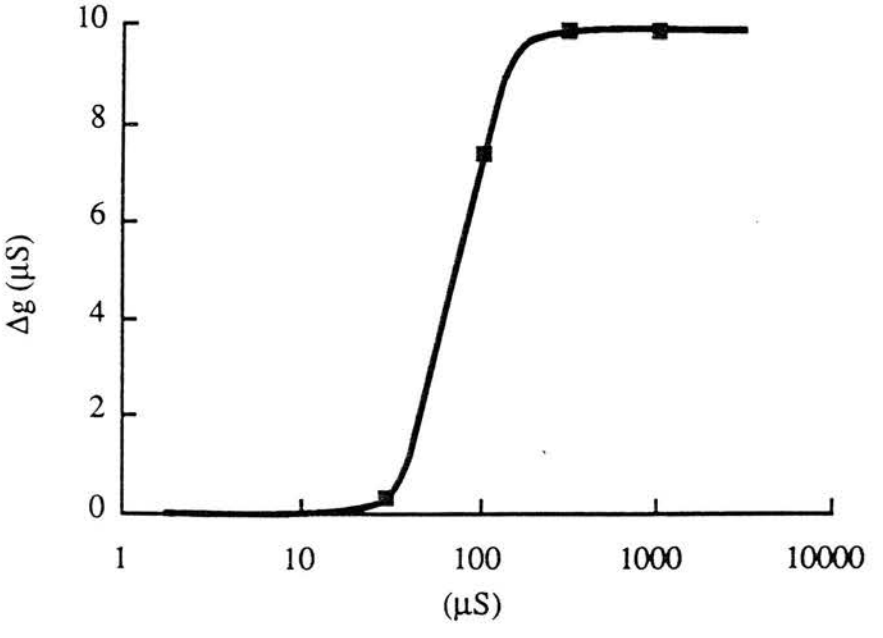
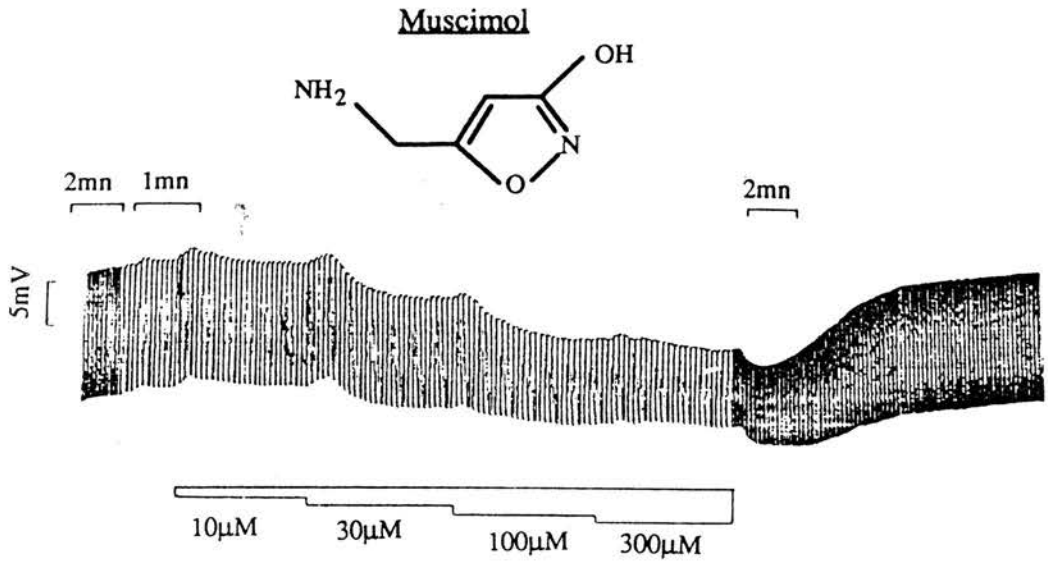


Figure 22: Muscimol

The upper trace illustrates the change in input conductance during cumulative application of muscimol (Injected current: I=40nA). In this experiment, the resting input conductance was 2.8μS. In the presence of muscimol 10μM, Δg=0.0μS; 30μM, Δg=0.6μS; 100μM, Δg=1.4μS; 300μM, Δg=2.1μS. The conductance returned to 100% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=2.4μS, EC50=75.6μM, N=1.4.


Muscimol
OH
NH2
N
O
2mn
1mn
2mn
5mV
10µM
30µM
100µM
300µM

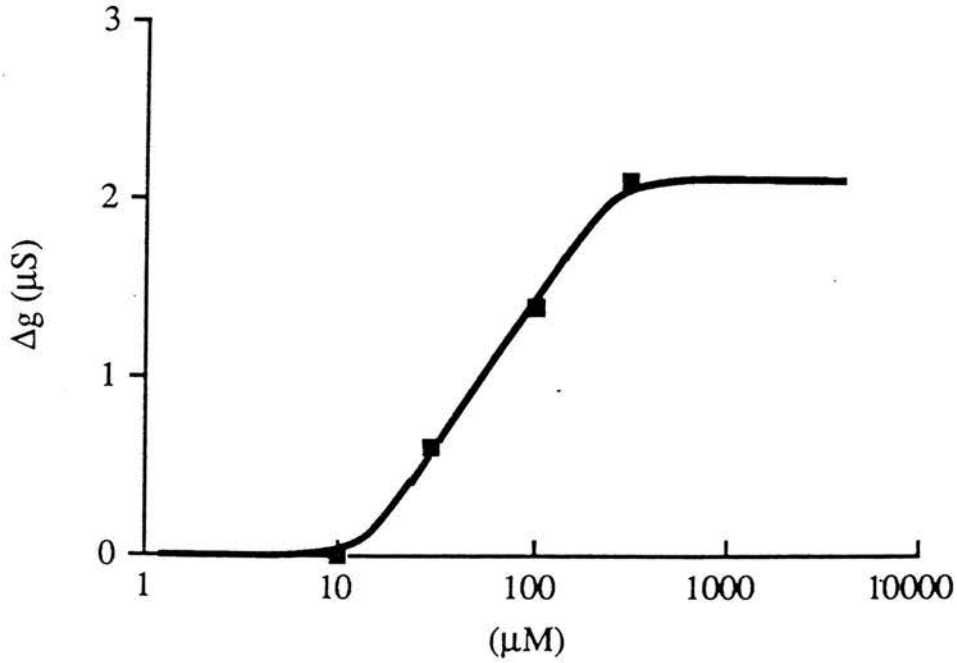
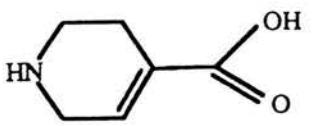
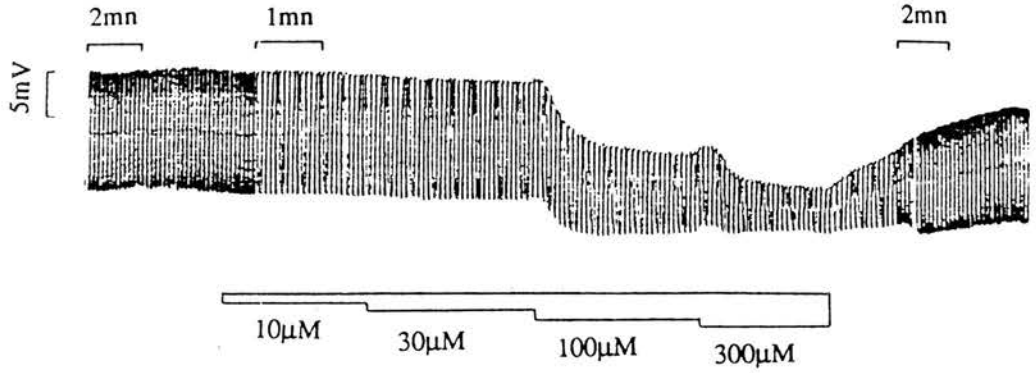

3
2
1
0
Δg (µS)
1
10
100
1000
10000
(µM)

Figure 23: Isoguvacine

The upper trace illustrates the change in input conductance during cumulative application of isoguvacine (Injected current: I=40nA). In this experiment, the resting input conductance was 2.9μS. In the presence of isoguvacine 10μM, Δg=0.0μS; 30μM, Δg=0.0μS; 100μM, Δg=1.1μS; 300μM, Δg=5.1μS. The conductance returned to 100% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=5.1μS, EC50=151.2μM, N=3.4.

Isoguvacine





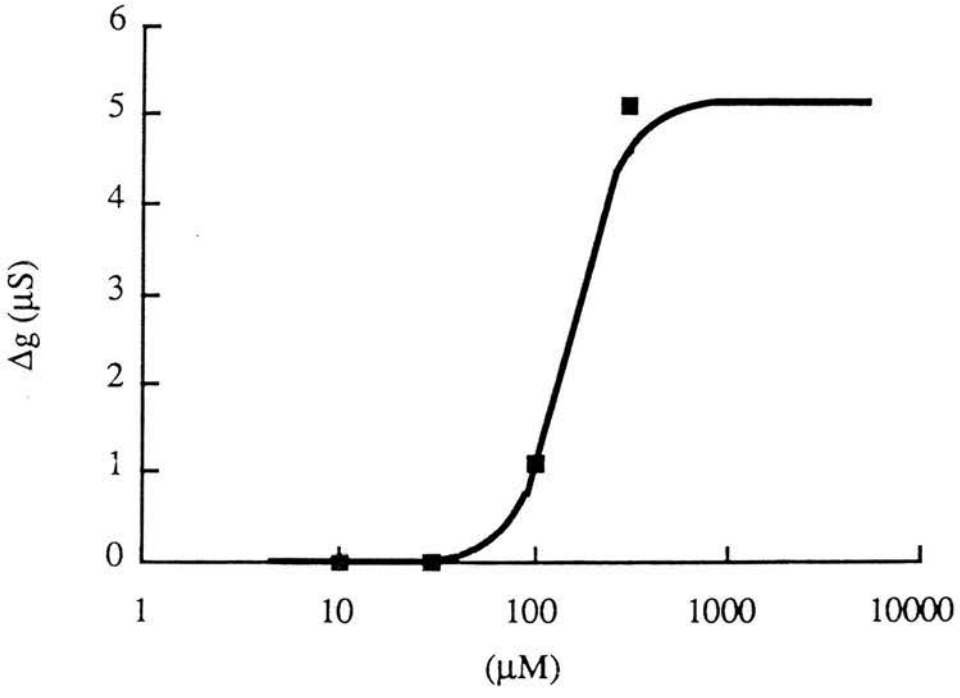
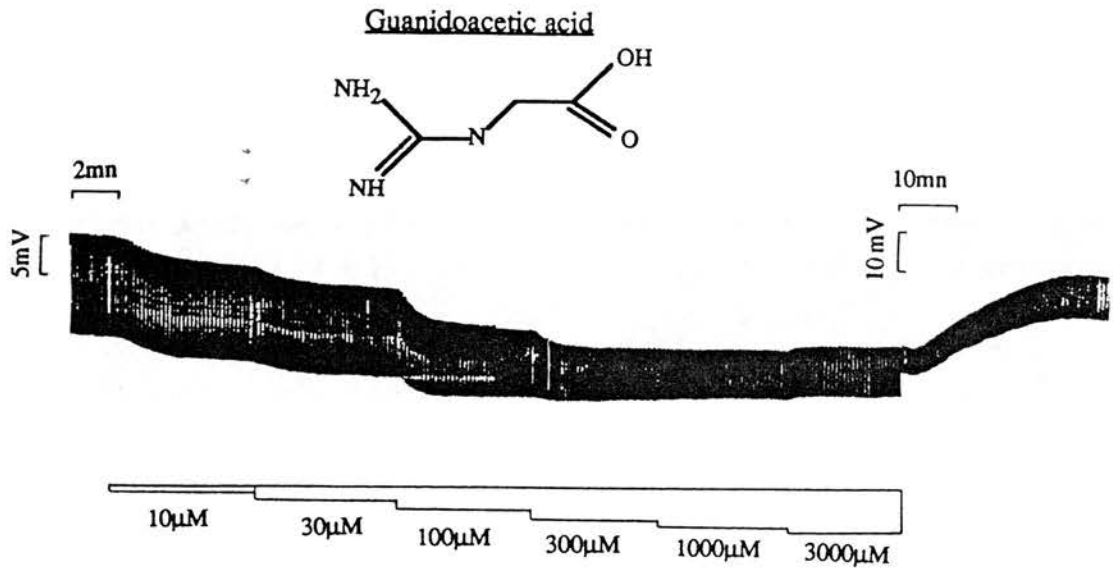


Figure 24: Guanido-acetic acid

The upper trace illustrates the change in input conductance during cumulative application of guanido-acetic acid (Injected current: I=40nA). In this experiment, the resting input conductance was 3.1μS. In the presence of guanidino-acetic acid, 10μM, Δg=0.2μS; 30μM, Δg=0.5μS; 100μM, Δg=1.6μS; 300μM, Δg=3.0μS; 1000μM, Δg=3.0μS. The conductance returned to 86% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=3.1μS, EC50=87.5μM, N=1.8.


Guanidoacetic acid
OH
NH2
N
O
NH
2mn
10mn
5mV
10 mV
10µM
30µM
100µM
300µM
1000µM
3000µM

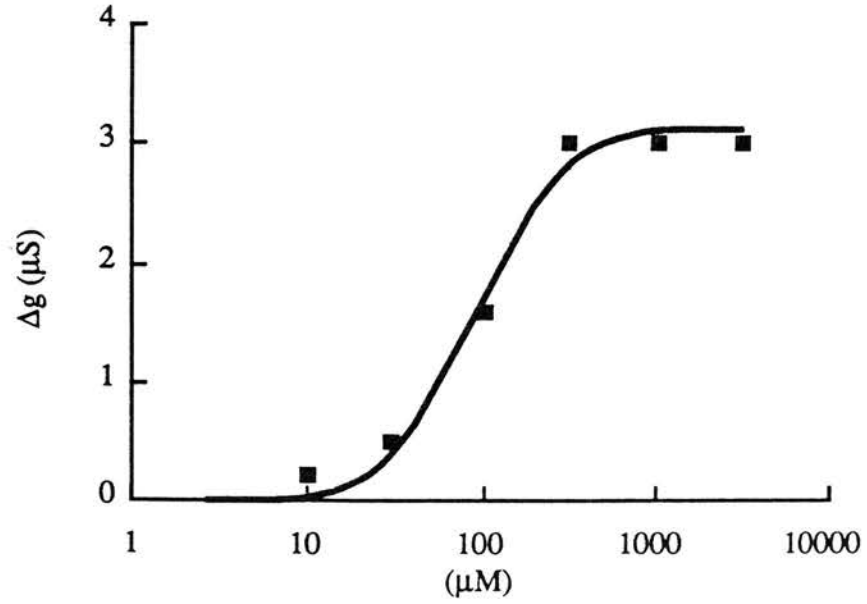
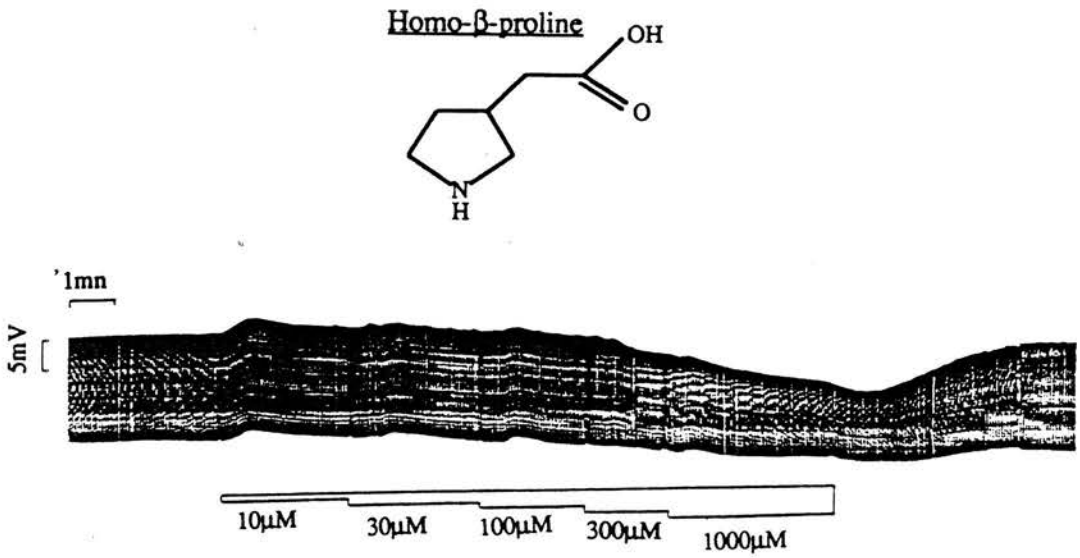

4
3
2
1
0
Δg (µS)
1
10
100
1000
10000
(µM)

Figure 25: Homo-β-proline

The upper trace illustrates the change in input conductance during cumulative application of H-β-P (Injected current: I=40nA). In this experiment, the resting input conductance was 2.3μS. In the presence of H-β-P, 10μM, Δg=0.0μS; 30μM, Δg=0.0μS; 100μM, Δg=0.2μS; 300μM, Δg=0.8μS; 1000μM, Δg=1.5μS. The conductance returned to 91% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=1.6μS, EC50=292.5μM, N=2.0.


Homo-β-proline
OH
O
N
H
1mn
5mV
10μM
30μM
100μM
300μM
1000μM

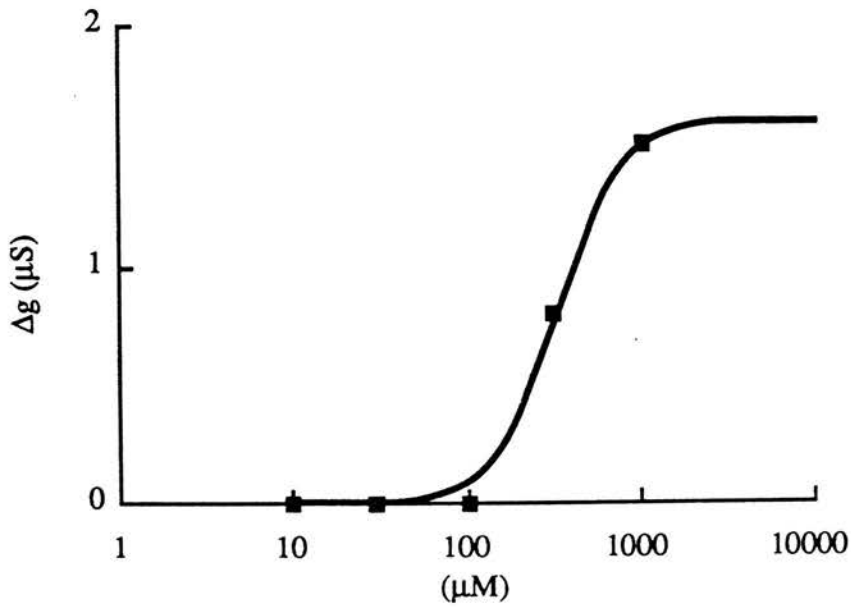
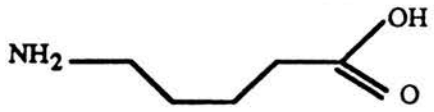
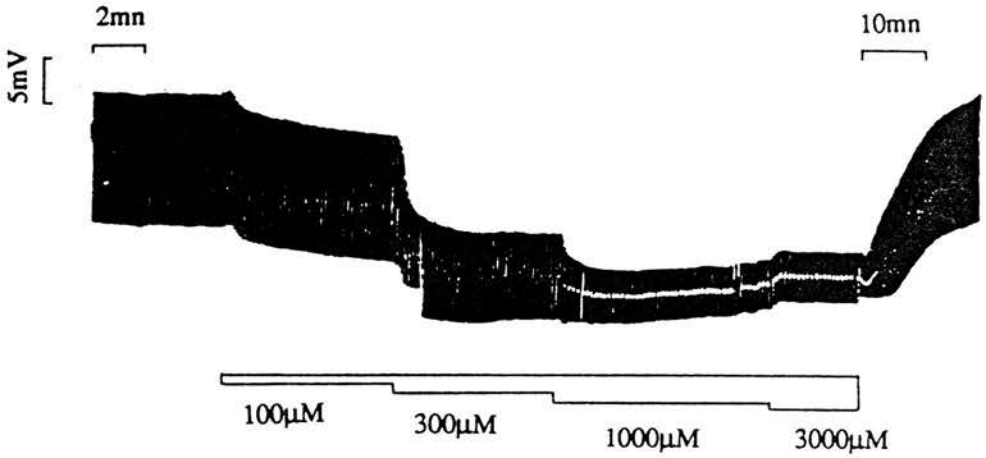

2
1
0
Δg (μS)
1
10
100
1000
10000
(μM)

Figure 26: δ-aminovaleric acid

The upper trace illustrates the change in input conductance during cumulative application of DAVA (Injected current: I=40nA). In this experiment, the resting input conductance was 2.7μS. In the presence of DAVA, 100μM, Δg=0.0μS; 300μM, Δg=1.3μS; 1000μM, Δg=3.9μS; 3000μM, Δg=3.9μS. The conductance returned to 100% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=4.0μS, EC50=375.0μM, N=3.5.

δ-aminovaleric acid





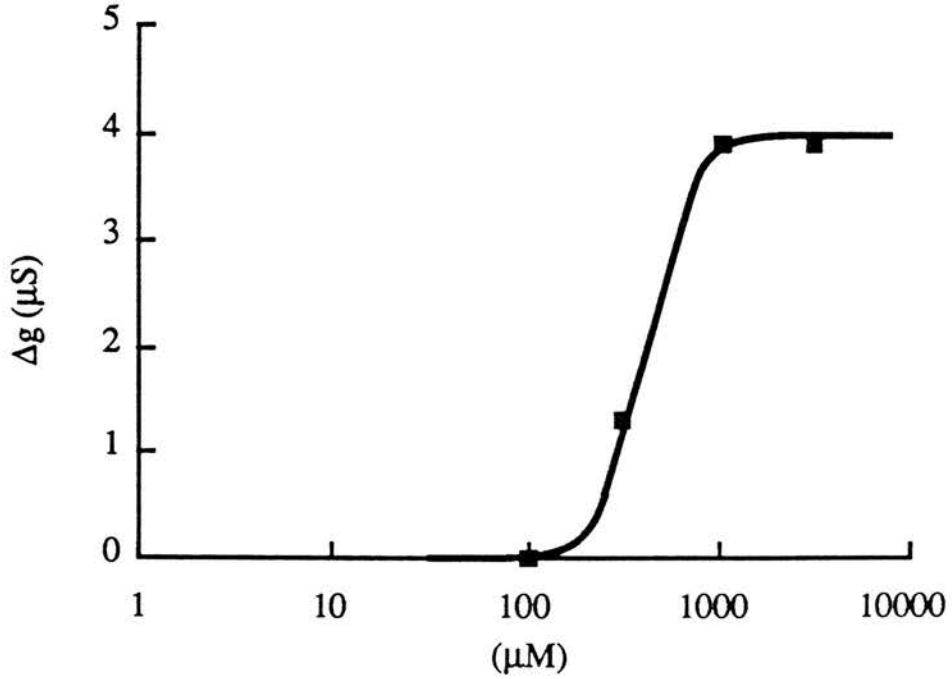
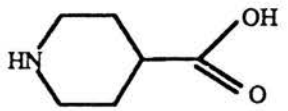
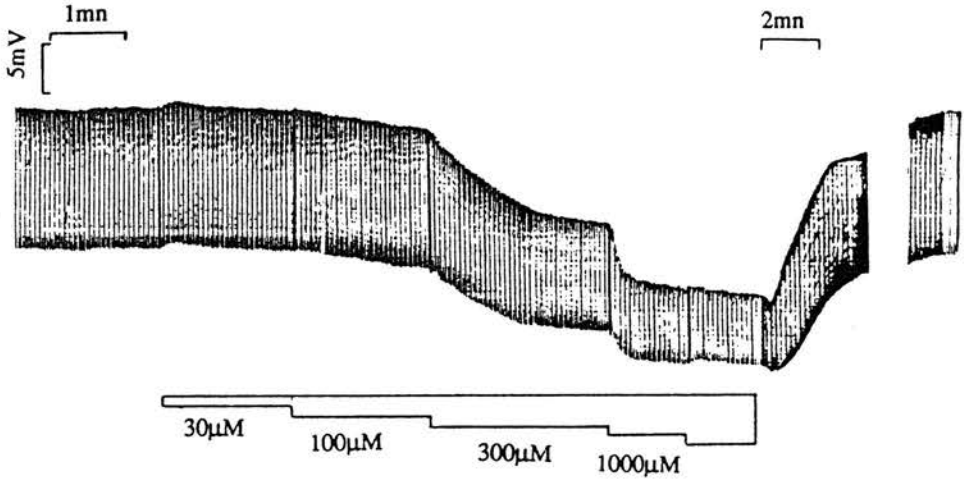


Figure 27: Isonipecotic acid

The upper trace illustrates the change in input conductance during cumulative application of INA (Injected current: I=40nA). In this experiment, the resting input conductance was 2.3μS. In the presence of INA 10μM, Δg=0.0μS; 30μM, Δg=0.0μS; 100μM, Δg=0.0μS; 300μM, Δg=0.4μS; 1000μM, Δg=1.5μS. The conductance returned to 100% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=5.3μS, EC50=581.2μM, N=3.8.

Isonipecotic acid





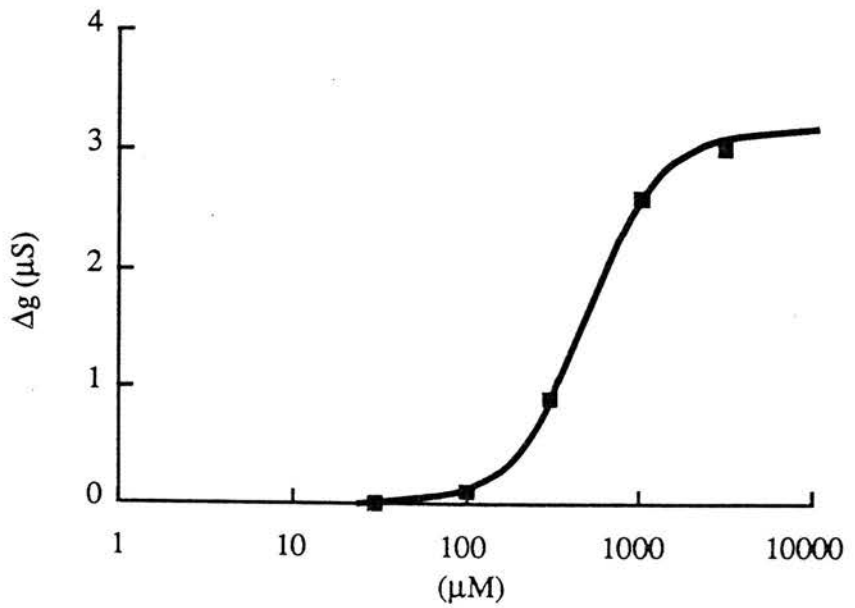
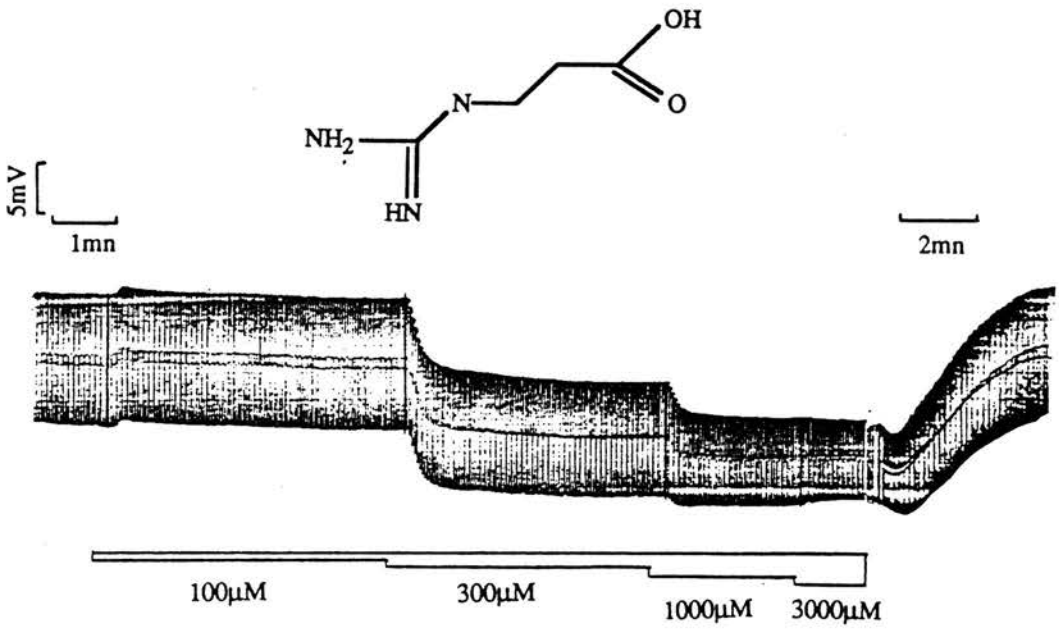


Figure 28: β-guanidinopropionic acid

The upper trace illustrates the change in input conductance during cumulative application of β-GP (Injected current: I=40nA). In this experiment, the resting input conductance was 3.2μS. In the presence of β-GP, 100μM, Δg=0.1μS; 300μM, Δg=0.6μS; 1000μM, Δg=1.8μS; 3000μM, Δg=2.0μS. The conductance returned to 90% of the resting value after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented (■); the solid line represents the fitted modified Hill equation, Δgmax=2.2μS, EC50=479.7μM, N=3.2.

β-guanidinopropionic acid



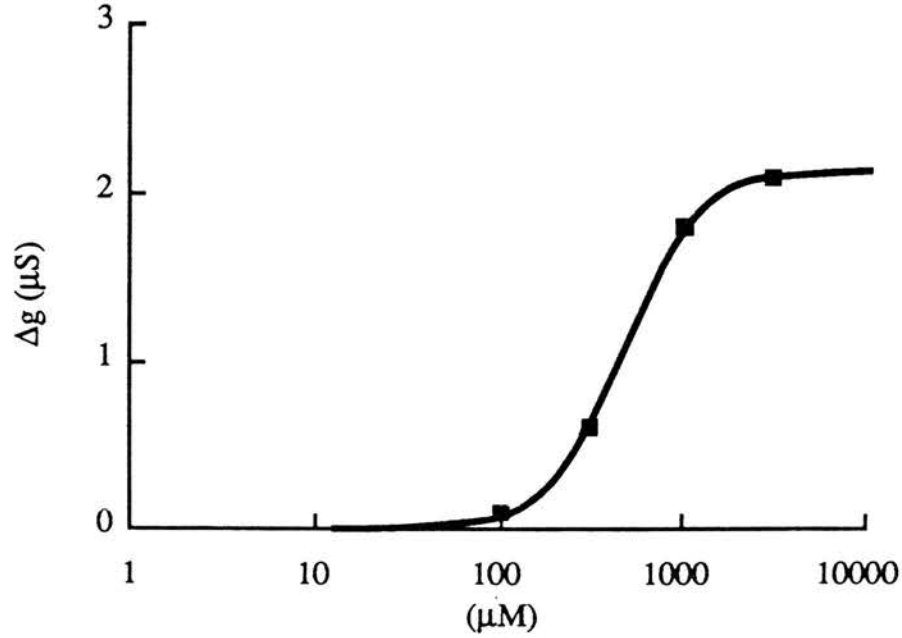


Table 9: Summary of GABA agonists potencies in *Ascaris*

n=number of experiments
RP=relative potency GABA=1
RP^{H}= relative potency on *Ascaris* muscle, from Holden-Dye et al 1988
RP^{K}= relative potency in displacing [^{3}H]GABA from rat brain synaptic membranes, from Krogsgaard-Larsen 1988.
RP^{A}= relative potency on guinea-pig ileum (Allan et al 1986)

For DHM, number in italic correspond to the mean calculated value for R(-) and S(+) isomers, assuming the racemic mixture is 50-50%.
value significantly different from the corresponding GABA value at ** $p \leq 0.05$, T-test or * $p \leq 0.01$

Table 9: Summary of GABA agonists potencies in *Ascaris*

Agonist	Δgmax (μS)	EC50 (μM)	N	n	RP	RP^H	RP^K	RP^A
DHM	4.48±0.36	8.3±1.5**	3.0±0.4	5	3.70	*4.19*	*3.80*	—
ZAPA	3.56±0.34	24.1±4.6	1.9±0.2	3	1.87	1.46	—	3.50
GABA	4.15±0.32	30.2±1.9	2.7±0.2	38	1.00	1.00	1.00	1.00
TACA	4.93±1.01	65.7±14.5**	2.7±0.4	11	0.47	0.55	—	—
IAA	6.77±1.46*	76.1±11.3**	3.0±0.5	4	0.40	0.20	—	—
Muscimol	2.48±0.77	82.5±20.4**	1.8±0.3	5	0.37	0.20	5.00	—
Isoguvacine	3.60±1.00	124.2±10.1**	1.9±0.5	4	0.25	0.19	0.75	—
GuAc	4.32±1.35	135.1±18.0**	2.5±1.0	4	0.22	0.16	—	—
H-β-P	2.96±0.39	233.6±37.2**	2.2±0.1	3	0.13	—	0.1	—
DAVA	3.77±0.94	366.5±17.6**	2.7±0.5	4	0.08	0.08	—	—
CACA	6.83±2.84	391.8±38.6**	2.7±0.3	3	0.08	0.03	—	—
INA	2.50±0.70	409.2±83.2**	2.4±0.3	6	0.07	—	—	—
β-GP	4.30±0.82	536.5±49.7**	1.9±0.1	4	0.07	0.08	—	—

II DISCUSSION

1. Quantitative evaluation of GABA and GABA agonist effects

As highlighted in Section 1 C., two points are critical in the measurement of drug-receptor interaction: the drug concentration and the type of response measured. These problems and the quantitative analysis of the data are discussed below.

1.1. Drug-application

Bath application of the drugs has the advantage of a known and more even concentration around the receptor than local administration. Uptake and metabolizing systems and diffusion barriers, can locally reduce the drug concentration around the cell, so that the bath concentration is not equivalent to the concentration around the receptor. In the *Ascaris* muscle preparation there is no evidence for a GABA-uptake system (Hewitt, 1987, quoted by Holden-Dye et al., 1989). The presence of connective tissue on the bag surface may, however, present a barrier for the bath applied drugs, so that the bath concentration is not strictly equivalent to the local concentration around the receptor. The existence of such barriers may partly explain why lower concentrations of GABA are effective in the patch-clamp experiments, where an enzymatic digestion removes the connective tissue.

1.2. Measurement of the response

In the case of a receptor directly coupled to an ion channel, such as the *Ascaris* GABA receptor, the primary event following agonist binding to the receptor is the opening of the Cl channels, leading to an increase in membrane conductance (Del Castillo et al., 1964b; Martin, 1985; Holden-Dye et al., 1988). The measurements of the change in membrane conductance is therefore directly related to the number of receptors activated; the measurement of the change in membrane potential is a secondary event following receptor activation and depends on the equilibrium potential for Cl (E_{Cl}) as well, as the conductance change:

$$\Delta V = \left(\frac{\Delta g}{\Delta g + g_m}\right) x\ (E_{Cl} - E_m)$$

where ΔV is the change in membrane potential, Δg is the change in membrane conductance, g_m is the resting conductance, E_{Cl} is the Nernst potential for Cl, E_m is the resting membrane potential.

After several applications of GABA or agonist, Cl ions accumulate intracellularly and the E_{Cl} is shifted to less negative values. This shift in equilibrium potential is illustrated in the Figure 34 (Section 5), where GABA 30μM has been applied several times on the same cell and shows that the responses are similar in terms of membrane conductance but not in terms of membrane potential. The change in membrane conductance is therefore a more appropriate measure of receptor activation than the change in membrane potential. To estimate the change in membrane conductance, a current-clamp technique was used.

1.3. Quantification of the results

Agonist activity is therefore quantified by three parameters: EC50, Δgmax and N.

a. Δgmax:

Δgmax is a parameter difficult to estimate. The first problem encountered is its measurement. g, the membrane conductance, is the inverse of the resistance. Errors made on g measurement increase when the resistance decreases, so that the error on Δgmax is large compared to Δg produced by lower concentrations of agonist. The second problem is the interpretation of Δgmax. According the occupancy theory, on which is based the modified Hill equation, Δgmax represents the effect produced by the activation of all the receptors available in the membrane. However, desensitization of the receptors is a frequent phenomenon. Desensitization may be the result of various mechanisms, and leads to a diminution of the number of functional receptors, in other words to a reduction in Δgmax. Patch-clamp experiments have shown that *Ascaris* muscle GABA receptors undergo desensitization when GABA concentrations higher than 5μM were applied (Martin, 1985). However in current-clamp studies, even when a high-GABA concentration (greater than 100μM), is applied for a long time (up to 20 minutes), the membrane conductance remains stable and does not recover progressively, as it would be expected if desensitization occurred. One plausible explanation is that desensitization occurs very rapidly: the receptors are already desensitized before a steady-state is reached, and therefore before the membrane conductance is stabilized. Patch-clamp experiments in *Ascaris*, are in favour of a rapid onset of desensitization. In this case, the electrophysiological responses measured in *Ascaris* muscle are desensitized responses, and should be similar whether GABA is applied in a cumulative or a non-cumulative fashion. There was no statistical significant difference observed between non-cumulative and cumulative dose-response curves to GABA. But the number of experiments with non-cumulative application of GABA

(three) was small, given the large variation of Δgmax between cells. Holden-Dye et al. (1988, 1989), have used a microperfusion technique and non-cumulative drug application. This technique is supposed to limit desensitization of the response, since the drug is applied locally and for a short time only. However, the maximal responses they obtained with GABA were smaller than with the bath application used here. This discrepancy may result from the short duration of GABA application and the presence of a diffusion barrier: steady-state is not reached with the receptors at the cell surface and therefore, only a proportion of the expected response is measured.

The fact that Δgmax did not vary significantly for all the compounds tested (except imidazole-4-acetic acid), does not mean that there is no partial agonist; the variation of Δgmax between the cells for a same compound is too large to allow the detection of partial agonism. The case of the rigid GABA analogues is however interesting; although the differences did not reach statistical significance, they all showed a lower Δgmax. They are also much weaker than at GABA-A receptors. Low Δgmax and high EC50, often characterize partial agonists. For example, piperazine, which is 100 times less potent than GABA, has been shown to act as a partial agonist at the *Ascaris* GABA receptor (Martin, 1982). But here, high EC50's are not always associated with low Δgmax, since some very weak agonists have a high Δgmax: β-guanidinopropionic acid (Δgmax=4.30±0.82μS, mean±SE, n=4), cis-aminocrotonic acid (Δgmax=6.83±2.84μS, mean±SE, n=3). To detect partial agonism, the compounds should be applied at high concentration on the same cell simultaneously, and then separately. The maximal response should be smaller when the two agonists are applied simultaneously, than when they are applied separately. The partial agonist partly antagonizes the effects of the full agonist.

Only imidazole-4-acetic acid showed a higher Δgmax than GABA (Δgmax=6.77±1.46μS, mean±SE, n=4, T-test $p \leq 0.05$). Imidazole-4-acetic acid is somehow a semi-rigid analogue of GABA as muscimol, and has a delocalized positive charge, as the ω-guanidino acids (Krogsgaard-Larsen et al., 1986). There is therefore no plausible explanation available yet for this result. This isolated result illustrates the difficulty in interpreting Δgmax in this preparation.

Although, Δgmax cannot be interpreted as a reliable estimate of the total number of receptors on the cell surface, it gives, nevertheless, a quantitative information on the physiological response of the cell to high concentrations of GABA or GABA agonist.

b. Hill coefficient:

There may be theoretical reasons why the Hill equation (or modified Hill equation) may not be appropriate (see Section 1.C), and it would be wrong to interpret the Hill

coefficient strictly as the number of agonist molecules required to activate the receptor. Several factors can influence the Hill coefficient. For example, the existence of an uptake system or a metabolizing system, would increase the apparent Hill coefficient. In *Ascaris* muscle, Hewitt (1987) (quoted by Holden-Dye et al., 1989) found no evidence for a GABA-uptake system when using nipecotic acid, a specific vertebrate neural uptake inhibitor. However this only means that there is no nipecotic acid sensitive uptake system. If an uptake or a metabolizing system exist, one expects a certain amount of specificity and only GABA and may be a few agonists will be substrates for these systems. It would be unlikely that all the agonists were substrates for the uptake as for metabolizing systems, and therefore differences between the Hill coefficients of GABA and the various agonists would be expected. This is not the case here. In conclusion, the existence of an uptake system could not be demonstrated in this system using the current-clamp technique, and positive cooperativity between agonist and the *Ascaris* GABA receptor is the likely explanation.

c. EC50:

EC50, in contrast to Δgmax, is the most useful parameter and its interpretation does not depend upon any theoretical model: it represents the concentration of GABA or agonist required to produce 50% of the maximal response. Of course problems arise when EC50 has to be related to the dissociation constant of the agonist K_D. Comparison of EC50's is, here, the basis of the discussion on the agonist profile of the *Ascaris* GABA receptor.

1.4. Depolarization in the presence of low-GABA concentrations

Depolarization of the membrane was also occasionally seen after the application of Ringer alone (2 cells). The mechanical disturbance occasioned by the drug application could be responsible for the membrane depolarization. However, the depolarizations occurring at the end of the recovery phase cannot be explained by the mechanical disturbances. It is interesting to note that Del Castillo et al. (1964b), also noticed that low GABA concentrations produced, small but statistically significant, depolarization of the membrane.

These depolarizations are unlikely to be due to acetylcholine receptor activation, since in that case a greater consistency of the response is expected.

The depolarizations seen in this present work, illustrated in Figure 10, resemble the modulation activity and the square waves described by Weisblat et al. (1976). Low-Ca and high-Mg solutions induced square waves carried by Na, similar to the spontaneous

modulation activity carried by Ca or Na (Weisblat et al., 1976). Here, in low-Ca, depolarization can only involve the entry of Na. Assuming that the current-voltage relationship is linear at depolarizing potentials, the estimation of the reversal potential gives a value of +29mV. This assumption may not be valid (see Figure 8), but nevertheless, even if inaccurate, the extrapolation suggests that a Na conductance is likely to be involved since the estimated reversal potential for Na is +27mV (values of intracellular ion concentrations from Brading and Caldwell, 1971: Na: 99.4mM, K: 48.6mM). ~~The conductance involved during the depolarization is permeable to Na and K; or that two different conductances: Na and K, are activated at the same time.~~

A transient decrease in conductance, associated with a small depolarization was sometimes noticed just before the peak depolarization. This suggests the closing of normally open K or more unlikely Cl channels. One can relate this to the inward rectifier K channels (Hille, 1984a). The inward rectifier K channels close with depolarization, producing further depolarization. If large enough, this depolarization will in turn, activate voltage-sensitive cations channels, producing a long lasting depolarization (Hille, 1984a). Inward rectifier K channels are involved in long lasting depolarizations and participate to the spontaneous activity in some neurones (Hille, 1984a). Here in *Ascaris*, the initial closing of these putative K channels may be of crucial importance in starting the square wave. Weisblat et al. (1976), suggested as well, that a conductance to an opposite ion, may be K, was important in generating the square waves. The apparent desensitization of the response is not explained, and may involve another ion conductance associated with a second messenger for example. One attractive hypothesis is that the effect of GABA, at low concentration, may involve a second messenger, via an action at a different receptor, like the vertebrate GABA-B type.

Further investigation of these depolarizations or square waves, may bring interesting informations on the generation of spontaneous activity associated with contraction, the role of Ca, and the interactions with neuro-transmitters, such as GABA.

2. Agonist profile of the *Ascaris* muscle GABA receptor

In Section 1, it has been shown that vertebrate GABA-A receptors are not pharmacologically homogeneous, and so it would be inappropriate to compare the *Ascaris* GABA receptor to only one subtype of GABA-A receptor. For this reason, the following discussion is based on some general characteristics of the GABA-A receptors, which does not imply that all the GABA-A subtypes present these properties.

A comparison of vertebrate GABA-A and *Ascaris* muscle GABA receptors, will be made here, in terms of structure-activity relationships, and the pharmacological implications will be discussed. Several interesting features can be pointed out from this present study; it will be shown that *Ascaris* GABA receptors resemble the vertebrate GABA-A receptors: baclofen, a specific GABA-B agonist is inactive; the optimal chain length between the positive and negative charges is approximately 5Å; agonists are more potent in an extended conformation; and the stereoselectivity for a certain number of compounds is similar. It is also shown that *Ascaris* GABA receptors differ from the vertebrate GABA-A receptors with respect to the potency of the sulphonic derivatives 3-APS, P4S; the potency of some rigid analogues: muscimol, isoguvacine, isonipecotic acid, THIP; and the inactivity of diazepam, pentobarbitone and cortisol in potentiating GABA responses.

2.1. GABA-A agonist properties

a. Inactivity of baclofen

GABA-A and GABA-B receptors are the two main classes distinguished in vertebrate. Baclofen is a specific agonist at the GABA-B receptors in vertebrate and is inactive at 1mM on membrane conductance in *Ascaris* muscle suggesting the absence of vertebrate GABA-B type receptors in this preparation. This confirms the results of Holden-Dye et al. (1988). Previous work has shown that the *Ascaris* GABA receptor is associated with Cl channels (Del Castillo et al., 1964b; Martin, 1985; Holden-Dye et al., 1988) like the GABA-A receptors. The lack of activity of baclofen further illustrates that the *Ascaris* GABA receptor is more like the vertebrate A type than to the vertebrate B type.

b. Optimal chain length between the positive and negative charges

GABA has four carbons between the positive charge of the amine function and the negative charge of the carboxylic function; the charges are separated by a distance of 4.7Å (Krogsgaard-Larsen and Johnston, 1978; Wermuth and Rognan, 1987). Aminoacids with a shorter chain length, such as in glycine (3C) and β-alanine (2C) are totally inactive at 1mM. Amino-acids with longer chain, are not potent either: ε-aminocaproic acid (6C) is inactive, δ-aminovaleric acid (5C) is a weak agonist. Compounds with longer chains but with the positive charge delocalized, possess a

some activity, as in the case of the ω-guanidinoacids: β-guanidinopropionic acid (4C, 1N), guanidoacetic acid (3C, 1N). These results confirm previous findings obtained in *Ascaris* (Holden-Dye et al., 1988), and are in good agreement with the observations in other invertebrates (Takeuchi and Takeuchi, 1975; see Nistri and Constanti 1979, for a review) and vertebrate preparations (see Nistri and Constanti, 1979; Krogsgaard-Larsen, 1988, for a review). The optimal chain length between the positive and negative charges on *Ascaris* GABA receptor is similar to that of the vertebrate GABA-A receptor and corresponds to a distance close to 4.7Å.

c. Extended conformation of GABA

GABA has great flexibility and can adopt various conformations. Structure-activity studies have suggested that GABA interacts with the GABA-A recognition site in a extended conformation (Steward, Player, Quillian et al., 1971; Johnston and Allan, 1984). In *Ascaris*, the use of the two isomers of the aminocrotonic acid, suggests that, like the GABA-A receptor, the *Ascaris* muscle GABA receptor is activated preferentially by the extended form (trans-aminocrotonic acid, EC50=65.7±14.5μM, n=11), the folded form is very weak (cis-aminocrotonic acid, EC50=391.8±38.6μM, n=3). These results are in good agreement with previous work on *Ascaris* (Holden-Dye et al., 1988). It must be noted that in vertebrate preparations, some baclofen-insensitive GABA receptors do not prefer the folded conformation. Johnston and Allan (1984), reported the GABA-like neuronal depressant activity of cis-aminocrotonic acid and other folded compounds via receptors named GABA-C (Johnston, 1986). These GABA-C receptors are furthermore insensitive to the GABA-A antagonist bicuculline.

d. Stereoselectivity

Holden-Dye et al. (1989), studied the relative potencies of stereoisomers in *Ascaris*. They showed that the *Ascaris* GABA receptor has a stereoselectivity similar to that of the central GABA-A receptors for RS(±)dihydromuscimol and RS(±)4-methyl trans-aminocrotonic acid. However, the stereoselectivity shown by the *Ascaris* GABA receptor for RS(±)3-hydroxyGABA, was similar to that seen at the spinal GABA-A receptors. S(+)dihydromuscimol, R(-)3-hydroxy GABA and S(-)4-methyl trans aminocrotonic were the most potent stereoisomers in *Ascaris*.

2.2. Differences with the GABA-A receptors

a. Inactivity of sulphonic derivatives

In this study, the two sulphonic acid derivatives 3-APS and P4S were found inactive at 1mM in *Ascaris*, as reported previously by Holden-Dye et al. (1988). These sulphonic acid derivatives are very weak or inactive in other invertebrate preparations (see Simmonds, 1983; and Walker and Holden-Dye, 1989, for review). In vertebrate brain preparation, P4S, in contrast to GABA, does not stimulate benzodiazepine binding (Krogsgaard-Larsen, 1988); this difference has been explained by the existence of a distinct type of GABA-A receptor coupled to benzodiazepine receptor (Karobath, Placheta and Lippitsh, 1979). Krogsgaard-Larsen (1988), however, proposed a different explanation and suggested that:

> "*...the mechanisms underlying the effects of GABA-A agonists containing the tetrahedrally oriented sulphonate groups are somehow different from other agonists containing planar carboxylate groups...*".

It is pointed out that Krogsgaard-Larsen's conclusion is partly based on Curtis's personal communication; and these views remain to receive further support. Obviously, the structural and electron differences between sulphonic acid and carboxylic acid moieties play a role in the differences of properties between GABA and P4S; but the existence of different populations of GABA-A receptors, more or less sensitive to sulphonic acid derivatives, can explain, in itself, the differences in agonist properties. Moreover, sulphonic acid derivatives are not the only compounds with little activity on benzodiazepine binding: isoguvacine and THIP were reported as weak stimulators of [^{3}H]diazepam binding while being potent antagonists of [^{3}H]GABA binding (Karobath et al., 1979). The existence of structural differences in the receptor itself is more likely to explain the inactivity of sulphonic acid derivatives at the *Ascaris* GABA receptor, and more generally at invertebrate GABA receptors.

The low potency of the sulphonic acid derivatives 3-APS and P4S, can be considered as a characteristic of invertebrate GABA receptors, and not as a special feature of the *Ascaris* muscle GABA receptor.

b. Loss of activity by rigid GABA-A agonists

May be, the most important observation here is the loss of activity, in *Ascaris*, of some of the potent and rigid GABA-A agonists: muscimol, thiomuscimol, isoguvacine,

isonipecotic acid and THIP. In vertebrates, these rigid or "*conformationally frozen*" compounds (Krogsgaard-Larsen et al., 1986), are considered as potent and specific GABA-A agonists. Because of their rigidity they are restricted to a certain conformation and therefore, are prevented from interacting with binding sites accepting GABA in a slightly different conformation. For example, these compounds do not interact, or weakly interact, with GABA uptake sites or GABA-B sites (see Krogsgaard-Larsen, 1988, for review). Muscimol and thiomuscimol were more potent than GABA itself, in inhibiting [^{3}H] GABA binding at rat brain synaptic membranes, and in inhibiting the firing of cat spinal interneurones (Krogsgaard-Larsen, 1988). In the same experiments, isoguvacine was found to be equipotent with GABA; isonipecotic acid and THIP were found less potent than GABA (Krogsgaard-Larsen, 1988). In invertebrate preparations, there is a great variability between species and even between tissues of the same species. For example, in arthropods, isonipecotic acid is more potent than GABA in *Limulus* central neurones, but not in *Periplaneta* (Roberts, Krogsgaard-Larsen and Walker, 1981). In *Limulus*, GABA receptors in the central nervous system (Roberts et al., 1981), and GABA receptors found in the heart (Benson, 1989), have different characteristics: THIP is as potent as GABA in *Limulus* central neurones, but inactive at eliciting hyperpolarization in *Limulus* heart (Roberts et al., 1981).

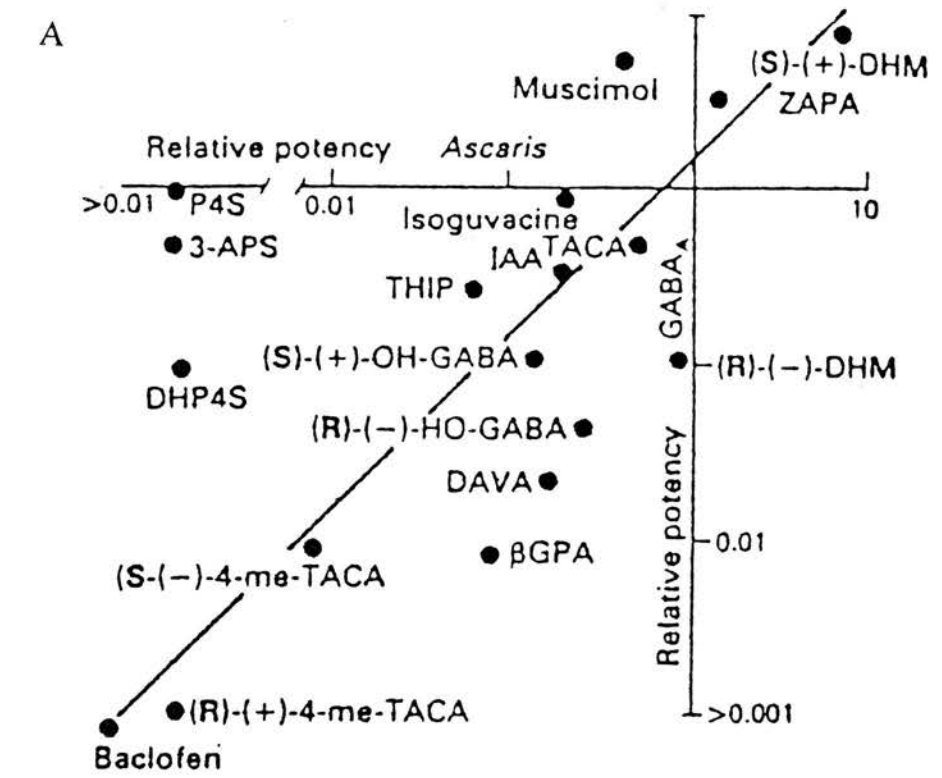
These variations of agonists potencies, reflect well the heterogeneity of vertebrate GABA-A and invertebrate GABA receptors already highlighted in the Section 1 B.

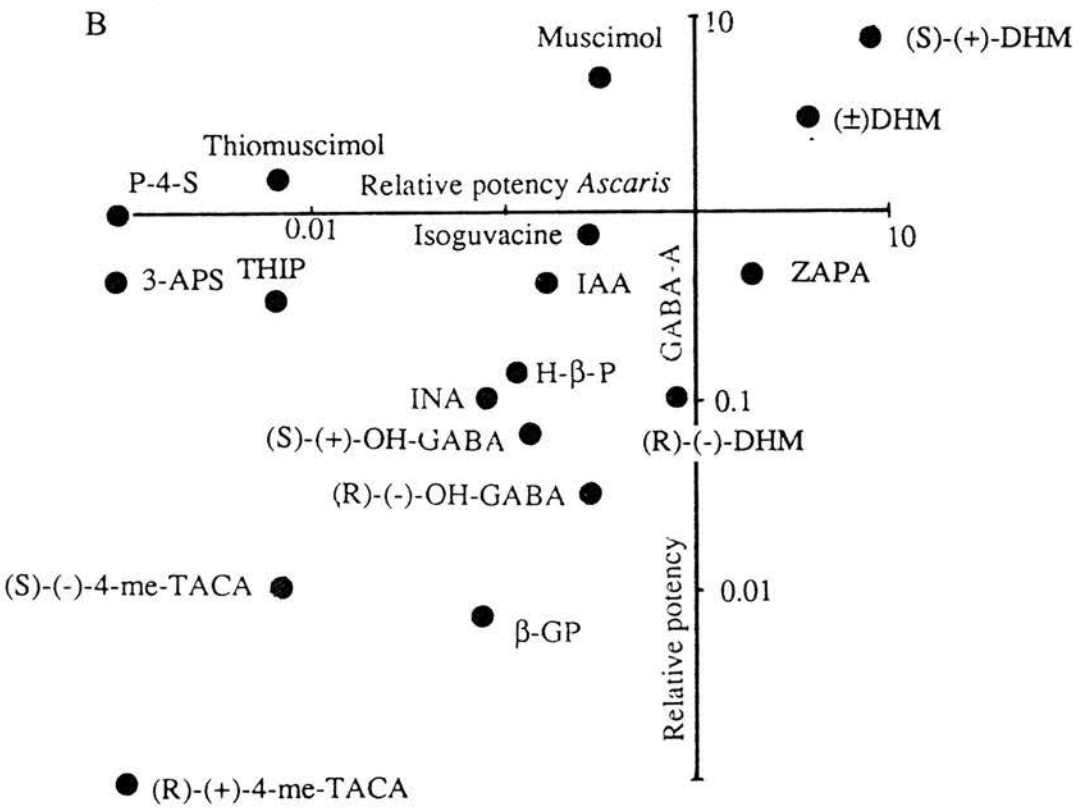
Holden-Dye et al. (1989), compared the relative potencies of various GABA agonists in *Ascaris* muscle to their relative potencies in vertebrate preparations: binding studies using rat synaptic membranes (Krogsgaard-Larsen, 1988), or at the guinea-pig ileum (Allan, Dickenson, Hiern et al., 1986a). They suggested that the order of potencies in both systems are positively correlated (Figure 29A). The relative potencies of the sulphonic derivatives, 3-APS and P4S, were not included in the calculation of the correlation coefficient, on the basis of a fundamentally different mechanism of interaction between these compounds and the GABA receptor (Krogsgaard-Larsen, 1988). The sulphonic group confers different properties to these compounds; but the structural differences in binding sites are more likely to be responsible for the inactivity of the sulphonic acid derivatives in *Ascaris*, and in invertebrate more generally. The rejection of these compounds from the comparison between the two systems does not seem justified. In addition, several vertebrate systems were used used for the calculation of the correlation coefficient: displacement of [^{3}H]GABA from rat brain membranes for most of the compounds (Krogsgaard-Larsen, 1988); and contraction of the guinea-pig ileum for ZAPA (Allan et al., 1986a). These two systems are not equivalent, especially in respect to ZAPA; the relative potency of ZAPA in the guinea-

Figure 29: Correlation between the relative potency of some agonists in *Ascaris* and in vertebrate preparations

A. From Holden-Dye et al., (1989)
Abscissa: relative potency in *Ascaris* muscle
Ordinate: relative potency in vertebrate preparations; displacement of [^{3}H]GABA from rat brain membranes (Krogsgaard-Larsen, 1988), except for ZAPA: contraction of the guinea-pig ileum (Allan et al., 1986). Slope=1.02, correlation coefficient r=0.74, $p<0.01$.

B. This present study.
Abscissa: relative potency in *Ascaris* muscle (this study for: H-β-P, IAA, muscimol, INA, isoguvacine, thiomuscimol, β-GP, ZAPA, RS(±)dihydromuscimol, and Holden-Dye et al., 1988; 1989, for: S(-)-me-TACA, R(+)-me-TACA, S(+)-OHGABA, R(-)-OHGABA, S(+)-dihydromuscimol, R(-)-dihydromuscimol, P4S, 3-APS, THIP)
Ordinate: relative potency in vertebrate; displacement of [^{3}H]GABA from rat brain membranes (Krogsgaard Larsen et al., 1986; Krogsgaard Larsen,1988).
Correlation coefficient, r=0.80, slope=0.87, $p<0.01$, regression coefficient R^2=0.64.
Note that because of the use of logarithmic coordinates, the linear regression was not superimposed on the diagram.


A
Muscimol
(S)-(+)-DHM
ZAPA
Relative potency
Ascaris
>0.01
P4S
0.01
Isoguvacine
10
3-APS
IAA
TACA
THIP
GABA$_A$
(S)-(+)-OH-GABA
(R)-(−)-DHM
DHP4S
(R)-(−)-HO-GABA
DAVA
βGPA
0.01
(S-(−)-4-me-TACA
Relative potency
(R)-(+)-4-me-TACA
>0.001
Baclofen


B
Muscimol
10
(S)-(+)-DHM
(±)DHM
Thiomuscimol
P-4-S
Relative potency Ascaris
0.01
Isoguvacine
10
3-APS
THIP
IAA
ZAPA
GABA-A
H-β-P
INA
0.1
(S)-(+)-OH-GABA
(R)-(-)-DHM
(R)-(-)-OH-GABA
(S)-(-)-4-me-TACA
0.01
β-GP
Relative potency
(R)-(+)-4-me-TACA

pig ileum, is: 3.50 (Allan et al., 1986a), whereas in binding studies, it is only: 0.43-0.50 (Krogsgaard-Larsen et al., 1986; Allan et al., 1986a). Figure 29B shows the correlation between the relative potencies of GABA agonists at the *Ascaris* muscle GABA receptor (this study, and Holden-Dye et al., 1988; 1989) and in binding studies on rat brain membranes (Krogsgaard-Larsen et al., 1986; Krogsgaard-Larsen, 1988). The sulphonic acid derivatives P4S and 3-APS were included in the calculation. The relative potency of ZAPA to displace [^{3}H]GABA was chosen, instead of its potency in eliciting contractions in the guinea-pig ileum. Both systems are found also positively correlated, with a slope of 0.87±0.15 (mean±SE, n=19, F-test, r=0.80, p≤0.01, R^2=0.64), indicating also that the *Ascaris* GABA receptor is related to vertebrate central GABA-A receptors, in terms of agonist potencies.

c. Inactivity of diazepam, pentobarbitone and cortisol

Diazepam (1μM), pentobarbitone (10μM) and cortisol (10μM), did not significantly potentiate, or antagonize the response to GABA. The concentrations used are low, especially for the barbiturate, pentobarbitone, which is not normally a strong potentiator of the GABA response. Holden-Dye et al. (1989), used higher concentrations of pentobarbitone: 100μM, and of the benzodiazepine, flurazepam: 100μM. But they could not find any significant enhancement of the GABA response. This is in agreement with the fact that, among invertebrates, benzodiazepine or barbiturate potentiation of GABA responses, is only found in insects (see Section 1,B). Cortisol had only been recently shown to interact with the GABA-A receptor complex in vertebrates. The concentrations required are much lower than for barbiturates. However, cortisol possesses a dual action: potentiator of the GABA response at low concentrations, and GABA-antagonist at high concentrations (Ong et al., 1987). To investigate more seriously the actions of cortisol, or of other putative modulators, on GABA response in *Ascaris*, a wider range of concentrations should be applied.

The small increase in Δgmax seen with pentobarbitone and cortisol, was not statistically significant. This has to be related to the difficulty in measuring Δgmax, and to the small number of experiments. The use of a more sensitive technique, such as the patch-clamp technique, for example, should be considered before any final statement on the presence or not of modulatory sites in *Ascaris*.

CONCLUSION

Whether or not the *Ascaris* GABA receptor belongs to the vertebrate A receptor type cannot not be decided only on a basis of agonist potencies. More practically, the similarities between *Ascaris* GABA receptor and the GABA-A receptors indicate that the exploration of *Ascaris* GABA receptor agonist properties is unlikely to lead to the discovery of a potent and selective agonist suitable for a new generation of anthelmintics.

In the next sections, the antagonist properties of the *Ascaris* muscle GABA receptor will be investigated. It will be shown that they are fundamentally different from the vertebrate and other invertebrate GABA receptors, they might therefore be exploited in anthelminthic drug research.

SECTION 4:
INACTIVITY OR WEAK POTENCY OF CLASSICAL GABA-A ANTAGONISTS AT THE *ASCARIS* MUSCLE GABA RECEPTOR

INTRODUCTION

In the present section the activity of some classical GABA-A antagonists such as (+)bicuculline, picrotoxin, the amidine steroid RU5135, were examined. The activity of some related compounds has also been examined: (-)bicuculline, usually less potent than the (+) isomer, and (±)d-tubocurarine, a classical acetylcholine nicotinic antagonist with some GABA antagonist properties (Simmonds, 1982; Krogsgaard-Larsen, 1988).

I METHODS

1. Muscle preparation

The muscle dissection has been described in the general methods Section and in Section 3. As for the experiments with agonists, the muscle flap was bathed in high-Cl, low-Ca Ringer (solution B), and maintained at 22ºC.

2. Drug application

Because of the limited quantities of antagonist available, the muscle was not perfused continuously. The experimental chamber (2.5ml) was carefully drained, and then flushed with 5ml of the next drug solution. When dose-response curves were required, drugs were bath-applied in a cumulative manner, without intermediate washing between the concentrations (see Section 3).

3.Current-clamp

3.1. Technique

The technique used here, has been described previously in Section 3. In some experiments, signals were recorded with an Axoclamp 2A (Axon Instruments) which permits the use of higher resistance electrodes for current injection: typically 20-50MΩ.

3.2. Analysis

a. GABA

The effect of GABA was determined by measuring the change in input conductance. When dose-response relationships were required, they were described by the modified Hill equation (Nistri and Constanti, 1979), and estimates for Δgmax, EC50 and N were obtained using a non linear regression programme (Patternsearch Colquhoun, 1971). See Section 3 for details.

b. Antagonists

- Screening

Initially, the action of 1mM antagonist was examined on the change in membrane conductance induced by GABA 30μM. The percentage antagonism was then estimated:

$$\% \text{ antagonism} = 100.(1-\frac{\Delta g_{ant}}{\Delta g_c})$$

where Δg_c is the change in membrane input conductance produced by GABA 30μM, and Δg_{ant} is the change in membrane input conductance produced by GABA 30μM + antagonist 1mM, measured after ten minutes application of the antagonist. Compounds detected as reasonably potent antagonists with this method, were then tested at several concentrations on GABA dose-response curves.

- Evaluation of IC50

The effect of RU5135 has been examined on GABA dose-response curves. Because of the natural variation of Δgmax and N, a control GABA dose-response curve was usually obtained from the same cell, before, or sometimes after the antagonist application. The IC50 was estimated according Schild's method (Arunlakshana and Schild, 1959).

4. Statistical test

Means and standard errors were calculated. Statistical significance was assessed using a two-tailed independent t-test.

II RESULTS

The results presented here are based on the analysis of experiments conducted on 17 cells from 8 preparations. Cells selected for recording and analysis had resting membrane potentials greater than -20mV and resting input conductances between 2.2μS and 3.2μS (2.9±0.1μS, mean±SE, n=17). Recordings were also rejected if the resting input conductance failed to return to at least 80% of the control.

1. Inactive compounds

(+) bicuculline 100μM (n=4), picrotoxin 300μM (n=3), (-)bicuculline methiodide 200μM (n=2), (±)d-tubocurarine 1mM (n=2), applied for ten minutes, were found inactive on the change in membrane conductance induced by GABA 30μM. Because of its instabillity in solution (Curtis et al., 1970), (+)bicuculline was used within two hours. Figure 30 illustrates the inactivity of (-) bicuculline, picrotoxin and d-tubocurarine. The inactivity of GABA-A competitive antagonist: (±)bicuculline methiodide (10μM) in *Ascaris* muscle had been previously reported (Holden-Dye et al., 1988). Picrotoxin, the non competitive Cl channel blocker, had been also shown unable to block GABA responses in *Ascaris* muscle at 10μM (Holden-Dye et al., 1988) and at 100μM (Wann, 1987). These results contrast strongly with the vertebrate GABA-A receptors where picrotoxin and (+)bicuculline are potent antagonists.

(-)bicuculline methiodide and tubocurarine were ineffective, but they are also weak antagonists at the vertebrate GABA-A receptors (Simmonds, 1982; Krogsgaard-Larsen, 1988).

2. RU5135 antagonizes GABA (30μM) induced change in membrane conductance

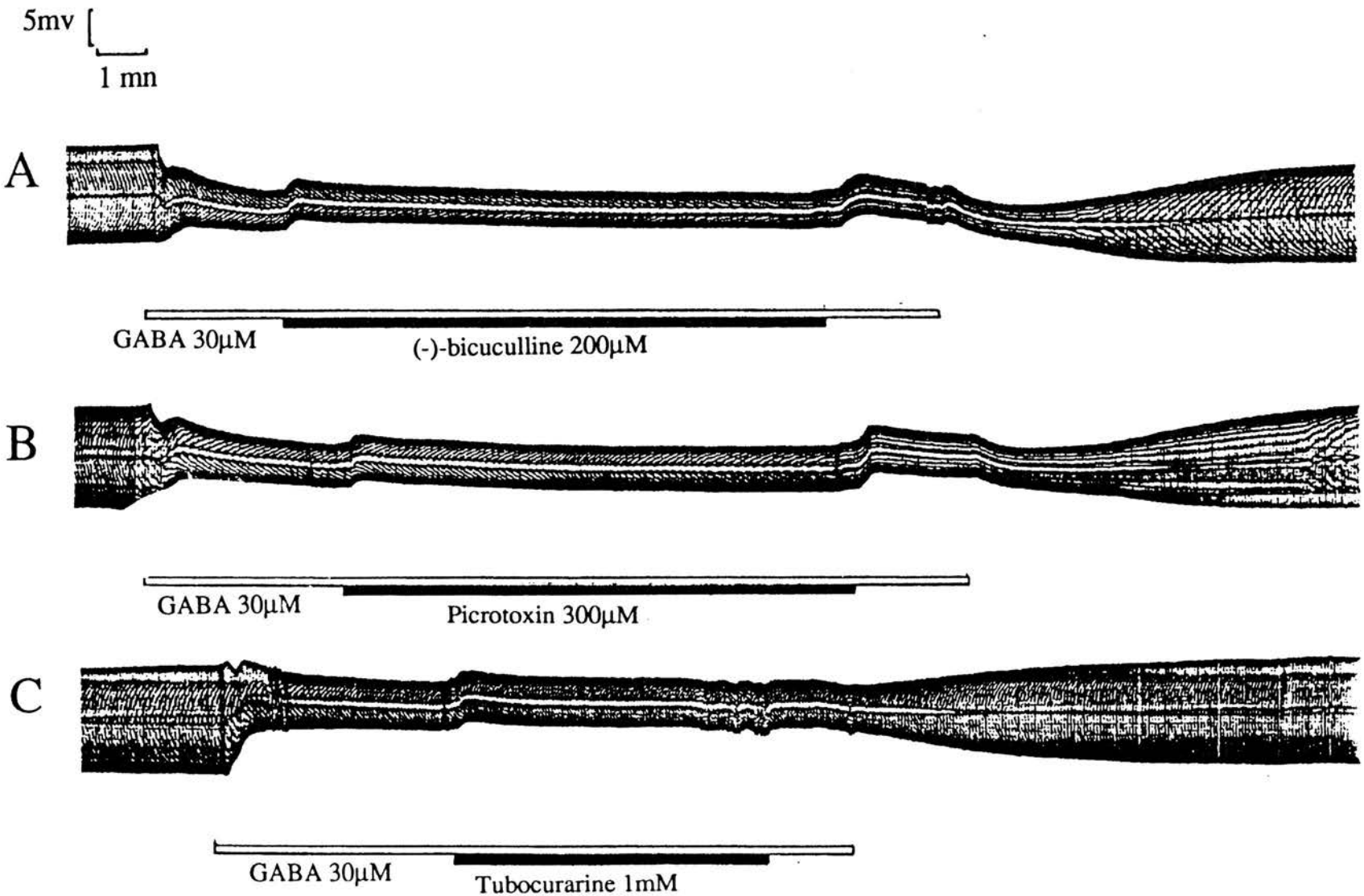
RU5135 (Hunt and Clements-Jewery, 1981), is the most potent competitive GABA-antagonist known at the vertebrate GABA-A receptor. To detect any antagonistic activity, the effects of 1mM RU5135, on the conductance change induced

Figure 30: Inactivity of (-)bicuculline, picrotoxin and d-tubocurarine as GABA antagonists in *Ascaris* muscle

A. Effect of (-)bicuculline 200μM (I=40nA):
go=3.1μS; GABA 30μM, Δg=4.2μS, GABA 30μM + (-)bicuculline 200μM, Δg=4.2μS; %antagonism=0.

B. Effect of picrotoxin 300μM (I=40nA):
go=2.9μS; GABA 30μM, Δg=3.8μS; GABA 30μM + picrotoxin 300μM, Δg=3.8μS; %antagonism=0.

C. Effect of d-tubocurarine 1mM (I=40nA):
go=2.7μS; GABA 30μM, Δg=3.4μS; GABA 30μM + d-tubocurarine 1mM, Δg=3.4μS; %antagonism=0.


5mv
1 mn
A
GABA 30µM
(-)-bicuculline 200µM
B
GABA 30µM
Picrotoxin 300µM
C
GABA 30µM
Tubocurarine 1mM

by 30μM GABA, were examined. Figure 31, illustrates the antagonism of the GABA response by RU5135. In this cell, the resting membrane conductance was 2.3μS. In presence of GABA 30μM, the conductance increased to 5.3μS, giving a change in conductance of 3.0μS. The application of RU5135 decreased the conductance to 2.6μS, and Δg to 0.3μS. In this experiment, 90% of the GABA response was antagonized by RU5135 1mM. The effects of RU5135 were fully reversed by perfusion of GABA 30μM alone, Δg returned to 3.0μS. The resting input conductance returned to 2.4μS in drug free solution, indicating that the membrane had not been damaged.

3. Effect of RU5135 on dose-response curves to GABA

In order to further characterize the antagonism of RU5135, the effects of 70, 210 and 700μM RU5135 on GABA dose-response curves were examined. The effects of RU5135 were investigated in five different cells. The EC50 for GABA was 30.3±1.8μM (mean±SE); Δgmax was 4.9±0.5μS (mean±SE); and the Hill coefficient N was 2.2±0.3 (mean±SE).

As illustrated in Figure 32, GABA dose-response curves, in the presence of 210 and 700μM were shifted to the right in a non parallel manner with an apparent reduction of the maximum response, suggesting a non-competitive antagonism. In five different preparations, RU5135 70μM, did not significantly affect the GABA response, whereas 210 and 700μM significantly increased the EC50 to 98.2±19.4μM and 315.6±26.9μM respectively (mean±SE, n=5, $p \leq 0.01$, T-test). Δgmax was significantly reduced in the presence of 700μM RU5135 to Δgmax=3.3±0.4μS (mean±SE, n=5, $p \leq 0.05$, T-test). The Hill coefficient was also decreased, but not significantly. These results are summarized in Table 10, and show that RU5135 acts as a non competitive antagonist, with an estimated IC50=117μM.

II DISCUSSION

1. Inactivity of the classical GABA-A antagonists: picrotoxin,(±)bicuculline, and the related compound (±)d-tubocurarine

The results presented in this study confirm the lack of potency of bicuculline (Holden-Dye et al., 1988) and picrotoxin on *Ascaris* muscle (Wann, 1987; Holden-Dye et al., 1988). Bicuculline is an inactive or weak antagonist in most invertebrate

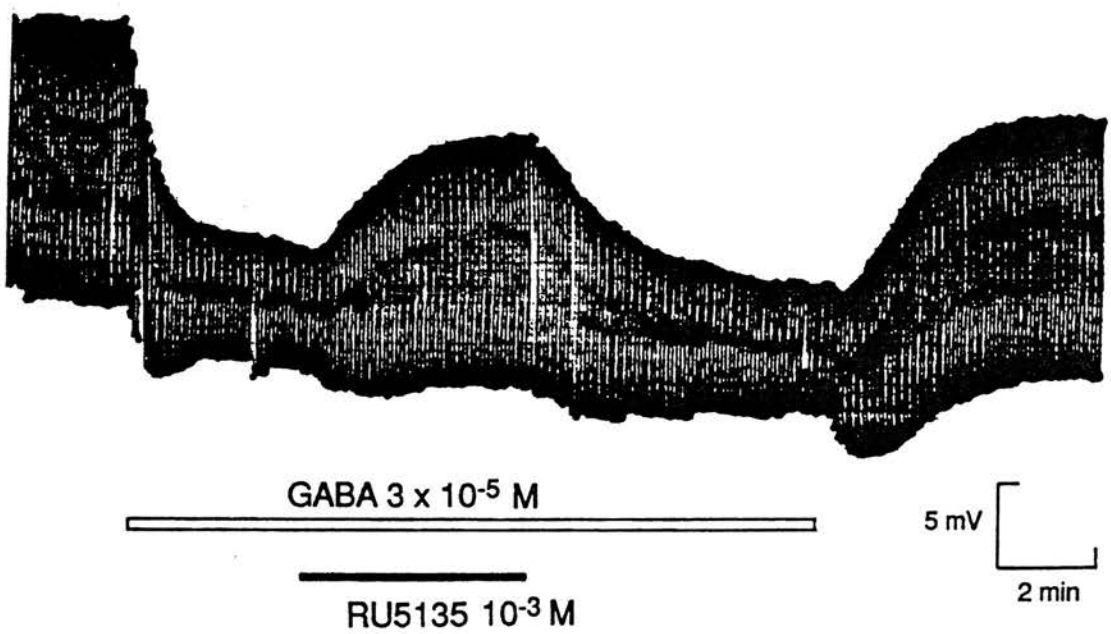

GABA 3 x 10-5 M
RU5135 10-3 M
5 mV
2 min

Figure 32: Dose-response curves to GABA and GABA + RU5135 at 70, 210 and 700µM.

Ordinate: change in membrane conductance Δg (µS).

Abscissa: GABA concentration (M), log scale.

Dose-response curves were obtained from the same cell and the experimental values were fitted by the modified Hill equation. The plain line describes the fitted modified Hill equation.

GABA control	EC50=28.1µM	Δgmax=3.8µS	N=2.1
GABA + RU5135 70µM	EC50=30.6µM	Δgmax=4.0µS	N=1.3
GABA + RU5135 210µM	EC50=100.4µM	Δgmax=3.7µS	N=1.5
GABA + RU5135 700µM	EC50=342.5µM	Δgmax=2.9µS	N=1.2

control
+ RU5135 70µM
+ RU5135 210µM
+ RU5135 700µM
Δg (µS)
4
3
2
1
0
-1
10
100
1000
Log[GABA] (µM)

preparations (see Section 1.B). But picrotoxin is usually potent at antagonizing GABA responses in invertebrates (see Section 1.B). An early voltage-clamp study reported that picrotoxin antagonized GABA applied by ionophoresis (Martin, 1982); anyhow the antagonism produced was small and not fully reversible.

Besides bicuculline and picrotoxin, other classical GABA-A antagonists were found inactive at the *Ascaris* GABA receptor: the competitive antagonists: securinine (10μM), pitrazepine (10μM), TBPS: (10μM) (Holden-Dye et al., 1988) and the channel blocker: dieldrin (100μM) (Colqhoun et al., 1989).

Picrotoxin and TBPS act at the channel level (see Section 1) and their inability to block the GABA responses in *Ascaris* suggests that the Cl channel itself differs from the vertebrate and even from most invertebrate GABA-associated Cl channels. These results indicate that the Cl channel in *Ascaris* may be also an interesting target for new anthelmintic compounds. Recently 5-nitro-2-(3-phenylpropylamino) benzoic acid (5-NPB), known to block Cl transport in renal tubules, has been shown to act as a non-competitive GABA-antagonist (IC50=69μM), in *Ascaris* (Colquhoun et al., 1989). However, 5-NPB is not a specific blocker of *Ascaris* GABA-gated Cl-channel, since it blocks also non-GABA gated Cl channels in vertebrates.

2. RU5135 acts as a weak non-competitive GABA antagonist in *Ascaris* muscle

RU5135 is known as a potent antagonist at the vertebrate GABA-A receptor: it is 500 times more potent than bicuculline in displacing [^{3}H]GABA from its binding site in rat brain membranes (Hunt and Clements-Jewery, 1981), and 200 times more potent in electrophysiological studies on rat cuneus nucleus (Simmonds and Turner, 1985). Like bicuculline, RU5135 inhibits [^{3}H]muscimol, [^{3}H]GABA binding and the enhancement of [^{3}H]diazepam binding by GABA, pentobarbitone and etazolate (Hunt and Clements-Jewery, 1981; Olsen, 1984; Simmonds and Turner, 1985). But RU5135 also interferes more strongly with the glycine receptor: <u>in vitro</u> with [^{3}H]strychnine binding (Hunt and Clements-Jewery, 1981; Simmonds and Turner, 1985), and <u>in vivo</u> on the firing of interneurones in the cat spinal cord (Curtis and Malik, 1985).

In *Ascaris* muscle, there is no evidence for glycine receptors (see Section 3 and Holden-Dye et al., 1988), RU5135 can therefore only interfere with the GABA receptors. This present study shows that RU5135 acts as a weak non competitive GABA antagonist at *Ascaris* GABA receptor. The concentrations required to block the GABA response in *Ascaris* muscle (IC50=117μM), are much higher than those required to block GABA responses in the rat cuneate nucleus (IC50=5nM) (Simmonds

Figure 31: Effects of GABA 30μM and GABA 30μM + RU5135 1mM on membrane input conductance.

Resting membrane input conductance go=2.3μS; GABA 30μM induced an increase in membrane conductance, Δg=3.0μS; when RU5135 is added, the membrane conductance decreased and stabilized at g=2.6μS, giving Δg=0.3μS; GABA recovered its full effect after RU5135 was washed away, Δg=3.0μS. (I=40nA).

Table 10: RU5135: Effect on dose-response curves parameters, EC50, Δgmax and N.

Mean and standard errors for EC50, Δgmax and N.
* and ** significantly different from the control GABA dose-response curve at $p \leq 0.05$ and $p \leq 0.01$ respectively.

	EC50 (μM)	Δgmax (μS)	N
GABA (n=5)	30.3±1.8	4.9±0.5	2.2±0.3
GABA+RU5135 70μM (n=5)	30.6±3.4	3.9±0.5	1.7±0.2
GABA+RU5135 210μM (n=5)	98.2±19.4**	4.4±0.3	1.6±0.3
GABA+RU5135 700μM (n=5)	315.6±26.9**	3.3±0.4*	1.4±0.1

and Turner, 1985).

The low potency of RU5135 as a GABA antagonist on *Ascaris* muscle, associated with the inactivity or low potency of other classical GABA-A antagonists (bicuculline, picrotoxin, TBPS, pitrazepine, securinine, SR95531 (Holden-Dye et al., 1988, 1989), confirms that the *Ascaris* GABA receptor differs pharmacologically from the vertebrate GABA-A receptor. Since on the basis of its agonist properties, the GABA recognition site in *Ascaris* is related to the GABA-A site, RU5135 should be able to bind the *Ascaris* GABA site and therefore should behave as a competitive antagonist. One possible explanation of its low potency is that its rigid GABA-like structure may not be well accepted by the GABA binding site in *Ascaris*.

CONCLUSION

All the classical GABA-A antagonists are inactive or have a low potency on the *Ascaris* GABA receptor. These results contrast markedly with the agonist properties, where *Ascaris* muscle GABA receptor and vertebrate central GABA-A receptors are correlated. The inactivity of non-competitive antagonists such as picrotoxin or TBPS can be explained by differences in the Cl channel and not in the GABA binding sites. However the inactivity of the competitive antagonists bicuculline, pitrazepine and securinine is surprising. One possible explanation is the existence of accessory binding sites (Ariëns, Beld, Miranda et al., 1979), responsible for the antagonists properties. Differences in accessory binding sites between *Ascaris* and vertebrate receptors might explain the inactivity of classical GABA-A competitive antagonists.

RU5135 was the only compound tested, found to antagonise GABA responses. But RU5135 acts as a weak non-competitive antagonist at the *Ascaris* muscle GABA receptor: IC50=117μM, being 20000 times less potent that in the vertebrate preparations (rat cuneate nucleus IC50=5nM, Simmonds and Turner, 1985). These observations suggest that the *Ascaris* GABA receptor differs pharmacologically from the vertebrate GABA-A receptors. These differences in antagonist properties might be exploited for therapeutic purposes.

The next Sections explore the antagonistic properties of pyridazine derivatives of GABA at the *Ascaris* GABA receptors, and further suggests the existence of different accessory binding sites responsible for antagonist properties in *Ascaris*.

SECTION 5:

THE EFFECTS OF THE ARYLAMINOPYRIDAZINE-GABA DERIVATIVES, SR95103 AND SR95531, ON THE *ASCARIS* MUSCLE GABA RECEPTOR: THE RELATIVE POTENCY OF THE ANTAGONISTS IN *ASCARIS* IS DIFFERENT TO THAT AT THE VERTEBRATE RECEPTOR.

INTRODUCTION

The previous sections have shown that, in terms of agonist potencies, the *Ascaris* GABA receptor is related to the vertebrate GABA-A receptors, in spite of some small pharmacological differences. However, in terms of antagonist profile, none of the classical GABA-A antagonists was potent. These results suggested that compounds with potential GABA-antagonist properties are certainly more promising in the search for a new and specific anthelminthic drug. This Section explores the antagonistic properties of two pyridazine derivatives of GABA, SR95531 and SR95103 (Figure 33), on GABA responses in *Ascaris* muscle using a current-clamp technique. The actions of arylaminopyridazine derivatives have been investigated in the mammalian central nervous system, where they are potent and selective competitive antagonists of GABA-A receptors (Chambon, Feltz, Heaulme et al., 1985); SR95531 is the most potent of the series. However, the action of this series of compounds remains to be fully explored in *Ascaris* and more generally in invertebrates.

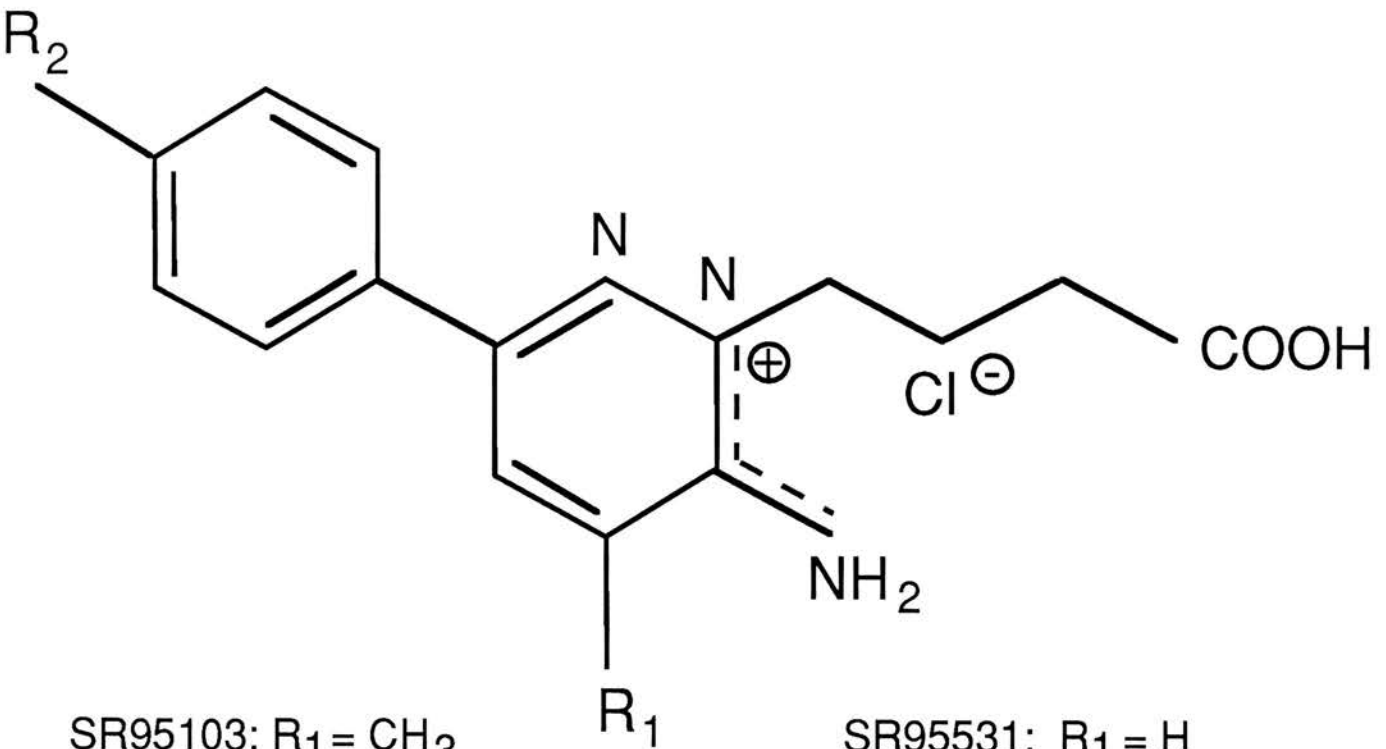
I METHODS

1. Muscle preparation

The muscle dissection has been described in the general methods and in Section 3. As for the experiments with GABA-agonists, the muscle flap was bathed in high-Cl, low-Ca Ringer (solution B), and maintained at 22ºC.

Figure 33: Chemical structure of SR95103 and SR95531

SR95103: R_1=-CH3, R_2=-H
SR95531: R_1=-H; R_2=-OCH3



SR95103: $R_1 = CH_3$
$R_2 = H$

SR95531: $R_1 = H$
$R_2 = CH_3O$

2. Drug application

2.1 Bath application

Because of the limited quantities of antagonist available, the muscle was not perfused continuously. The experimental chamber (2.5ml) was carefully drained, and then flushed with 5ml of the next drug solution. When dose-response curves were required, drugs were bath-applied in a cumulative manner, without intermediate washing between the concentrations (see Section 3).

2.2. Ionophoresis

In voltage-clamp experiments, GABA was applied ionophoretically as an cation from a micropipette (10-20MΩ) using a standard pulse of 300nA, 500ms (micro-ionophoresis programmer Model 160 WP instruments). GABA was used at concentration 2mM, pH=2.5 adjusted with HCl. A retaining current of 10nA was used. Ionophoresis does not allow an accurate estimation of the drug concentration; but it did permit an estimation of the voltage-sensitivity of GABA antagonism produced by an arylaminopyridazine derivative of GABA, SR95103. Ionophoretic application of GABA was chosen in order to deliver the GABA to a local area under control of the voltage-clamp, since the geometry of *Ascaris* muscle cells does not permit a total space clamping, and limits the area of the membrane clamped to the bag region (Martin, 1982).

3.Current-clamp

3.1. Technique

The technique used here, has been described previously in Section 3. In some experiments, signals were recorded with an Axoclamp 2A (Axon Instruments), which permits the use of higher resistance electrodes for current injection: typically 20-50MΩ.

3.2. Analysis

a. GABA

The effect of GABA was determined by measuring the change in input conductance. Dose-response relationships were described by the modified Hill equation (Nistri and

Constanti, 1979), and estimates for Δgmax, EC50 and N were obtained using a non linear regression programme (Patternsearch, Colqhoun, 1971). See Section 3 for details.

b. Antagonists

- Screening

Initially, the action of 1mM antagonist was examined on the change in membrane conductance induced by GABA 30μM. The percentage of antagonism was then estimated:

$$\% \text{ antagonism} = 100.(1-\frac{\Delta g_{ant}}{\Delta g_c})$$

where Δg_c is the change in membrane input conductance produced by GABA 30μM, and Δg_{ant} is the change in membrane input conductance produced by GABA 30μM + antagonist 1mM, measured after 10 minutes application of the antagonist. Compounds detected as reasonably potent antagonists with this method, were then tested at several concentrations on GABA dose-response curves.

- Quantitative evaluation

The effect of SR95103 was examined on GABA dose-response curves. Because of the natural variation of EC50, Δgmax and N, a control GABA dose-response curve was obtained from the same cell, before, or sometimes after the antagonist application. Dose-ratios were measured and, examined using a Schild plot (Arunlakshana and Schild, 1959) or a modified Schild plot (Williams et al., 1988).

. Schild analysis

When dose-response curves are parallel without apparent reduction in the maximal response, the dose-dependent nature of the antagonism is tested using a Schild plot (Arunlakshana and Schild, 1959):

$$\log(DR-1) = pK_B + \log(X_B)$$

where DR is the dose-ratio, pK_B is the negative logarithm of the dissociation constant of the antagonist-receptor complex and X_B is the concentration of the antagonist. However, this analysis does not account for cooperative interaction between GABA and its receptor (see Section 1.C).

. Modified Schild analysis

The dose-ratios were further examined using a modified Schild plot (Williams et al.,1988) which accounts for cooperativity between the agonist and its receptor:

$$\log(DR^N-1) = pK_B + M\log(X_B)$$

where N is the Hill coefficient, M is the number of antagonist molecules interacting with the receptor, and the other symbols are the same as before.

Although physically plausible models for the interaction of agonist and antagonists do not predict a simple or integral value for these plots, they provide a reasonable approximation if simplifying limitations can be applied (see Section 1, C.).

4. Voltage-clamp

Due to their morphology, the space-clamping in *Ascaris* muscle cells may not be complete (see Martin, 1982). Current responses following GABA application can be interpreted only if the activated receptors are in close proximity to the microelecrodes; this necessitates the use of a microapplication method.

4.1. Technique

The current electrode was pulled to a resistance of 10MΩ and the voltage electrode to 20-50MΩ. Microelectrodes were back-filled with Kacetate 2M, and were inserted into the bag region of a muscle cell. Signals were amplified by an Axoclamp 2A (Axon Instruments) used in voltage-clamp mode (TEVC). The gain was 100mV/mV. Capacity compensation and phase were adjusted for each cell to give the optimal voltage stability. The holding potential were -10mV, 0mV and +10mV. Voltage-steps to -80 or -90mV, were made for 500ms at 1Hz under control of an isolated stimulator Digitimer 2533. Signals were displayed on a Tectronix 2210 oscilloscope and on a Lectromed chart recorder.

4.2. Data-analysis

The peak responses at the holding potential and at the step potential were measured from the envelope of the current trace. The voltage sensitivity (Vs) of the antagonism was expressed as:

$$Vs = 1 - \frac{\%\text{ant } V_{Hold}}{\%\text{ant } V_{Step}}$$

where, $\%\text{ant}V_{Hold}$ = percent antagonism at the holding potential; and $\%\text{ant } V_{Step}$ = percent antagonism at the step potential

5. Statistical test

Means and standard errors were calculated for EC50,Δgmax, N values for GABA alone and in the presence of different concentrations of antagonist. Statistical significance was assessed using a two-tailed independent t-test.

II RESULTS

The results reported here are based on an analysis of experiments conducted on 18 muscle cells from 13 preparations. Cells selected for recording and analysis had resting membrane potentials greater than -20mV and resting input conductances between 1.9-4.0μS (2.5±0.1μS, mean±SE, n=18). Recordings were also rejected if the resting input conductance failed to return to at least 80% of the control.

1. SR95103 and SR95531 have no effect on membrane conductance

SR95103 and SR95531 (1mM) applied onto the muscle in the absence of GABA, did not produce any change in membrane conductance. The absence of effect of arylaminopyridazine GABA derivatives on membrane conductance is illustrated in Section 7, Figure 50.

2. SR95531 and SR95103 as GABA antagonists

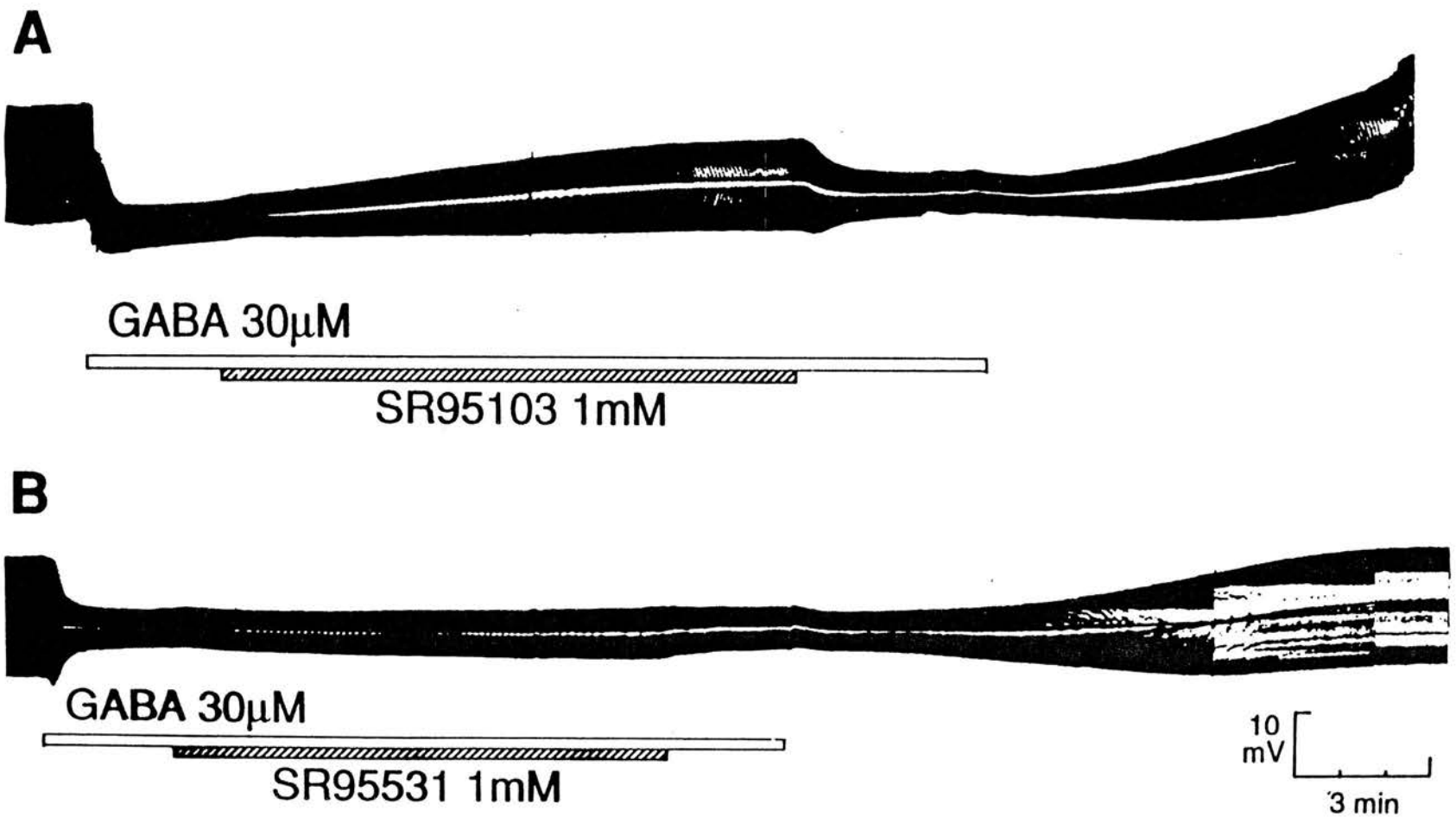
In mammalian preparations, SR95531 is the most potent arylaminopyridazine derivative GABA-A antagonist; it acts competitively and antagonizes electrophysiological responses to GABA (Chambon et al., 1985; Desarmenien, Feltz, Loeffler et al., 1987; Michaud, Mienville and Chambon, 1986; Hamann, Desarmenien, Desaulles et al., 1988), as well as inhibits GABA binding in rat brain preparations (Chambon et al., 1985; Heaulme, Chambon, Leyris et al., 1986a). In *Ascaris*, the application of 1mM SR95531 had little effect on conductance changes produced by GABA 30μM (Figure 34).

SR95103 is less potent than SR95531 at the GABA-A vertebrate receptor in rat brain membranes (Heaulme et al., 1986a; Heaulme, Chambon, Leyris et al., 1986b) and rat dorsal root ganglion cells (Chambon et al., 1985). This contrasts with the effects seen in *Ascaris* (Figure 34), where SR95103 1mM produces nearly complete antagonism. The antagonism reverses completely after washing. SR95103 reduced the

Figure 34: SR95103 is a more potent antagonist than SR95531

A: SR95103. The resting input conductance is 2.2μS. In the presence of GABA 30μM, Δg=4.4μS. SR95103 1mM antagonized 86% of the GABA response. The antagonism was completely reversed when the preparation was washed in 30μM GABA. (I=40nA).

B: SR95531. SR95531 was tested on the same cell. The resting input conductance was 2.2μS. In the presence of 30μM GABA, Δg=4.4μS. SR95531 1mM antagonized only 36% of the response to GABA. This effect was completely reversed after the preparation was washed in 30μM GABA. (I=40nA).


A
GABA 30μM
SR95103 1mM
B
GABA 30μM
SR95531 1mM
10
mV
3 min

response by 92.3±3.2% (mean±SE, n=5) and SR95531 reduced the response by 46.2±6.7% (mean±SE, n=5). To further characterize the properties of arylaminopyridazine derivatives of GABA on the *Ascaris* GABA receptor, the effects of various concentrations of SR95103 on GABA dose-response curves were examined.

3. SR95103 acts as a competitive antagonist

3.1.Dose-dependent nature of the SR95103 antagonism

The dose-dependent nature of the antagonism was tested by examining the effects of 100, 300 and 1000μM SR95103 on cumulative dose-response curves. Figure 35 shows the typical effects of 100μM SR95103 where there is a parallel shift to the right with no reduction in the maximum response. Both control and 100μM dose-response curves were obtained from the same cell. Table 11 shows pooled results with the mean and SE values for the parameters of the Hill equation used to describe the dose-response curves. It can be see that N and Δgmax were not significantly altered by the presence of SR95103, and EC50 was increased in a dose-dependent manner.

3.2. Schild-plots

Dose-ratios were determined from the change in EC50 values and a classical Schild plot obtained, Figure 36 (Arunlakshana and Schild, 1959). This was characterized by an apparent linear relationship between log(DR-1) and log(X_B), and an intersect at the abscissa giving an apparent pA_2 of 4.0. However this plot was characterized by a slope 0.45±0.15 (mean±SE, n=14) significantly less than one ($p \leq 0.05$, T-test). Thus the dose-ratios were smaller at higher antagonist concentrations than predicted by the Schild equation. One interpretation of this result is that SR95103 might not be acting in a competitive manner. However, the classical Schild analysis does not account for cooperative models of agonist action involving two agonist binding sites (Williams et al.,1988). A modified Schild analysis was therefore carried out, where it was assumed than two molecules of agonist were required to bind the receptor and the slope of the plot gives an indication of the stoichiometry for the interaction antagonist-receptor. The slope was 0.62±0.17 (mean±SE, n=14) and not significantly different from one ($p \leq 0.05$, t-test). These results are consistent with a model which involves the activation of the receptor by two molecules of agonist but only one molecule of antagonist is required to block this effect. Although these results do not establish such a mode of action for SR95103, it describes the interaction antagonist-receptor and permits the

Figure 35: Effect of SR95103 on the cumulative GABA dose-response relationship

(♦): control GABA responses and modified Hill fit: Δgmax=5.8μS, EC50= 24.4μM, N=1.7

(□): GABA dose response curve in the presence of 100μM SR95103, Δgmax=5.8μS, EC50=66.2μM, N=1.5.

Ordinate: change in input conductance. Abscissa: GABA concentration (log scale).

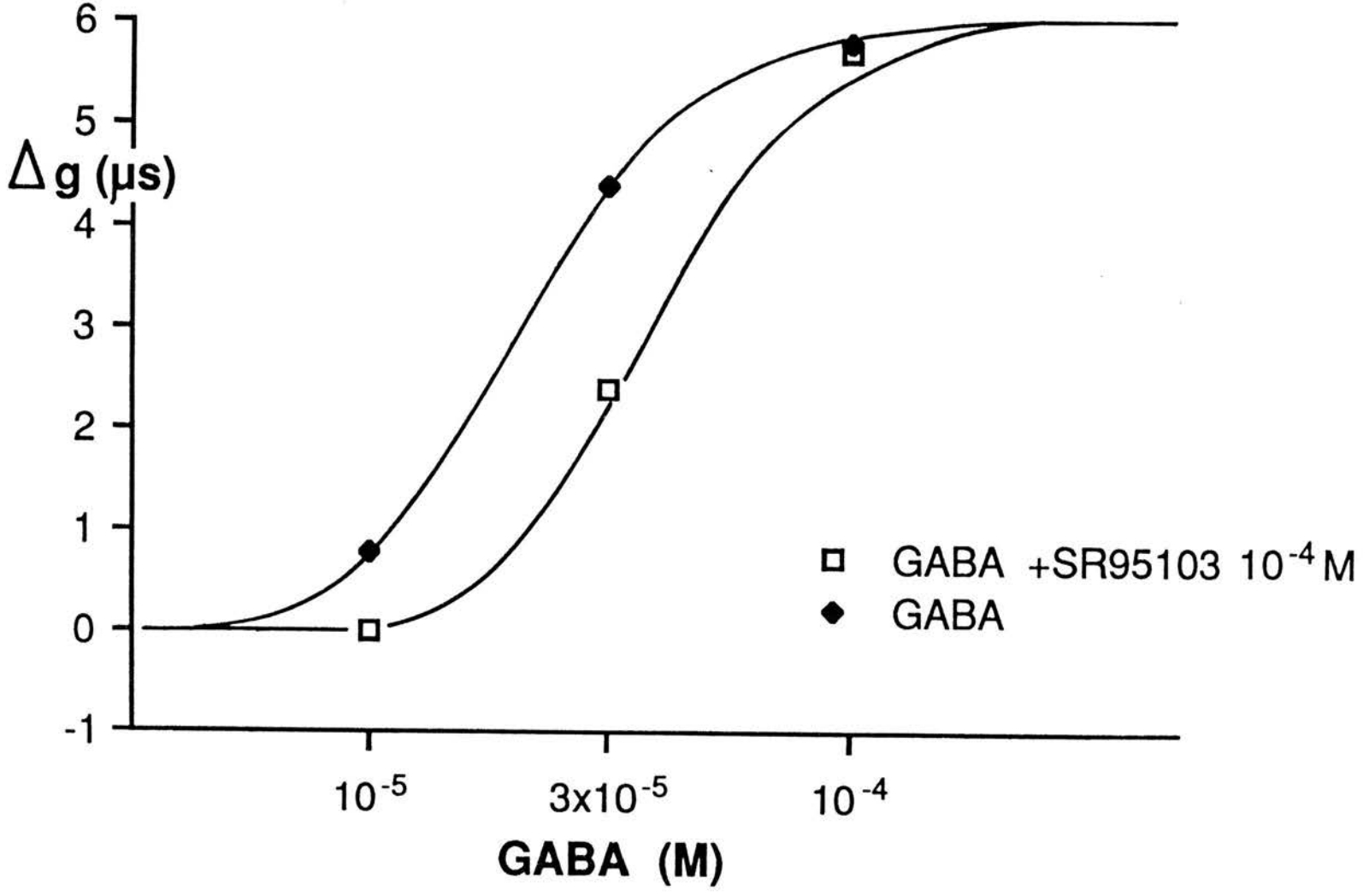

6
5
Δ g (μs)
4
3
2
1
0
-1
10-5
3x10-5
10-4
GABA (M)
GABA +SR95103 10-4 M
GABA

Table 11: SR95103: Effects on GABA dose-response curve parameters

Parameters for the modified Hill equation used to describe cumulative GABA dose-response curves in the presence of SR95103. ** indicates p≤0.05, T-test

	EC50 (μM)	Δgmax (μS)	N
GABA control (n=8)	24.9±3.3	6.1±0.1	2.6±0.3
GABA + 100μM SR95103 (n=6)	46.5±4.2**	5.1±0.6	2.3±0.3
GABA + 300μM SR95103 (n=3)	81.2±15.3**	5.5±0.6	1.7±0.7
GABA + 1000μM SR95103 (n=4)	119±5.7**	4.5±0.7	2.7±0.6

Figure 36: SR95103: Schild and modified Schild plots

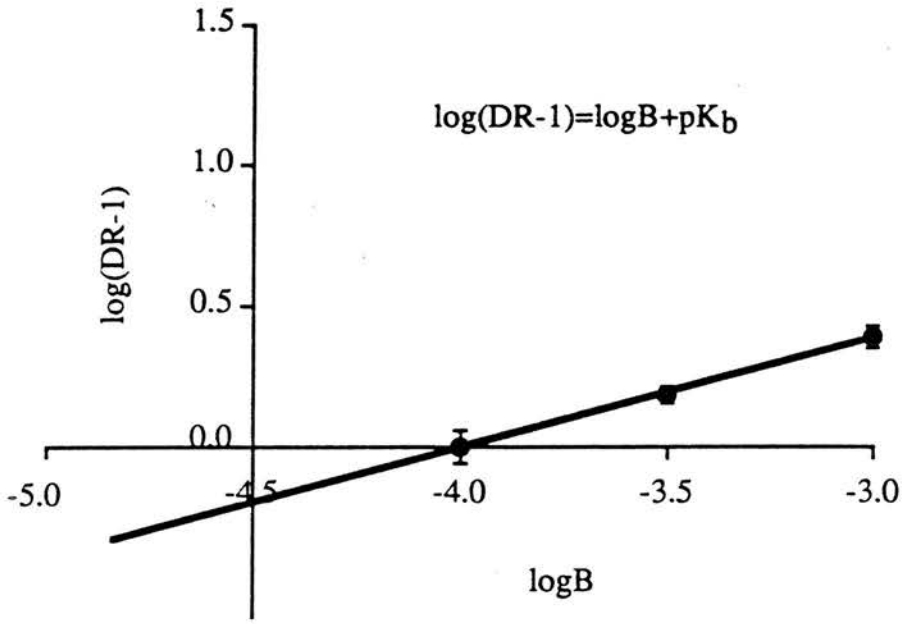
Schild plot:

The plot of log(DR-1) against $\log X_B$ appears linear with a slope of 0.45±0.15 (mean±SE, n=14). The slope is significantly less than one ($p \leq 0.01$, t-test). The linear regression value had a p value of 0.008 (F test). The goodness of the fit can be represented by the regression coefficient: $R^{\wedge 2}=0.46$.

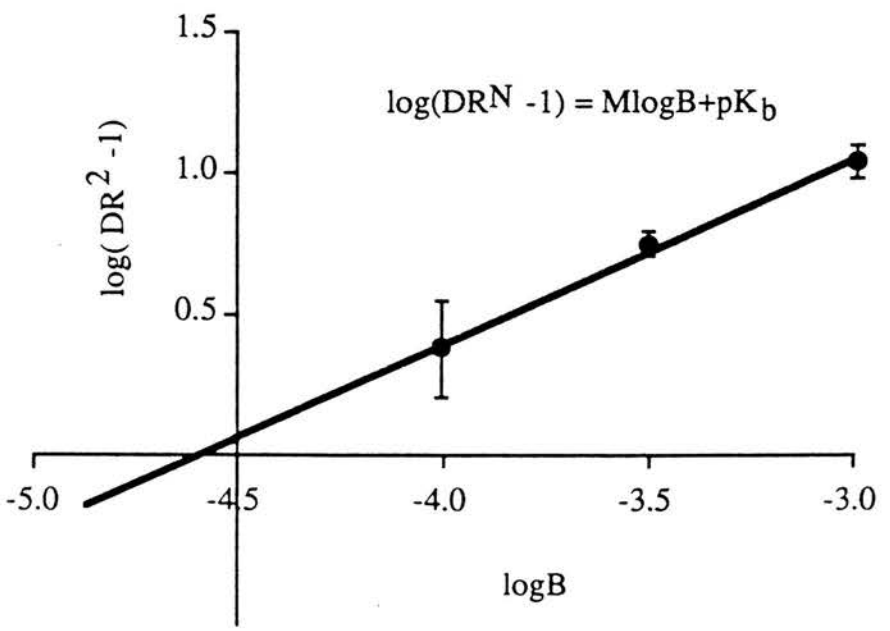
Modified Schild plot:

The plot of $\log(DR^2-1)$ against $\log X_B$ appears linear with a slope of 0.62±0.17 (mean±SE, n=14). The linear regression had a p value of 0.0041 (F test). The fit was better than for the Schild plot: $R^{\wedge 2}=0.51$.

Schild analysis



Modified Schild analysis



estimation of an apparent dissociation constant K_B, estimated from the modified Schild equation assuming N=2 and M=1:

$$K_B = \frac{DR^2-1}{X_B},$$

and K_B was 64±13μM (mean±SE, n=14).

The modified Schild plot also gave a better fit ($R^{\wedge 2}$=0.54), than the classical Schild plot ($R^{\wedge 2}$=0.51).

3.3. Voltage sensitivity

The two-microelectrode voltage-clamp technique with the ionophoretic application of GABA (Martin, 1982), was used to examine the voltage-sensitivity of the antagonism produced by bath application of 0.1-1mM SR95103. If SR95103 were acting predominantly as an anion to produce a channel block, then it would be expected that the degree of channel block would decrease at more negative potentials. However the antagonism showed little evidence of voltage sensitivity: Vs=0.05±0.17 (mean±SE, n=4).

II DISCUSSION

The main findings in this Section, are that: SR95103 is more potent than SR95531 as a GABA antagonist in *Ascaris* muscle with a pA_2 of 4.0 and an apparent K_B of 64μM. The study also shows that the potency of the arylaminopyridazine GABA analogues can vary at the *Ascaris* GABA receptor. In a previous study with SR95531 (Holden-Dye et al., 1988; 1989), it was also found that SR95531 was not a potent antagonist in *Ascaris*. However, it was suggested that this class of compounds does not recognize the *Ascaris* GABA receptor, although no other arylaminopyridazine GABA derivative was examined.

1. SR95103 is more potent than SR95531 in *Ascaris*: comparison with mammalian preparations

It has already been pointed out that GABA-A receptors may be heterogeneous (Levitan, Schofield, Burt et al., 1988; Pritchett, Luddens and Seeburg, 1989; see Krogsgaard-Larsen, 1988, for review). But it is still useful to compare the *Ascaris* receptor to a typical GABA-A receptor. The greater relative potency of SR95103 compared to SR95531 contrasts with the relative potency for the vertebrate GABA-A

receptor. In binding studies, SR95531 displaces [^{3}H]GABA from rat brain membranes with a global dissociation constant K_B=0.15μM (Heaulme et al., 1986a), and SR95103 has a K_B of 2.2μM (Chambon et al., 1985). In electrophysiological experiments on rat dorsal root ganglion cells (Chambon et al., 1985; Hamann et al., 1988), SR95531 has a pA_2 close to 7.0 and SR95103 a pA_2 close to 5.0. By comparison, SR95103 IC50=100μM, in *Ascaris* is only ten times less potent than in vertebrates (IC50=10μM estimated from Chambon et al., 1985).

SR95531 is similar in structure to SR95103 (Figure 33) but has a methoxy group on the 6-phenyl substituent of the pyridazine ring, and no 4-substituent. SR95103 has no substituent on the 6-phenyl, but has a methyl group in 4-position. The difference in structure and the greater relative potency of SR95103 on the *Ascaris* GABA receptor, indicates that its structural requirements for antagonist properties are different to the vertebrate GABA-A receptor. The lack of effect of the classical GABA-A antagonists: bicuculline (Holden-Dye et al., 1988), picrotoxin (Wann, 1987, Holden-Dye et al., 1988), pitrazepine, securinine, TBPS (Holden-Dye et al., 1988), also supports this view. Although GABA-A and *Ascaris* receptors appear to have similar structure-activity relationships for agonists (Holden-Dye et al., 1989).

Ariëns et al. (1979), have argued that the binding to accessory sites on hydrophobic regions of the receptor are responsible for the antagonists properties of competitive antagonists; the structure of the moiety binding to the GABA recognition site becomes less critical than for agonists. The structure-activity relationship for arylaminopyridazine GABA derivatives at the vertebrate GABA-A receptor (Wermuth, Bourguignon, Schlewer et al., 1987), is strongly in favour for the presence of accessory binding sites responsible for the antagonists properties of this series of compounds. The hypothesis of different accessory binding sites in *Ascaris* muscle would explain why the classical GABA-A antagonists are inactive whereas most of the GABA-A agonists are active. The evidence given here by SR95531 and SR95103 needs to be confirmed by a more complete structure-activity study of arylaminopyridazine GABA derivatives on *Ascaris* muscle. Further exploration of the pharmacology of these compounds in *Ascaris* GABA receptor may be useful for the design of new anthelmintic agents.

2. SR95103 acts as a competitive antagonist

The examination of the antagonism shown by SR95103 was not described by a classical Schild plot with a slope of one. However, this observation cannot be used to exclude a competitive mode of action (see Section 1.C). The modified Schild analysis

was consistent with two molecules of GABA, but one molecule of antagonist interacting with the receptor. Intuitively, this is reasonable, if binding of two molecules of GABA to different sites are required to open the channel and binding of a single molecule of antagonist at either site would be sufficient to prevent opening. Arylaminopyridazine GABA derivatives are competitive antagonists at the vertebrate GABA-A receptor. They displace [^{3}H]GABA or [^{3}H]muscimol from their binding sites (Chambon et al., 1985; Heaulme et al., 1986a; 1986b), and displace physiological dose-response curves to GABA to the right in a parallel manner without reduction of the maximal response (Chambon et al., 1985). [^{3}H]SR95531 labels specific binding sites, from which it is displaced by GABA, GABA-agonists and bicuculline, suggesting that the GABA binding site is involved. A patch-clamp study on bovine chromaffin cells, showed as well that SR95531 acted mainly as a competitive GABA-antagonist at the single channel current level (Hamann et al., 1988). Although SR95103 is less potent in *Ascaris* than in mammalian preparations, the same mechanism of action seems to be involved.

3. Only one molecule of SR95103 antagonizes the action of two molecules of GABA

Like the Hill equation, the Schild or modified Schild equations are based on simplified theoretical models of drug-receptor interaction. Such theoretical models may not be valid, but are believed to provide a reasonable approximation of antagonist-receptor interaction. Both equations involve the same hypothesis (see Section 1.C); the difference is that the modified Schild equation allows a stoichiometry greater than one for the agonist and/or antagonist interaction with the receptor. There is strong evidence from the agonists studies (see Section 3), that two molecules of GABA interact with the *Ascaris* GABA receptor. The modified Schild equation is therefore more appropriate than the classical Schild equation to describe the data. This seemed to be confirmed here by slope less than one for the classical Schild plot; and by the fact that the modified Schild analysis gave a better fit for the linear regression. The Schild plot and modified Schild plot were used in this study, to describe the interaction antagonist-GABA receptor. However, the K_B was estimated using the theoretical predicted equation, and not the fitted equation: i.e. with N and M fixed to integral values, according to the best estimates (Waud and Parker, 1971). This method limits the error made on K_B estimation.

Williams et al. (1988) applied the modified Schild equation to results obtained on the glutamate receptor. They reported that, apparently, only one molecule of the

glutamate antagonist 2-amino-5-phosphonopentanoic acid (AP-5) was required to block the effects of two molecules of quinolinate or NMDA. However, a stoichiometry of one means also that only one molecule of antagonist can actually bind the receptor. This is more difficult to explain especially since competitive antagonists are supposed to interact with the agonist binding site in the same manner than the agonist itself. One possible explanation is the non equivalence of the agonist binding sites. There is already biochemical evidence for the non equivalence of the two α subunits on the torpedo nicotinic receptor, and that an antagonist, MBTA, labels only one of the subunit (Raftery, Conti-Tronconi, Dunn et al., 1984). Purification of these subunits confirmed that they were not strictly equivalent (Raftery et al., 1984). In the case of the vertebrate GABA-A receptor, purification, sequencing, and cloning of the various subunits, predict that the GABA binding sites may be slightly different, since several types of subunits have been isolated, β1, β2, β3 (see Schofield, 1989, for review). However, if the two GABA binding sites were different in *Ascaris*, some agonists could be more selective for one site or the other. Such compounds would show a Hill number not greater than one. All the GABA agonists tested in Section 3, even the rigid ones, showed a Hill number greater than one, suggesting that they can bind both sites. However, this does not exclude the possibility of having two non-equivalent GABA-binding sites in *Ascaris*. It is however surprising that SR95103, which has a very flexible GABA moiety, can only bind one site. Structure-activity studies in vertebrate strongly suggested that the antagonist properties of arylaminopyridazine derivatives of GABA rely on the presence of accessory binding sites (Wermuth et al., 1987). It is conceivable that only one set of accessory binding sites is present on the receptor; so that only one molecule of antagonist can bind effectively the receptor. Another plausible explanation is that the presence of one large molecule of SR95103 bound to the receptor, blocks the access for another antagonist molecule to the second GABA binding site. All three explanations are plausible, but the existence of only one set of accessory binding sites on the receptor seems the most attractive. Identification and study of these putative sites, may be achieved by examining the protein domains of each subunits.

CONCLUSION

The *Ascaris* GABA receptor appears under two aspects: like the GABA-A receptor in terms of agonist properties, and unlike the GABA-A receptor in terms of antagonists properties. The Ariëns theory of accessory binding sites would explain the lack of

potency of the competitive antagonists bicuculline, pitrazepine, securinine and the order of different potency found with SR95531 and SR95103.

The quantitative analysis of SR95103 antagonism is consistent with a competitive mechanism where only one molecule of SR95103 is required to block the action of two GABA molecules. In order to obtain more informations on the type of antagonism involved, the actions of SR95103 were explored at the single channel level and are the subject of the next section.

SECTION 6:

EFFECTS OF SR95103 ON GABA-ACTIVATED SINGLE-CHANNEL CURRENTS FROM *ASCARIS SUUM*

INTRODUCTION

In the previous section the action of SR95103 on the GABA receptor of *Ascaris* muscle has been examined using a current-clamp technique. SR95103 was shown to produce a parallel shift to the right in conductance dose-response curves, but that the classical Schild plot (Arunlakshana and Schild, 1959) had a slope less than one. This observation however did not exclude competitive antagonism since it was possible to use a modified Schild plot (Williams et al., 1988) to describe the observations. In order to investigate further the action of SR95103 on *Ascaris* GABA receptors, a patch-clamp study was carried out to look at effects at single-channel level. It appears that most of the antagonism was competitive but evidence for a small non-competitive component is seen at high concentrations.

I METHODS

1. Preparation

The muscle dissection has been described in the general methods Section. Clean membranes were obtained by collagenase treatment (350UI/ml). The enzyme was applied for 20-30 minutes in extracellular solution without added calcium (solution D) and the temperature was maintained at 35°C. The enzyme treatment was stopped when vesicules started to form at the surface of the muscle cells membranes. The preparation was then maintained in extracellular solution (solution E) and at 22°C. Patch-pipettes were filled with intracellular solution (solution F). So an outside-out patch has a cation concentration of 149mM on both side of the membrane; an outside Cl concentration of 51mM (extracellular concentration), and an inside Cl concentration of 153mM (intracellular concentration), giving a Nernst potential for Cl of +27.9 mV. GABA-activated Cl channels were recorded with a membrane potential of -50mV, the resulting driving force for Cl ions corresponded to a difference in potential of -77.9mV. EGTA was used in the pipette solution to buffer the intracellular concentration of Ca and limit

the occurrence of Ca-activated Cl channels present in *Ascaris* muscle cell membrane (Thorn and Martin, 1987).

2. Drug-application

Drugs were applied via a perfusion system in the small experimental chamber containing the membrane patch. After the formation of an outside-out patch, the patch pipette was transferred to a small chamber which could be isolated from the rest of the preparation by a separation so that the whole muscle flap was not exposed to the drug.

3. Patch-clamp

3.1. Technique

a. Outside-out patch formation

Standard patch-clamp techniques were used to obtain outside-out patches (Hamill, Marty, Neher et al., 1981). The formation of an outside-out patch is illustrated in Figure 37. A giga-seal (resistance greater than 1 GΩ) was obtained by gentle suction applied to the electrode. A high voltage step (100mV) was then applied to the patch electrode, to break the membrane. The reading of a negative patch-pipette potential allows to check that the membrane had broken and the electrode had penetrated the cell (the potential was close to the value of membrane potential, -20 to -30mV). Once inside the cell, the patch-pipette was slowly lifted up and the membrane reformed a giga-seal but in an outside-out conformation.

b. Recording technique

Patch electrodes were made from micro-haematocrit capillary tubes (Garner glass 7052) pulled on a two stage vertical puller (David Kopf) and had a resistance of 1-5MΩ. Sylgard™ was used to coat electrodes, to improve the frequency responses (typically greater than 2kHz). Currents were recorded with a List EPC-7 and a modified Sony Betamax video system. An agar-plug electrode made with Agar 150mM KCl set in a polythene tube, was used to minimize junction potentials.

c. Data-processing

Records on tape were played back into a CED 1401 interface, connected to a DCS 286 PC computer and under the control of PAT software (Dr J.Dempster, Strathclyde University). Records were filtered by a laboratory made 8-pole Bessel filter, -3dB at

Figure 37: Diagram illustrating the making of an outside-out patch

(From Ogden and Stanfield, 1987) A gigaseal is formed by contact with the membrane and a slight suction applied to the micropipette. A high voltage pulse (100mV), is then applied to break the membrane. Once inside the cell, the micropipette is then carefully lifted until the membrane reseal in the outside-out conformation.

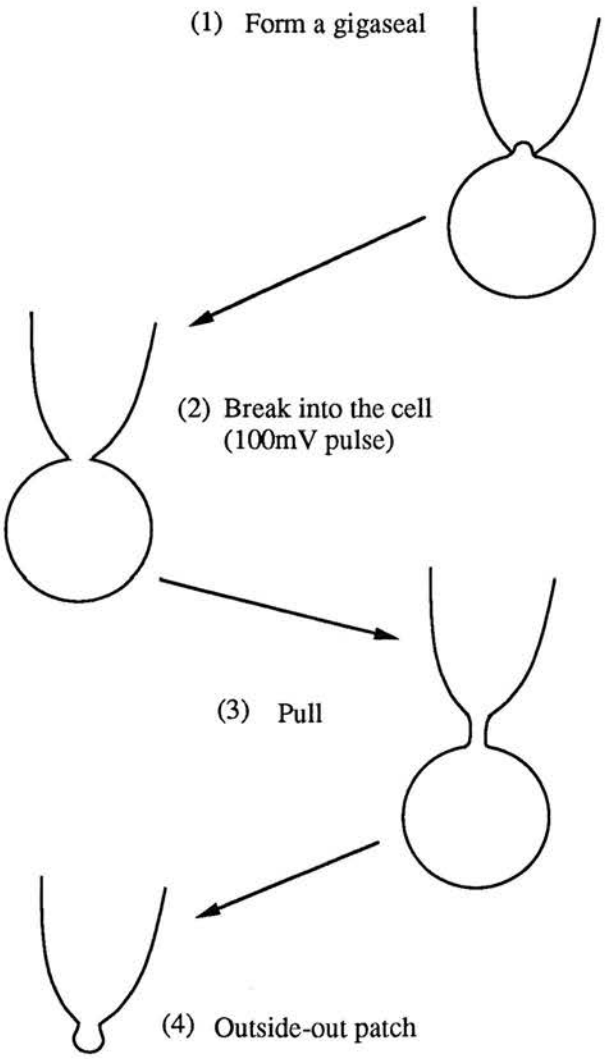
(1) Formation of a gigaseal by gentle suction

(2) Breaking of the membrane by applying a high-voltage pulse

(3) Reseal (whole cell conformation)

(4) The pipette is gently pulled and a membrane patch is formed in the outside-out conformation.

Formation of an outside-out patch



1kHz, and sampled at an interval of 100μs. When the signals were filtered at 1kHz (-3dB), the rise-time of the 8-pole Bessel filter was 0.36ms and the dead-time of the system was 0.20ms.

The PAT software was used to obtain current amplitude histograms which were used to measure proportions of the time when 1,2,3,...N channels were open. Records with less than 5% of all openings appearing as simultaneous openings were selected and used to measure channel open- and closed-times; the double openings were edited and not used for analysis. The threshold for detection was set at 50% of the unit channel current. This method of detecting the transition between open- and closed-times is different to that described previously on the same preparation (Martin, 1985). The "effective mean open times" described previously (Martin, 1985) approximates to the mean "long" bursts in this study. The PAT software produced files of open and closed durations, which were transferred to a mainframe computer for further analysis.

3.2. Data analysis

Before any kinetic analysis, the records must be checked for stationarity. Non-stationarity of channel behaviour can be defined as a systematic drift in the channel open- or closed- states durations. Channel behaviour by definition is a probabilistic process so that open and closed states durations vary, but they do so in a non systemic way.

a. Stationarity

It was possible to examine open times and closed times in sequence and to test for changes in their properties with time. Desensitization, for example, may produce after a delay, an increase in the mean closed-time and a reduction in the probability of the channel being open. CUSUM plots (Glasbey and Martin, 1986) were used to test for time dependent changes in open and closed durations:

$$Sj = \sum_{i=1}^{j} X_i - X_{mean}$$

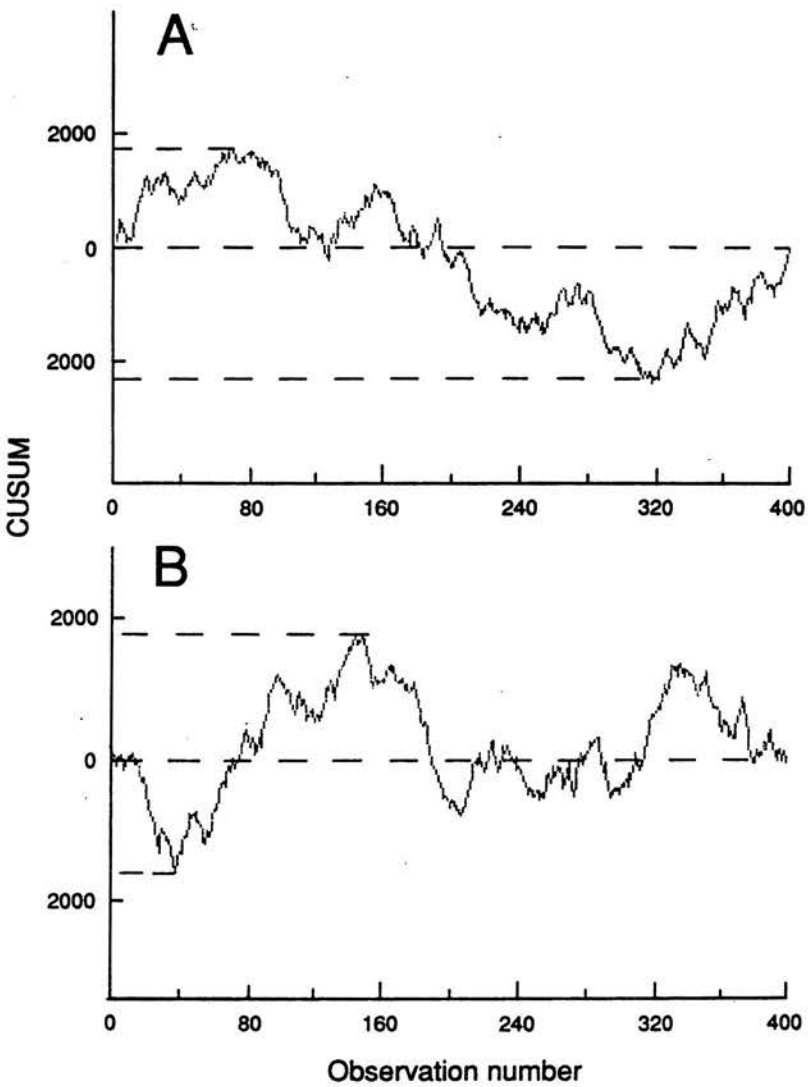
for j=1,...,n; where X_i is the rank order of the ith closed time, X_{mean} is the mean rank. A random fluctuation about 0 is expected for channels which show stationarity behaviour. The significance of the deviation may be tested by determining D_α, the maximum value of $\frac{S_j}{1+(\alpha-1).\frac{j}{n}}$ where α=9, 1 and 1/9 and then estimating the three p values for the maximum deviations: $p = 2 \exp[\frac{-24\alpha D_\alpha^2}{n^2(n+1)}]$ (see Glasbey and Martin,

Figure 38: CUSUM plot of closed and open times

A: Closed times; ordinate, CUSUM; abscissa, observation number. The p values were 0.17, 0.95 and 0.13. The closed times were considered to be in random order.

B: Open times; ordinate, CUSUM; abscissa, observation number. The p values were 0.12; 0.89; and 0.39. The open times were considered to be in random order.

CUSUM = normalized deviation of event duration


A
2000
0
2000
CUSUM
0
80
160
240
320
400
B
2000
0
2000
0
80
160
240
320
400
Observation number

1986). An example of these plots is shown in Figure 38; it can be seen that there is no evidence of change in channel properties throughout the record. This and similar observations on other patches, showed no evidence of non stationarity throughout the records.

b. Independence of channel open probability

If channels in a patch have the same probability of being open and are independent, then the distribution of the proportion of time, P_r, that 0,1,2,...N, channels are open will be described by the binomial distribution:

$$P_r = \frac{N!}{r!(N-r)!} p^r (1-p)^{N-r}$$

where p is the probability that an individual channel is open and N is the number of channels present. The probability (p) of an individual channel being open was calculated from the areas under the amplitude histograms. If the proportions of total recording time spent with 1,2,....N, channels open were $T_1, T_2, ...T_N$ then:

$$p = \frac{T_1 + 2T_2 + ... + NT_N}{N}$$

The fraction of time which r channels and only r channels were open was calculated from the T values. The binomial distribution was the used with the value of p to predict the proportion of time with only 0,1,2,...N channels open.

c. Exponential curve-fitting

In these experiments, individual channel activations were interrupted by short closed-times. Bursts of openings were therefore defined as groups of openings separated by closed times shorter than a specified time, T_{crit}. T_{crit} was chosen to make the proportion of long closing misclassified (as short) equal to the proportion of short intervals misclassified (as long), by solving numerically the equation:

$$1 - e^{(Tcrit/Ts)} = e^{(-Tcrit/Tm)}$$

(Colquhoun and Sakmann, 1985), where T_s and T_m are short and intermediate time constants in the distribution of all closed times. An interval of 1-2ms was used so that only brief closings were classified as gaps in bursts. After T_{crit} had been determined, the files of open and closed times were rearranged to give files of burst durations.

The next stage in the analysis was the fitting of exponentials to the distributions of closed and burst durations. Only events greater than 0.5ms (twice the dead time) were used to fit 1,2,3 or 4 exponentials to each data file to see which was the best fit. A maximum likelihood procedure was used to estimate the parameters of the probability density function (p.d.f.) which best describes the distributions:

$$\text{p.d.f.} = \sum_{i=1}^{k} \frac{ai}{Ti} . e^{(-t/Ti)}$$

where a_i represents the area of the i^{th} component ($\sum a=1$), T_i is the fitted time constant, t is the time in milliseconds and k is the number of exponentials fitted. A decrease of three log units with each additional component fitted was taken as a significant improvement in the fit.

d. Correction for missed events

After the exponentials had been fitted to the burst and closed times, it was possible to correct for missing events, using the method described below (Colquhoun and Sakmann, 1985). It was assumed that very short closings and openings will be missed in the usual analysis of open and closed times because of the limited resolution of the recording technique. The number of short events was estimated by extrapolation from the data which had been recorded.

- The total number of closed times and burst times were estimated from the observed number of events; this was done separately for the closed and burst times by the use of the following equation:

$$N = \frac{n}{\sum_{i=1}^{k} a_i (e^{(-tmin/Ti)} - e^{(-tmax/Ti)})}$$

where n is the number of observed events greater than the threshold time (t_{min}=0.5ms) during the recording period (t_{max}) and N is the estimated number of events; a_i and T_i are the area and time constant of the i^{th} component, respectively.

- The total time spent in gaps within bursts m_{TS}, i.e., gaps shorter than T_{crit}, including those undetected, was calculated using:

$$m_{TS} = N_{gaps} . [\sum_{i=1}^{k} a_i T_i - a_i (T_{crit} + T_i) . e^{(-Tcrit/Ti)}]$$

where N_{gaps} is the total number of closed times estimated previously; the other symbols have defined above and refer to the closed times.

- The corrected mean burst duration m_b, was calculated using:

$$m_b = \sum_{i=1}^{k} a_i.T_i$$

where a_i and T_i refer to the areas and time constants of the two components of the burst duration distribution.

- The corrected total open time C_{open}, was calculated using:

$$C_{open} = (N_{burst}.\ m_b) - m_{TS}$$

where N_{burst} is the estimated total number of bursts.

- The corrected mean open time C_{mot}, was calculated using:

$$C_{mot} = C_{open}/N_{gaps}$$

where N_{gaps} is the estimated total number of gaps. The corrected mean open time estimated in this way is shorter than the mean open time estimated in any other way because it allows for missed short events and reduces the mean value.

- If it is assumed that there are few gaps in the "short" bursts, the corrected mean length of an individual opening in a long burst is then:

$$m_{io} = \frac{N_{\text{"long" bursts}}.\ T_{\text{"long" bursts}} - m_{TS}}{N_{\text{short gaps}} + N_{\text{"long" bursts}}}$$

where $N_{\text{"long" bursts}}$ is the estimate number of long bursts, $T_{\text{"long" bursts}}$ is the mean duration of the long bursts and $N_{\text{short gaps}}$ is the estimated total number of short gaps.

II RESULTS

The following report is based on the analysis of experiments performed on 11 outside-out patches obtained from separate parasites.

1. GABA (3μM) activated channels

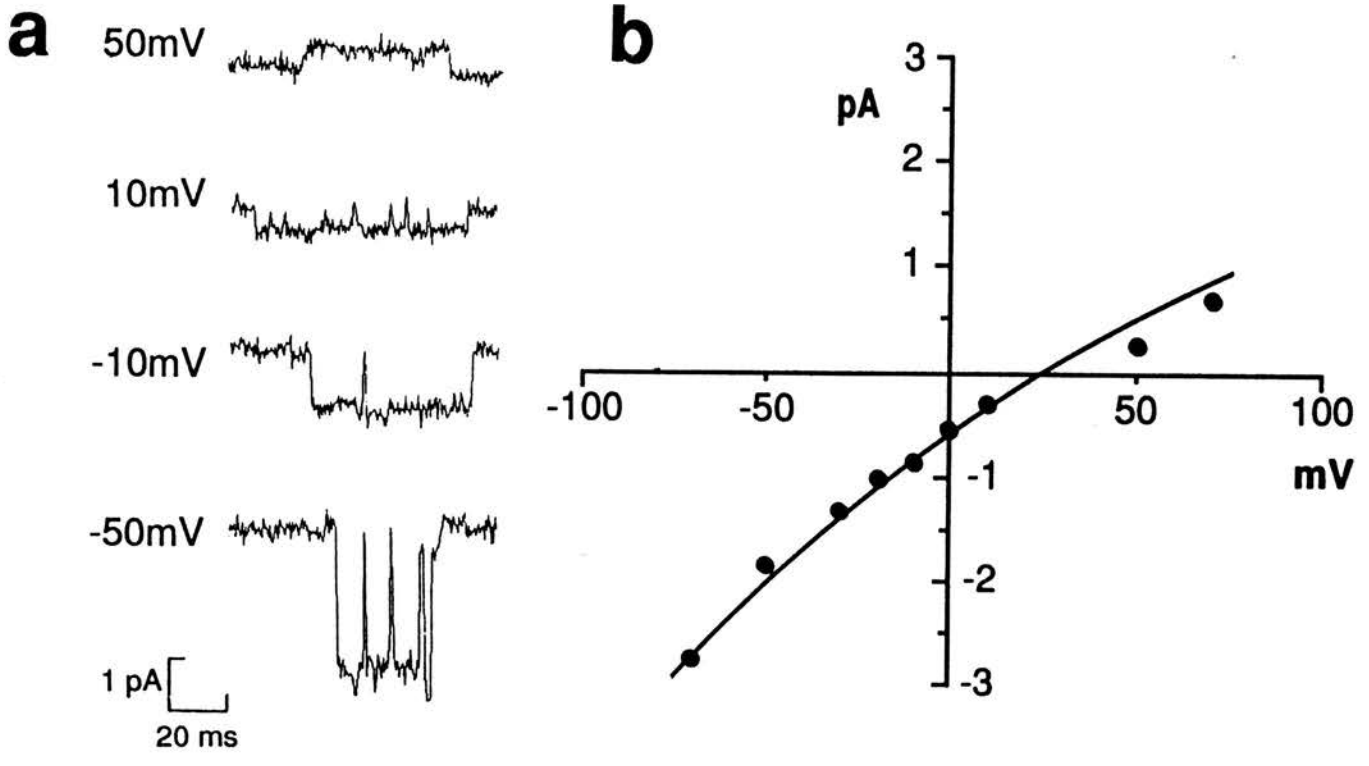
1.1. Single channel current-voltage relationship

Non-symmetrical Cl concentrations across the patch (41mM outside, 153mM inside) were used throughout this series of experiments to increase the amplitude of the single channel currents (at -50mV) and to further test the identity of the ions flowing through the channels. Figure 39 shows a representative I/V plot of the main (most frequent) conductance state seen in a particular patch. The plot shows inward rectification and a reversal potential of +27mV; the rectification and a reversal potential close to this is predicted by the Goldman constant field equation (Hille, 1984a) for a Cl

Figure 39: Main state GABA channel I/V relationship

A: Channel openings to the main state at four potentials from +50 to -50mV.

B: Plot of main state channel currents at different potentials. The reversal potential was near +27mV, the Nernst Cl potential. The solid line shows a plot of the I/V relationship predicted by the Goldman-Hodgkin-Katz constant fields equation with a single channel permeability of 6.3 $10^{-14}cm^3s^{-1}$. Note the inward rectification.


a
50mV
10mV
-10mV
-50mV
1 pA
20 ms
b
pA
3
2
1
-1
-2
-3
-100
-50
50
100
mV

ion-channel under these conditions. The Cl estimated permeability for Cl is $6.3x10^{-14}cm^3s^{-1}$, assuming that the permeability for other ions is insignificant.

1.2. Main state conductance

GABA channels in *Ascaris* are characterized by the presence of more than one conductance state (Martin, 1985). Figure 40 shows example of the main conductance state and two less frequently observed subconductance states. The main state at -50mV had an amplitude of 1.7pA, which corresponds to a chord conductance of 22pS, if a driving force of 77mV is assumed. The two subconductance states had amplitude of 1.0 and 0.5pA at -50mV (conductances of respectively 13 and 6.5pS). In the following analysis, the main state conductance was used because it occurred most frequently. The small subconductance state was automatically rejected during analysis, whereas records had to be edited manually in the case of the larger subconductance state since its amplitude: 1.0pA corresponded to the threshold set for open and closed states detection.

1.3. Channels have the same Popen

The application of 3μM GABA to outside-out patches produced the simultaneous opening of up to three channels. If GABA channels in a patch have the same probability of being open and are independent, then the distribution of the proportion of time, P_r, that 0,1,2,...N, channels are open will be described by the binomial distribution. Figure 41 shows predicted and observed values of P_r from a typical patch record with three channels present, and p=0.082. It can be seen that observed and predicted values are close; this supports the hypothesis that the channels behave independently and have the same open probability. Similar results were obtained from all the patches. As a result of these observations, the GABA channels were assumed to be homogenous.

2. Effects of SR95103 on GABA activated channels

2.1.Reduction of channel opening frequency

a. SR95103 decreases the opening frequency

Figure 42 shows the inhibitory effect of SR95103 on opening frequency of GABA-activated channels at -50mV. At this time scale, openings are seen as clusters of inward

Figure 40: GABA channel main state and subconductance states

The closed state (C); the 0.44pA subconductance state, (S_1); the 1.45pA subconductance state, (S_2); and the 2.24pA main state conductance (at -50mV).

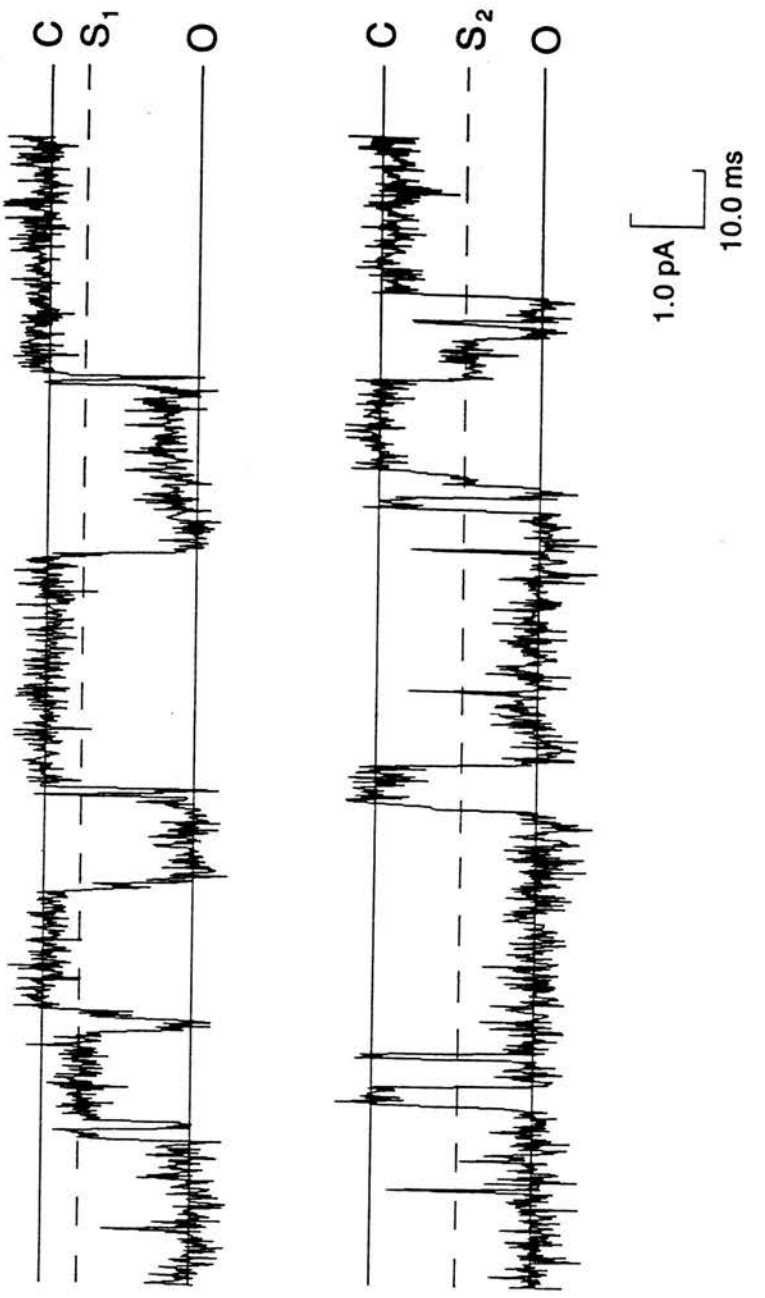

C
S1
O
C
S2
O
1.0 pA
10.0 ms

Figure 41: Independence of the channels

Observed and binomial distribution of the proportion of time channels were, predicted to be, open in a patch. The horizontal bars are the observed values for the % time spent with 0,1, 2 and 3 channels open. The arrows indicate the values predicted by the binomial distribution with n=3, the total number of channels present in the patch, and p=0.082, the probability of channel opening.

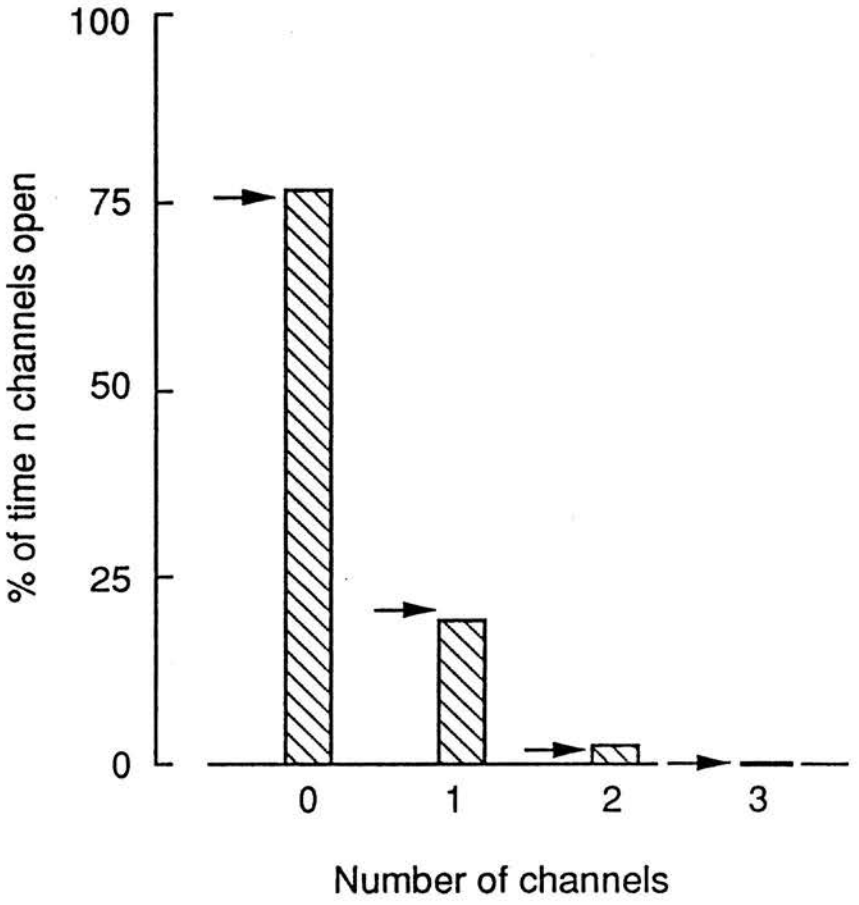

100
75
50
25
0
% of time n channels open
0
1
2
3
Number of channels

Figure 42: Inhibitory effect of SR95103 on GABA channel opening

A: control; GABA 3μM, Popen=0.036

B: GABA 3μM + SR95103 30μM, Popen=0.025

C: GABA 3μM+SR95103 100μM, Popen=0.001

D: wash, GABA 3μM alone

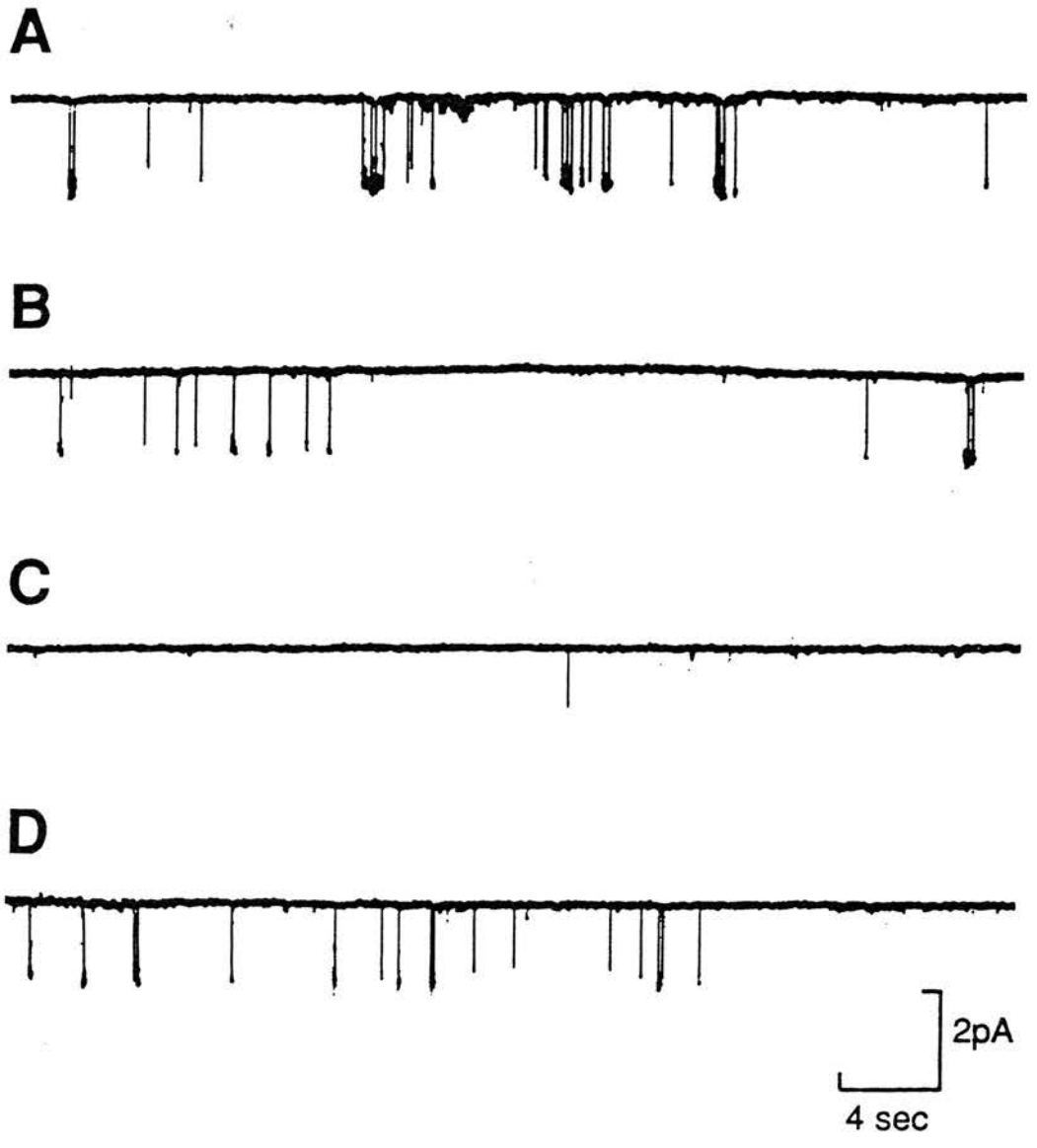
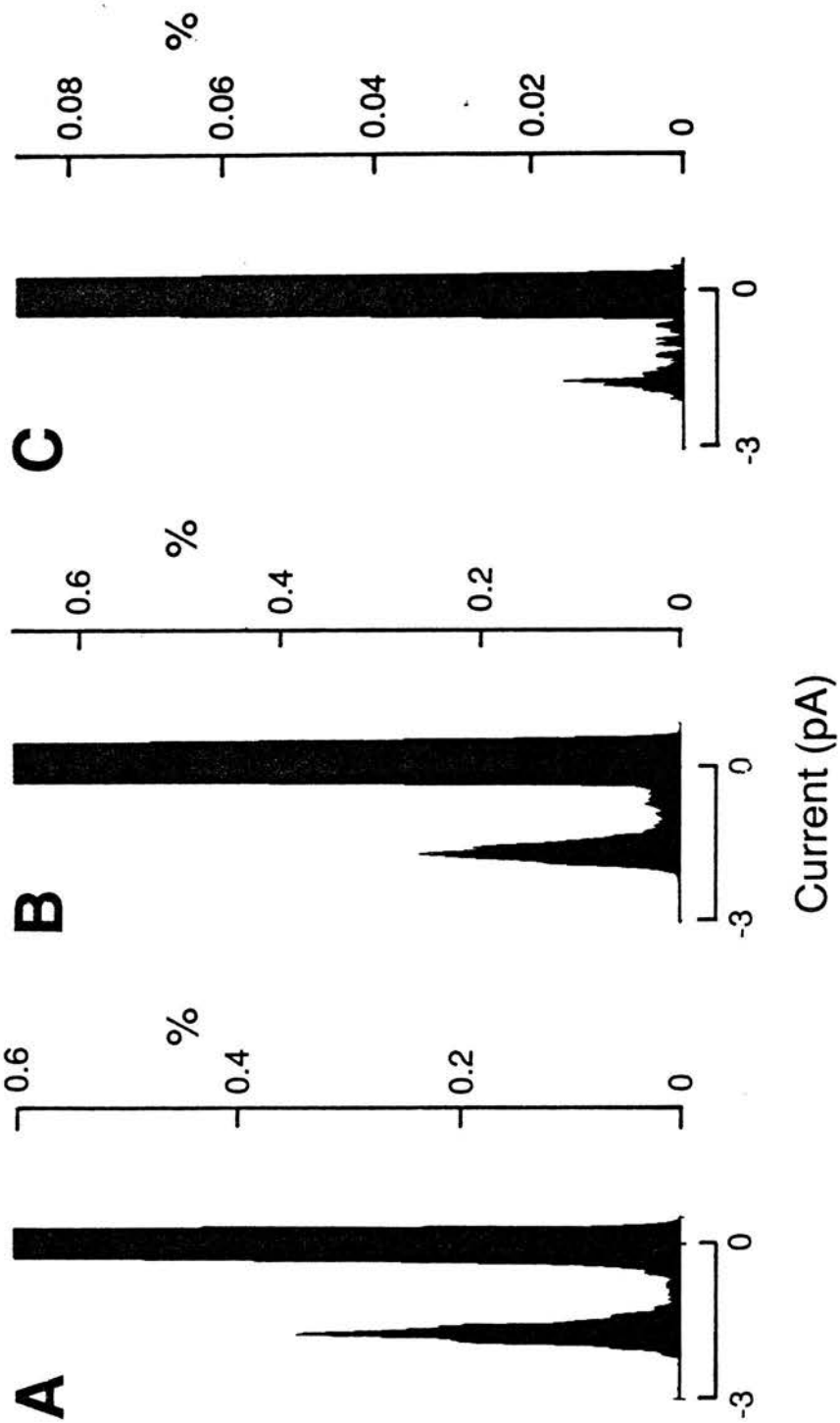

A
B
C
D
2pA
4 sec

Figure 43: GABA channel current-amplitude histograms and the lack of effect of SR95103

A: Control, GABA 3μM, I=-1.74±0.02pA (mean±SE)

B: GABA 3μM + SR95103 30μM, I=-1.64±0.04pA (mean±SE)

C: GABA 3μM + SR95103 100μM, I=-1.72±0.03pA (mean±SE)


A
B
C
%
0.6
0.4
0.2
0
0.08
0.06
0.04
0.02
-3
0
Current (pA)

current spikes (Figure 42A). SR95103 30μM reduced the frequency of opening (Figure 42B). The addition of SR95103 100μM further reduced the frequency of opening (Figure 42C). When the antagonist was washed from the preparation, the opening frequency returned to normal (Figure 42D). The proportion of time an individual channel is open can be estimated from the current histogram (see above). In this experiment illustrated in Figure 43, the control p was 0.036 (Figure 43A); in the presence of 30μM antagonist p was reduced to 0.025 (Figure 43B) and in the presence of 100μM antagonist, p was 0.001 (Figure 43C). In order to compare the level of antagonism between the experiments, the percentage antagonism was defined as:

$$\%\text{antagonism} = 100.(1 - \frac{P_{sr}}{P_c})$$

where P_{sr} is the probability of an individual channel being open in the presence of SR95103, and P_c is the control probability in the absence of antagonist. In the presence of SR95103 30μM, the percentage of antagonism was 53±11.6% (mean±SE, n=4); in presence of SR95103 100μM, it was 77.8±8.2% (mean±SE, n=5). The degree of antagonism was shown to be dose-dependent.

b. The modified Hill equation describes the action of SR95103

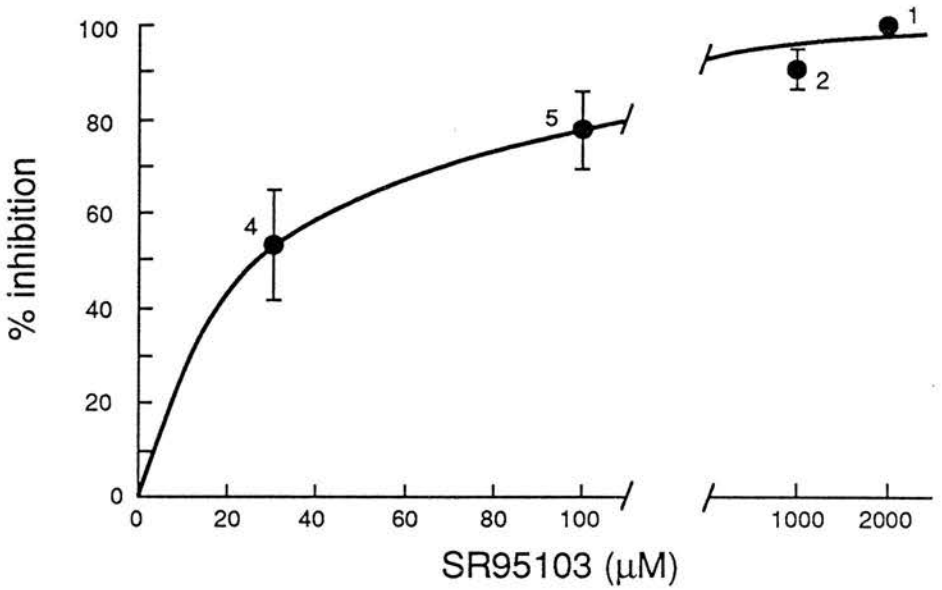
Figure 44 shows the dose-response relationship for the percentage of antagonism and the fitted modified Hill equation. With 3μM GABA as the agonist, the Hill coefficient was 0.98, suggesting that only one molecule of SR95103 combines with the GABA receptor; the concentration producing half saturation, IC50, was, 24μM. The estimated percentage of inhibition at saturating concentrations of antagonist was 97%. The fact that the maximum antagonism is near 100% suggests that most, but perhaps not all of the antagonism is produced by an action at a single site on the GABA receptor.

2.2. Effect on channel amplitude

The lack of effect of SR95103 on the amplitude of GABA single-channel currents is illustrated in Figure 43, which shows the current amplitude histograms for the traces shown in Figure 42. The current amplitude histograms were fitted and described by Gaussian distributions. The control amplitude was -1.74±0.02pA (mean±SE) (Figure 43A); in the presence of SR95103 30μM it was -1.67±0.04pA (mean±SE) (Figure 43B); in the presence of SR95103 100μM, it was -1.72±0.03pA (mean±SE) (Figure 43C). Similar results were obtained for all the other experiments. It was concluded that SR95103 does not affect single-channel conductance.

Figure 44: Percent inhibition dose-response curve

Ordinate: percent inhibition in the presence of 3μM GABA of opening probability
Abcsissa: SR95103 concentration.
The line fitted was a least-squares estimate of parameters for the modified Hill equation: maximum percent inhibition, 97%; IC50, 24μM; N, 0.98. The number besides each mean value is the number of experiments performed with this concentration of antagonist.


% inhibition
100
80
60
40
20
0
0
20
40
60
80
100
1000
2000
SR95103 (μM)
1
2
4
5

2.3. Effects of SR95103 on burst distributions

Bursts were defined as the total duration of a sequence of openings and closings, with the openings separated by closings less than Tcrit, a burst always starting and ending by an opening. With a higher time resolution it can be seen that the burst duration was reduced in the presence of SR95103 100μM, but not at 30μM (Figure 45). The main apparent effect was a reduction in the mean burst length at 100μM concentration of the antagonist.

The effects of SR95103 on burst-times were further explored by examining the burst time distributions. The main result is that 30μM has no significant effect on burst duration and burst distribution, but 100μM significantly reduced the burst duration by increasing the proportion of brief bursts. This effect was described quantitatively by fitting exponential distributions. In the experiment illustrated in Figure 46, the control GABA 3μM distribution was best described by the sum of two exponentials:

$$pdf_{burst} = \frac{0.57}{3.1} e^{(-t/3.1)} + \frac{0.43}{33} e^{(-t/33)}$$

where pdf_{burst} is the burst probability density function and t is time in milliseconds. Thus there appears two types of burst: "short" and "long"; 56.5% of the bursts were of the short type with a mean duration of 3.1ms; the long type of burst had a mean duration of 33.2ms, and comprised 43.5% of the bursts. The short bursts appeared to be mostly single short openings, whereas the long bursts were mostly multiple openings. the effect of SR95103 30μM on this distribution was small (Figure 46B), in contrast to the effect of SR95103 100μM (Figure 46C), where the burst distribution was best fitted by:

$$pdf_{burst} = \frac{0.82}{3.1} e^{(-t/3.1)} + \frac{0.18}{41.9} e^{(-t/41.9)}$$

The main effect was to increase the proportion of short bursts, in this experiment from 57 to 82%. Similar results were obtained in other experiments and are summarized in Table 12. The Table 12 shows that there is a statistically significant increase in the proportion of short bursts with 100μM but not 30μM SR95103, and that there is no significant effect on the mean duration of the short or long bursts at either concentrations. It is evident that the increase in the proportion of short bursts in presence of 100μM SR95103, is responsible for the reduction in mean burst duration.

This increase in the proportion of short bursts can be reversed by increasing the agonist concentration. In one experiment, SR95103 60μM, produced an increase in the proportion of short bursts from 25% to 83%; an increase in GABA concentration from 3μM to 6μM then, partially reversed the effect of the antagonist and decreased the proportion of short bursts from 83 to 60%. Further details of these results are shown in

Figure 45: Effects of SR95103 on channel currents seen at high time resolution.

A: Control GABA 3μM

B: GABA 3μM + SR95103 30μM

C: GABA 3μM + SR95103 100μM, note the short bursts

D: Wash, GABA 3μM alone

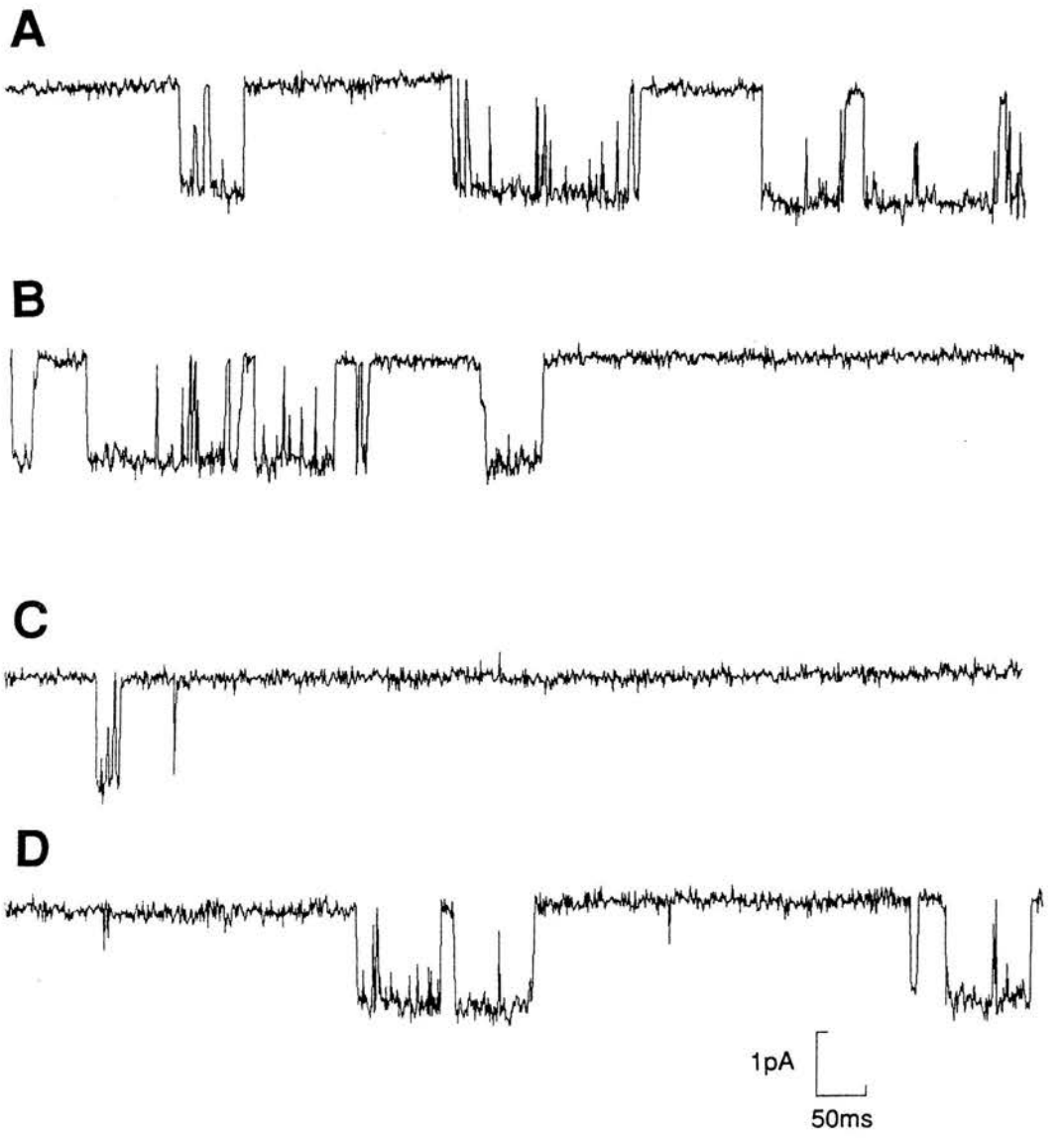

A
B
C
D
1pA
50ms

Figure 46: Frequency histograms: burst distributions

A: Control GABA 3μM, pdf = $\frac{0.56}{3.1} e^{(-t/3.1)} + \frac{0.44}{33.2} e^{(-t/33.2)}$, mean burst duration=16.1ms

B: GABA 3μM + SR95103 30μM, pdf = $\frac{0.30}{1.6} e^{(-t/1.6)} + \frac{0.70}{26.7} e^{(-t/26.7)}$, mean burst duration=19.4ms

C: GABA 3μM + SR95103 100μM, pdf = $\frac{0.82}{1.6} e^{(-t/1.6)} + \frac{0.18}{41.9} e^{(-t/41.9)}$, mean burst duration=10.2ms

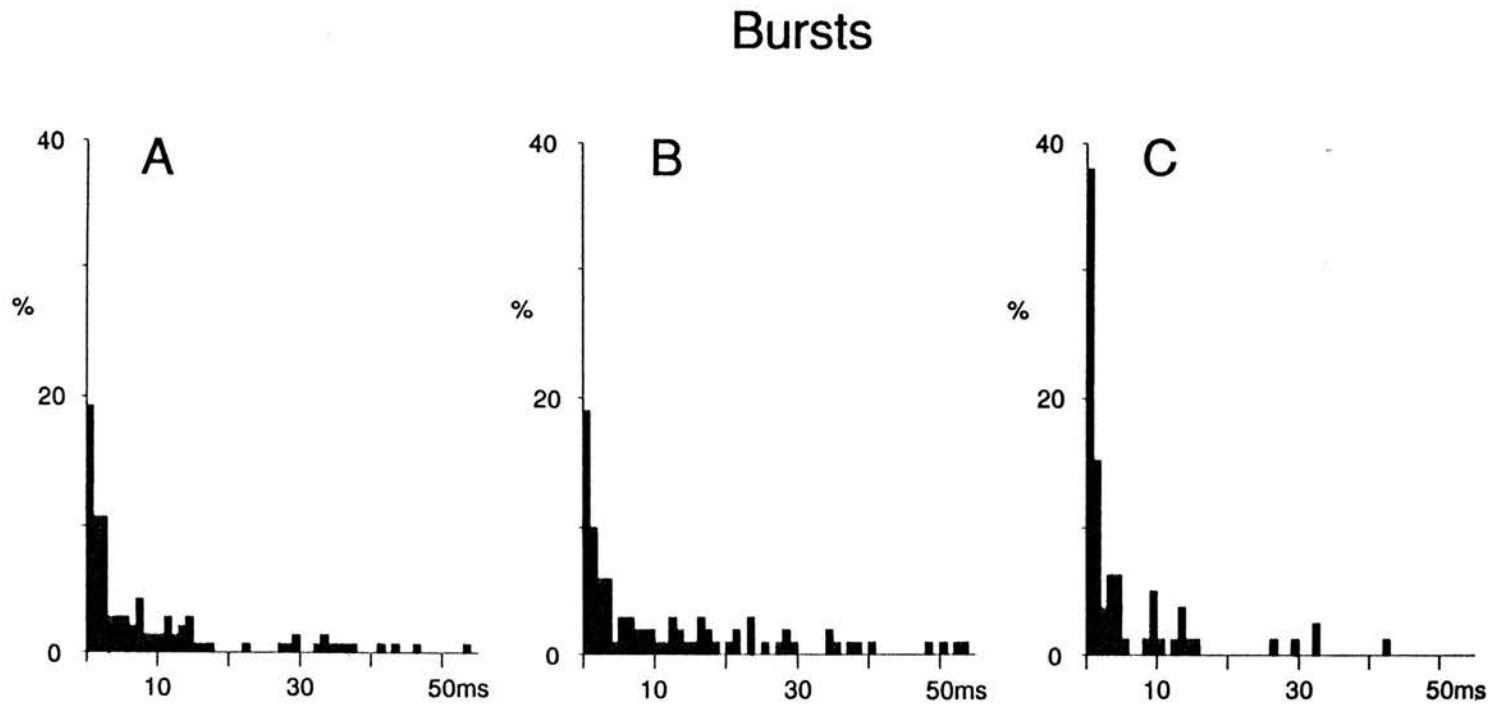

Bursts
A
B
C
40
20
0
%
10
30
50ms

Table 13. These observations were consistent with a surmountable action of SR95103 at the single channel level.

2.4.Effects of SR95103 on closed times distribution

The antagonism of SR95103 is associated with a dose-dependent increase in long closed times as illustrated in Figure 42. The effects of SR95103 on closed-times distributions were examined by fitting exponentials to describe the p.d.f.. Control GABA 3μM were best described by three exponentials. In one representative patch the result was:

$$\text{p.d.f.}_{\text{closed}} = \frac{0.29}{0.7} e^{(-t/0.7)} + \frac{0.16}{6.0} e^{(-t/6.0)} + \frac{0.55}{63} e^{(-t/63)}$$

where $\text{p.d.f.}_{\text{closed}}$ is the closed time probability density function and t the time in ms. This result is comparable to that reported previously (Martin, 1985), and shows that there are at least three closed states giving rise to the brief (mean duration 0.7ms), intermediate (mean duration 6.0ms) and long (mean duration 63ms) types of closings. The effect of 30 and 100μM SR95103 on the distribution of closed times of this patch was to increase the duration of the intermediate and long closings. The closed time distribution for the same patch with 30μM SR95103 was best described by:

$$\text{p.d.f.}_{\text{closed}} = \frac{0.2}{1.7} e^{(-t/1.7)} + \frac{0.36}{15.4} e^{(-t/15.4)} + \frac{0.54}{133} e^{(-t/133)}$$

The mean closed time increased from 35.8ms to 77.7ms; the mean duration of the intermediate closings increased from 6 to 15.4ms and the mean duration of the long closings increased from 63 to 133ms.

At 100μM, the mean closed time increased to 99.6ms; the mean intermediate closed time to 21.8ms and the mean long closed time to 215ms. The closed time distribution was best described by:

$$\text{p.d.f.}_{\text{closed}} = \frac{0.34}{0.7} e^{(-t/0.7)} + \frac{0.22}{21.8} e^{(-t/21.8)} + \frac{0.44}{215} e^{(-t/215)}$$

No increase in the number of components was required to describe the closed times distributions in this or other patches; therefore no increase in the number of closed states in presence of the antagonist could be detected. The effects of SR95103, 30μM and 100μM on the other patches was very similar and was characterized by an increase in the duration of intermediate and long closings. However, there was a large variation in mean estimates for the intermediate closings and long closings, due to the variation in the number of channels present in the patch. In contrast, SR95103 had no consistent effect on the duration of the brief closings (Table 12). In the presence of GABA alone, an increase in the two long closed time constants is expected when the GABA concentration is reduced (Martin, 1985) and reflects the decrease in open probability.

Table 12: Summary of GABA-activated channel properties in the absence and presence of SR95103. Each observation is the mean from experiments on at least four patches. * $p \leq 0.05$ T-test.

		Control GABA 3μM	GABA 3μM + SR95103 30μM	GABA 3μM+ SR95103 100μM
Popen		0.053±0.011	0.028±0.008	0.009±0.004
Channel conductance (pS)		22.4±1.0	22.6±1.0	23.3±0.6
Mean open time (ms)		8.45±1.11	9.90±2.44	4.54±1.41*
Mean burst duration (ms)		16.94±2.54	20.15±3.93	7.96±3.63
pdf_{burst}	a_{bf}	0.418±0.083	0.389±0.090	0.745±0.088*
	T_{bf}(ms)	2.3±0.6	1.6±0.5	1.3±0.5
	a_{bs}	0.581±0.083	0.610±0.090	0.255±0.880*
	T_{bs}(ms)	26.6±2.0	27.4±2.2	22.0±7.2
Brief closed times				
	a_{cf}	0.459±0.090	0.477±0.122	0.440±0.067
	T_{cf}(ms)	0.66±0.20	0.85±0.30	0.66±0.27

Table 13: Effects of SR95103 on GABA channel burst distributions and the partial reversal by increasing GABA concentration

	GABA 3μM control	GABA 3μM SR95103 30μM	GABA 3μM SR95103 60μM	GABA10μM SR95103 60μM
Corrected mean burst duration (ms)	23.8	20.2	2.3	8.7
pdfburst				
abf	0.246	0.241	0.829	0.591
T_{bf} (ms)	0.5	0.7	0.4	1.0
a_{bs}	0.754	0.759	0.171	0.409
T_{bs} (ms)	31.4	26.4	11.7	19.8

2.5. Effects of SR95103 on mean open times

One difficulty in measuring open times directly is that unresolved brief closings lead to bursts appearing as long single openings (see Colquhoun and Hawkes, 1982). This artefact increases the observed open times. However it is possible to compensate for these unresolved closings by estimating the true number of closings by extrapolating from the closed time distributions. The estimated total open time is divided by the true number of closings to obtain the mean open time. The control mean open time with GABA 3μM for one typical patch was 11.9ms; this was unchanged in presence of SR95103 30μM : 12.0ms; and reduced to 5.8ms in presence of SR95103 100μM. Table 12 shows the mean±SE values obtained from the different patches and shows that there is a significant decrease in the corrected mean open time in the presence of 100μM antagonist but not in the presence of 30μM.

III DISCUSSION

This patch clamp study of SR95103 was carried out to further investigate the mode of antagonism. The percentage of antagonism dose-response curve was described by the modified Hill equation with a coefficient close to one. The lower antagonist concentration (30μM), reduced the probability of the channel being open without significantly affecting the burst distribution, the corrected mean open time or the channel main conductance. The higher (100μM) antagonist concentration increased the proportion of short bursts, reduced the corrected mean open time but the reduction in mean burst duration did not reach statistical significance. As with 30μM, the channel conductance was not altered. In the subsequent discussion, it is suggested that SR95103 acts mostly as a simple competitive antagonist; but at high concentration there is an additional small non competitive action, possibly a channel block.

1. Possible kinetic scheme for the antagonism

A variety of patch-clamp studies in different preparations have been carried out on GABA-activated channels where the open time distributions were described by two exponentials and the closed time distributions described by three exponentials: on *Ascaris* (Martin, 1985), mouse spinal cord neurones (Jackson, Lecar, Mathers et al., 1982; Sackmann, Hamill and Borman, 1983; Mathers, 1985), on chick cerebral neurones (Weiss and Magleby, 1989), and bovine chromaffin cells (Bormann and

Clapham, 1985). More recently, MacDonald, Rogers and Twyman (1989), have described an additional open time component, which suggests a greater complexity in the interaction GABA-receptor-channel. Molecular biology studies on the GABA channel structure (Schofield et al., 1987) have shown that the receptor-channel complex is formed by two α and two β protein transmembrane subunits. The β subunits were shown to bind GABA (Barnard, Darlison and Seeburg, 1986), so it was concluded that two agonist molecules combine with the receptor channel complex. In reality, the situation is more complex and new subunits have been discovered (see Section 1 B.); moreover monomeric receptor-channel complexes are also able to be activated by GABA, suggesting that not only the β subunits can bind GABA. In contrast to GABA, the results of the current-clamp experiments presented in Section 5, and the percent inhibition dose-response curve presented here, suggest that only one molecule of SR95103 binds the receptor and is responsible for most of the antagonism. Figure 47 shows a minimal kinetic scheme which may explain these observations. It predicts (Colquhoun and Hawkes, 1977; 1982) three components for the closed time distribution and two components for the open time and burst distributions.The effect of a competitive antagonist would be associated with: an increase in the number of components in the closed time distribution; a reduction in the probability of the channel being open and no change in the open or burst distributions (if the open states and the closed states which are part of the bursts are the same in the absence as well as in the presence of the antagonist).

2. SR95103 antagonism

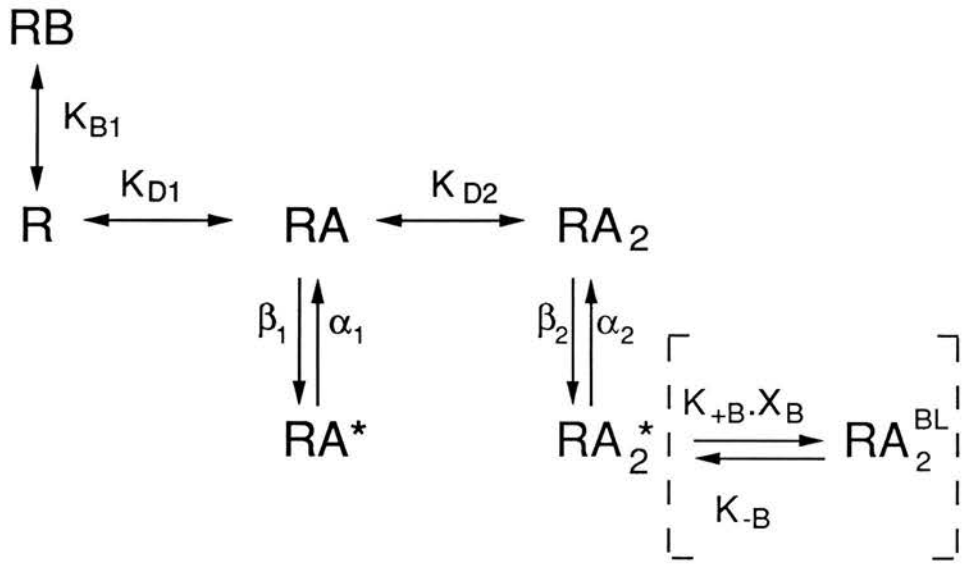
These experiments did not reveal an increase in the number of components in the closed time distribution in the presence of the antagonist. But, the long duration of many closed times seen in the presence of the antagonist reduced the number of events available for analysis and the ability to separate components. There was also the further complication of different numbers of channels present in each patch; this complication gave rise to considerable variation in estimates of the mean duration of "intermediate" and "long" closed-times. Both of these factors would make the detection of any extra long closed-states in the presence of the antagonist difficult.

2.1. SR95103 (30μM) behaves as a competitive antagonist

The channel main conductance was not altered in the presence of the antagonist. On the basis of the Monod-Wymann-Changeux model (Colquhoun and Hawkes, 1977,

Figure 47: Minimal kinetic scheme for agonist and antagonist action

The receptor channel complex (R) has two sites to which the agonist (A), but one site to which the antagonist (B), combine. Open states (*) may occur only after isomerization of the single-bound or double-bound agonist receptor-channel complex. The dissociation constant for the first agonist molecule to combine (K_{D1}) is much larger than for the second agonist molecule (K_{D2}); the dose-response relationship would then be approximated by the modified Hill equation with N=2. The channel closing rates are $\alpha 1$ and $\alpha 2$. $\alpha 1$ is larger than $\alpha 2$ so that RA_2^* is the long open state. The channel opening rates are $\beta 1$ and $\beta 2$. The antagonist dissociation constant is K_{B1}. The putative blocked channel, RA_2^{BL}, is also shown in brackets.


RB
K_{B1}
K_{D1}
K_{D2}
R
RA
RA_2
β_1
α_1
β_2
α_2
RA*
RA_2^*
$K_{+B}.X_B$
RA_2^{BL}
K_{-B}

1982) for GABA-receptor interaction, the addition of a simple competitive antagonist predicts no change in the channel mean open time or burst distribution. There was no significant effect of SR95103 (30μM) on corrected mean open times and burst distributions, so it is argued that at this concentration, this antagonist behaves as a competitive antagonist. Moreover a non-competitive mechanism, predicts at the current-clamp level a reduction in the maximum response and a reduction in the slope dose-response curves at higher antagonist concentrations; such an effect with concentrations of SR95103 up to 1mM (Section 5) was not detected.

A variety of experimental preparations have been used to look at the action of arylaminopyridazine GABA derivatives on the vertebrate GABA-A receptor. Binding studies (Chambon et al., 1985; Heaulme et al., 1986a, 1986b) and electrophysiological studies (Chambon et al., 1985; Michaud et al., 1986; Desarmenien et al., 1987) have all indicated that arylaminopyridazine derivatives of GABA act as competitive antagonists at the vertebrate GABA-A receptor. SR95531, the potent analogue of SR95103 in vertebrate, has been examined at single-channel level in bovine chromaffin cells (Hamann et al., 1988). It was found that SR95531 reduced the open probability of GABA channels without affecting the channel main amplitude and open times and burst durations kinetics, an effect compatible with competitive antagonism. Therefore the antagonism produce by SR95103 (30μM) is compatible with a competitive mechanism at the channel level.

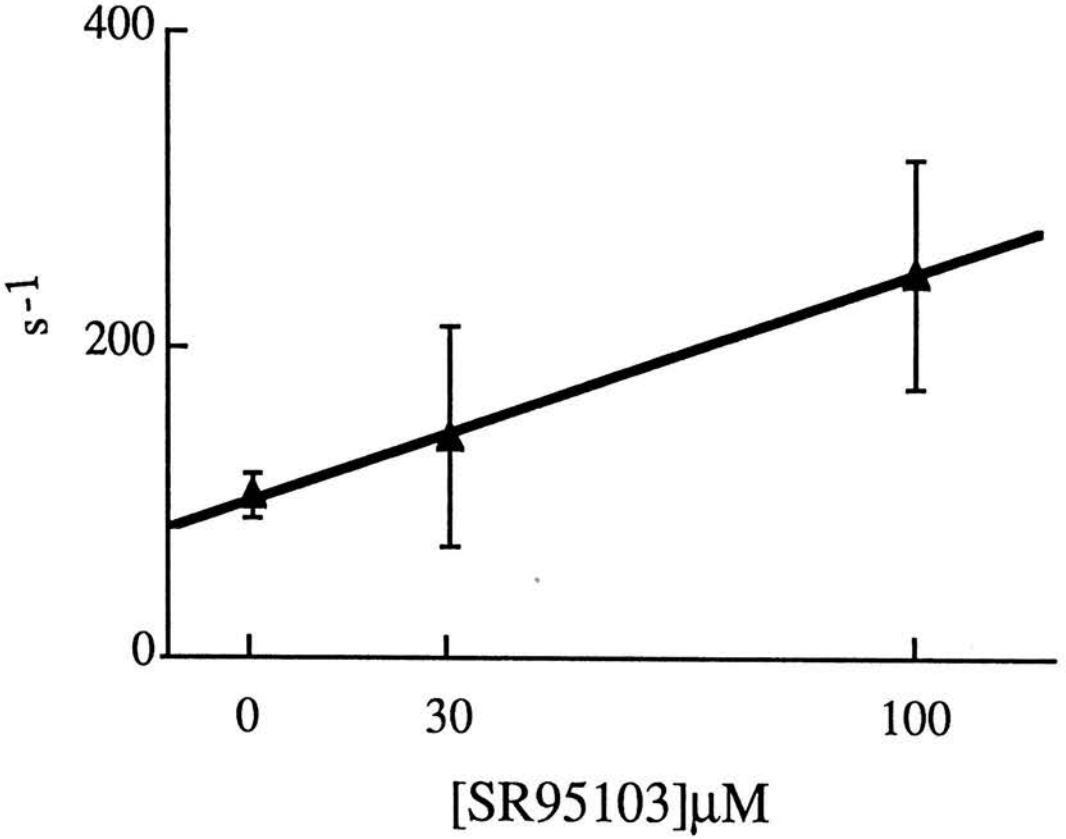
2.2. SR95103 (100μM) antagonism shows a non-competitive component

In contrast to 30μM, the effects of 100μM SR95103, are not compatible with a simple competitive mechanism, because they are associated with significant reduction of the corrected mean open time and increase in the proportion of short bursts.

At the single-channel level, the effects of a channel block on the burst duration depends on the duration of the blocked state. If the blocked state is brief (less than T_{crit}), the burst duration and the number of gaps per burst would increase, but that was not detected However if the blocked state is longer than T_{crit}, the observed mean long burst duration would get shorter and the mean open time in the long burst would decrease. Although the observed reduction in mean long burst duration did not reach statistical significance, there was a significant reduction in the corrected mean open time in the long bursts: $\frac{1}{\alpha_2}$. To estimate the corrected mean open time in the long bursts, all brief closings were assumed to occur in the long bursts (see Methods). Figure 48 shows the observed linear relationship between the reciprocal of this duration and the

Figure 48: Effect of SR95103 on mean open time in long bursts

Ordinate: reciprocal of mean open times in long bursts.
Abscissa: concentration of antagonist. The slope (obtained by least-squares regression was $1.44M^{-6}s^{-1}$ and could be interpretated as the channel bloccking rate at -50mV.


400
200
0
s-1
0
30
100
[SR95103]µM

concentration of SR95103. The reciprocal of this duration approximates to the sum of the rate constants leading from the open state, providing there is little interconversion between the monomeric open state and the dimeric open state (Colquhoun and Hawkes, 1982). The slope of the plot in Figure 48 is then an estimate of the channel blocking rate constant: $1.44M^{-6}s^{-1}$. Although these findings may be in favour for a channel block mechanism at this concentration, they do not predict the observed increase in proportion of short bursts.

SR95531 has shown an antagonism compatible with competitive mechanism at single channel-current level, however, an additional channel block mechanism could not be excluded at high concentration of SR95531, but the potent action of SR95531 did not allow further analysis (Hamann et al., 1987).

Arylaminopyridazine derivative of GABA are said to be highly specific for the GABA-A receptor, but a small inhibitory effect on glycine receptors as well as on the GABA receptor, of rat cortical neurones has been described for SR95103 by Michaud et al., (1986), but not for other analogues such as SR95531. GABA and glycine both conduct Cl, Barker and Mac Burney (1979) suggested that the Cl channels were identical, so that the channel could be a common site of action. These two reports raise the question of a channel block mechanism in addition to the competitive antagonism of SR95103. In light of the molecular biology results, it appears that recognition site and channel are not two separate entities which can be coupled or uncoupled, but are formed by the complex organization of protein subunits. This, however does not exclude similarities between GABA- and glycine-receptor-channel complex (Schofield et al., 1987).

Conclusion: although SR95103 may act in part as non competitive antagonist, this cannot play a big role in the antagonism of GABA conductance responses, since high concentrations of SR95103 did not depress the maximum response seen in current clamp experiments, and no voltage-sensitivity was detected in voltage-clamp experiments (see Section 5). This study shows that SR95103 antagonism is compatible with a competitive mechanism but there is an additional non competitive element at high concentration, which shares some of the properties of a channel block.

This Section shows further evidence that SR95103 is mainly a competitive antagonist at the *Ascaris* GABA receptor. Thus, SR95103 appears to have a mechanism of action similar to that at vertebrate GABA-A receptors.

Current-clamp and patch-clamp studies of the antagonism produced by SR95103 strongly suggest a competitive mechanism. This result is important, since SR95103 and

more generally arylaminopyridazine derivatives of GABA were shown to be competitive at vertebrate GABA-A receptors. The same mechanisms seem to be involved in *Ascaris* and vertebrates.

The next Section explores the antagonist properties of various pyridazine GABA derivatives and study their structure-activity relationship on *Ascaris* muscle. It will be shown that the structure-activity relationships of pyridazine GABA derivatives are different in *Ascaris*, and that all the potent compounds behave like SR95103.

SECTION 7:

ANTAGONIST PROPERTIES OF AMINOPYRIDAZINE DERIVATIVES AT THE *ASCARIS* MUSCLE GABA RECEPTOR

INTRODUCTION

In Section 5, the arylaminopyridazine GABA derivative SR95531 was shown to be very weak at antagonizing GABA responses in *Ascaris*, but its close analogue SR95103 was more potent with a K_B=64μM. This contrasts with the vertebrate GABA-A receptor, where SR95531 is the most potent antagonist, approximately 20 times more potent than SR95103 (Heaulme et al., 1986b). It was suggested then, that arylaminopyridazine GABA derivatives may show structure-activity relationships in *Ascaris* different from those found at the vertebrate GABA-A receptor. In this section, other arylaminopyridazine derivatives obtained from SANOFI were tested, and their relative potency in *Ascaris* muscle and in mammalian were compared. The results obtained with these compounds reinforced the hypothesis of the presence of different accessory sites on the *Ascaris* GABA receptor. To further explore the structure-activity relationships of arylaminopyridazine derivatives in *Ascaris*, a collaboration was set up with Professeur C.G.Wermuth from the "Laboratoire de Neurochimie du CNRS", Strasbourg University, France. New pyridazine derivatives were synthesized in Strasbourg by J.M.Sitamze and Dr M.Schmitt, and then tested by myself on *Ascaris* muscle preparation. These results are discussed in terms of accessory binding site theory.

I METHODS

1. Muscle preparation

The muscle dissection has been described in the general method (Section 2) and in Section 3. As for the experiments with GABA-agonists, the muscle flap was bathed in high-Cl, low-Ca Ringer (solution B), and maintained at 22ºC.

2. Drug application

Because of the limited quantities of antagonist available, the muscle was not perfused continuously. The experimental chamber (2.5ml) was carefully drained, and then flushed with 5ml of the next drug solution. When dose-response curves were required, drugs were bath-applied in a cumulative manner, without intermediate washing between the concentrations (see Section 3).

3.Current-clamp

3.1. Technique

The technique used here, has been described previously in Section 3, all signals were recorded with an Axoclamp 2A (Axon Instruments) in current-clamp mode, which permits the use of higher resistance for the electrodes: typically 20-50MΩ.

3.2. Analysis

a. GABA

The effect of GABA was determined by measuring the change in input conductance. When dose-response relationships were required, they were described by the modified Hill equation (Nistri and Constanti, 1979), and estimates for Δgmax, EC50 and N were obtained using a non linear regression programme (Patternsearch, Colqhoun, 1971). See Section 3 for details.

b. Antagonists

- Screening

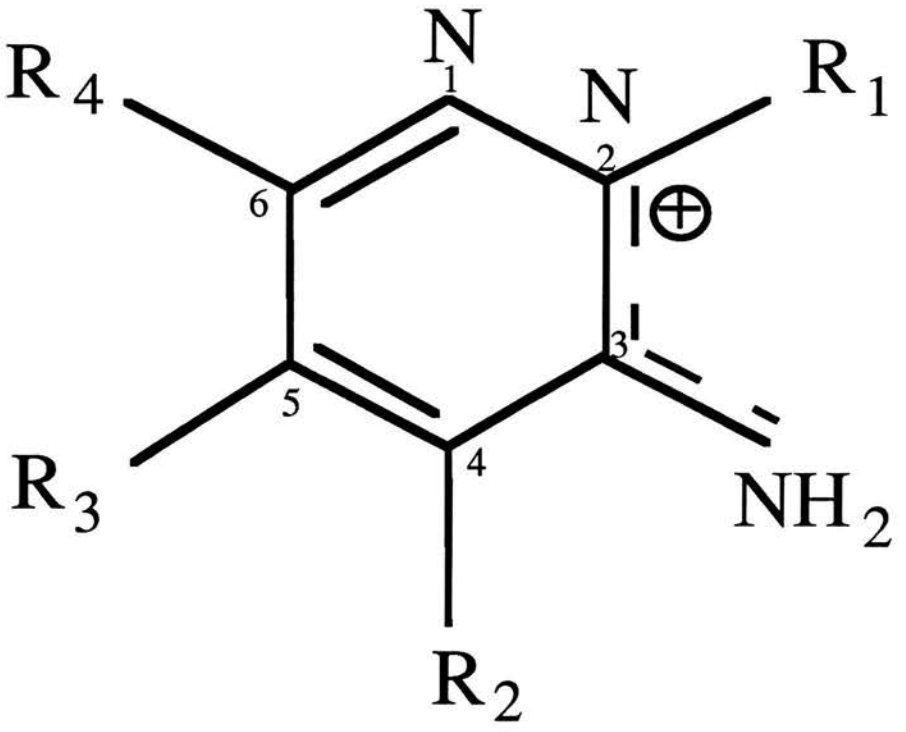
Initially, the action of 1mM antagonist was examined on the change in membrane conductance induced by GABA 30μM. The percentage of antagonism was then estimated:

$$\% \text{ antagonism} = 100.(1-\frac{\Delta g_{ant}}{\Delta g_c})$$

where Δg_c is the change in membrane input conductance produced by GABA 30μM, and Δg_{ant} is the change in membrane input conductance produced by GABA 30μM + antagonist 1mM, measured after 10 minutes application of the antagonist. Compounds detected as reasonably potent antagonists with this method, were then tested at several concentrations on GABA dose-response curves.

Figure 49: Structure of pyridazine derivatives

Code name	R1	R2	R3	R4
SR95103	$-C_3H_6COOH$	$-CH_3$	-H	$-C_6H_5$
SR95531	$-C_3H_6COOII$	-H	-H	$-C_6H_4OCH_3$
SR42666	$-C_3H_6COOH$	$-CH_3$	-H	$-C_6H_4OCH_3$
SR95132	$-C_3H_6COOH$	$-C_6H_5$	-H	$-C_6H_5$
SR42640	$-C_3H_6COOH$	-H	$-CH_3$	$-C_6H_5$
SR42627	$-C_3H_6COOH$	$-CH_3$	-H	$-C_6H_4CH_3$
SR95133	$-C_3H_6COOH$	-CH3	-H	$-C_{10}H_8$
NCS194-83	$-C_4H_8COOH$	$-CH_3$	-H	$-C_6H_5$
NCS247-90	$-C_3H_6COOH$	$-CH_2CH_3$	-H	$-C_6H_5$
NCS248-90	$-C_3H_6SO_3$	-H		$-C_9H_{10}$
NCS249-90	$-C_3H_6SO_3$	$-CH_3$	-H	$-C_6H_5$
NCS250-90	$-C_3H_6SO_3$	$-CH_2CH_3$	-H	$-C_6H_5$
NCS251-90	$-C_3H_6COOH$	$-CH_2C_6H_6$	-H	$-C_6H_5$
NCS252-90	$-C_3H_6COOH$	$-CHCH_3CH_3$	-H	$-C_6H_5$


R4
N
1
N
R1
2
6
⊕
3
5
4
R3
NH2
R2

- Quantitative evaluation

The effect of SR42666, SR95132 and NCS247-90, were examined on GABA dose-response curves. Because of the natural variation of EC50, Δgmax and N, a control GABA dose-response curve was obtained from the same cell, before, or after the antagonist application. Dose-ratios were measured and, examined using a Schild plot (Arunlakshana and Schild, 1959) or a modified Schild plot (Williams et al., 1988), see Section 5 for more details and Section 1.C for discussion.

4. Statistical test

Means and standard errors were calculated. Statistical significance was assessed using a two-tailed independent t-test.

5. Chemicals

Details can be found in Section 2. For the pyridazine derivatives, the full chemical names are also given in Section 2; but Figure 49 summarizes their chemical structures.

IIRESULTS

The results reported here are based on the analysis of experiments recorded from 59 cells from 40 preparations. Cells selected for recording and analysis had a resting membrane potential greater than -20mV and a resting input conductance: 2.34±0.06μS, mean±SE, n=59. Recordings were also rejected if the resting input conductance failed to return to at least 80% of the control

1. Pyridazine derivatives are inactive on membrane conductance

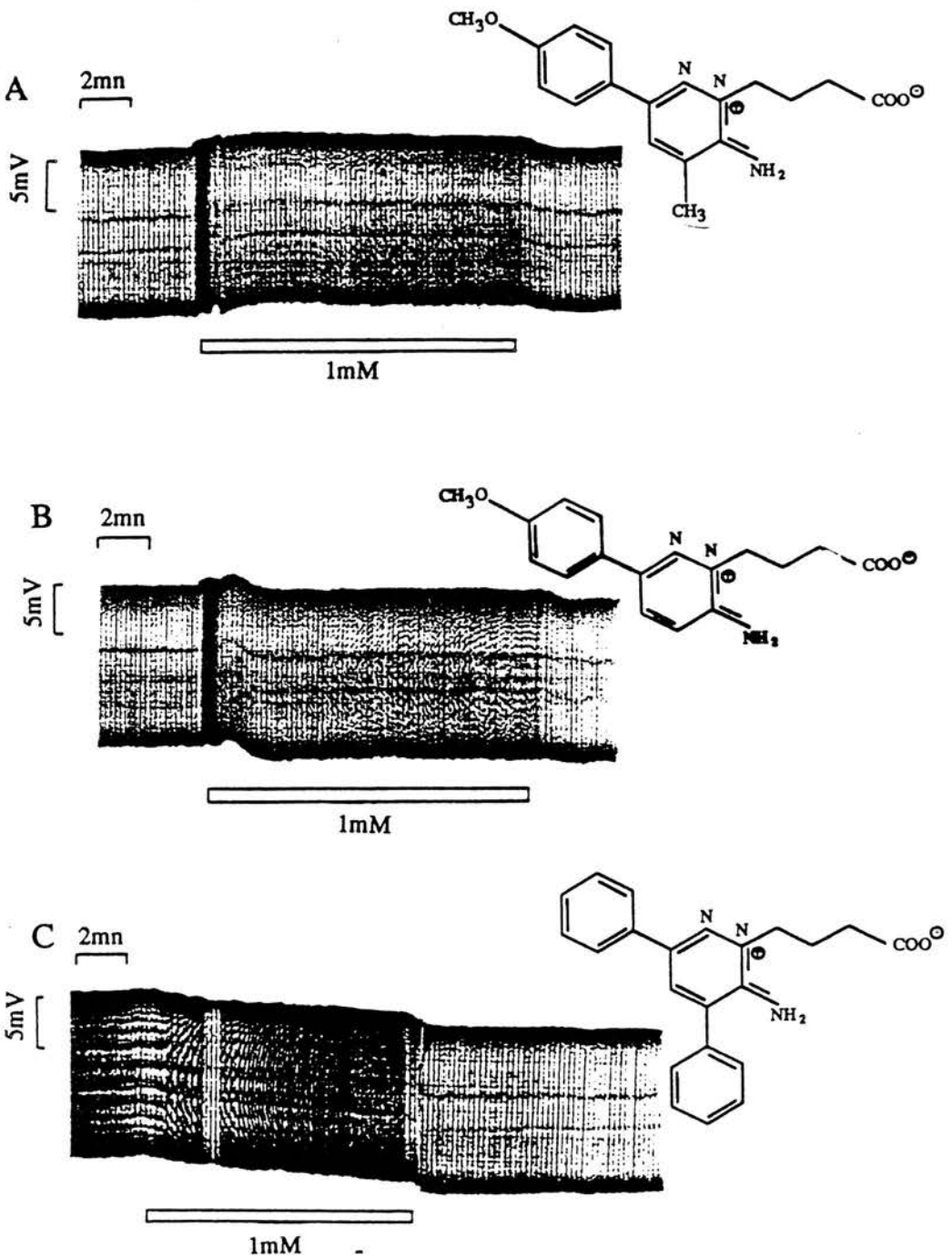
SR95103, SR95531, SR42666, SR42640, SR42627, SR95133, NCS194-83, NCS247-90, NCS248-90, NCS249-90, NCS250-90, NCS251-90 and NCS252-90, were initially tested at 1mM, in the absence of GABA or any GABA-agonist. No change in membrane conductance was produced. Figure 50 illustrates the inactivity of 1mM: SR42666 (A), SR95531 (B), SR95132 (C), on membrane conductance.

Figure 50: Inactivity of arylaminopyridazine derivatives of GABA on membrane conductance

A. SR42666: g_0=2.2μS; in the presence of 1mM SR42666 for ten minutes, Δg=0μS.

B. SR95531: g_0=2.2μS; in the presence of 1mM SR95531 for ten minutes, Δg=0μS.

C. SR95132: g_0=2.2μS; in the presence of 1mM SR95132 for ten minutes, Δg=0μS.


A
2mn
5mV
1mM
B
2mn
5mV
1mM
C
2mn
5mV
1mM

2. Potency of a series of arylaminopyridazine GABA derivatives in *Ascaris* muscle

Figures 51 and 52, illustrate the effects produced by 1mM SR95133, SR95132, SR42666, SR42627, and SR42640, on the GABA (30μM) response, .

SR42640 was the weakest compound, and antagonized 7.5±7.5% of the GABA response (mean±SE, n=2). SR42627 was not very potent either, with only 21.0±8.0% of the GABA response antagonized (mean±SE, n=2).

SR95133, the α-naphtyl derivative, antagonized 58.0±8.0% of the GABA response (mean±SE, n=4), and was therefore more potent than SR95531 (44.2±6.1%, mean±SE, n=5, see Section 5).

The two most potent antagonists were, SR42666, and the 4-phenyl derivative, SR95132, with an antagonism of 76.6±6.6% (mean±SE, n=5), and 89.0±3.4% (mean±SE, n=5), respectively. All these results are summarized in Table 14.

Figures 53, 54, 55 and 56, illustrate the effects of 1mM of the newly synthesized compounds: NCS194-83, NCS247-90, NCS248-90, NCS249-90, NCS250-90, NCS251-90, and NCS252-90, on the change in membrane conductance produced by 30μM GABA.

The two sulphonic acid derivatives: NCS249-90 (n=2) and NCS250-90 (n=2), were totally inactive, both producing 0% antagonism. NCS248-90 is very weak, 1mM antagonized 13.7±5.1% of the response produced by GABA 30μM (mean±SE, n=6).

NCS194-83, the δ-aminovaleric derivative, antagonized 45.5±9.3% (mean±SE, n=4) of the response produced by 30μM GABA. NCS194-83 is therefore not a potent antagonist at the *Ascaris* GABA receptor, but retains some activity considering the change in the structure of the side-chain.

NCS247-90 1mM antagonizes nearly 100% of the response produced by 30μM GABA, 95.2±3.1% (mean±SE, n=5).

NCS252-90 and NCS251-90 antagonized respectively, 80.9±2.5% (mean±SE, n=4) and 99.2±0.8% (mean±SE, n=3), of the response to GABA (30μM).

Thus, SR95132, NCS247-90, NCS251-90 and NCS252-90 are the most potent compounds, being apparently as potent as SR95103. SR42666 also retains a certain degree of antagonism.

3. SR95132, SR42666 and NCS247-90, like SR95103, antagonize GABA responses in an apparent competitive manner

In vertebrates, arylaminopyridazine derivatives act as competitive antagonists, they displace [^{3}H]GABA and [^{3}H]muscimol from their binding sites (Chambon et al., 1985; Heaulme et al., 1986a; Heaulme et al., 1986b) and decrease [^{3}H]diazepam binding stimulated by GABA (Heaulme et al., 1986a). Section 5 shows that in *Ascaris*, SR95103, 100µM, 300µM and 1000µM, displaces GABA dose-response curves to the right in a parallel manner without a decrease in the maximum response. The dose-dependency was best described by the modified Schild equation (Williams et al., 1988), suggesting that the mechanism was competitive and that only one molecule of SR95103 interacted with the GABA receptor. The actions of the three more potent compounds: SR95132, SR42666, and NCS247-90, were therefore examined at 100, 300, and 1000µM, on GABA cumulative dose-response curves.

SR95132, SR42666 and NCS247-90, displaced GABA dose-response curves to the right in a parallel manner without a reduction in the maximal response, suggesting a competitive mechanism.

Figure 57 illustrates the antagonism produced by SR95132, A is the control GABA responses, B and C are the GABA responses in the presence of respectively 100µM and 300µM SR95132. All three recordings were obtained from the same cell. Dose-ratio were calculated for each cell, as the ratio of EC50s estimated for the GABA dose-response curves in the absence and presence of antagonist. Figure 58 shows the dose-response curves for the traces shown in Figure 57.

The dose-dependent antagonism produced by SR42666 is illustrated in Figure 59, where A is the control GABA response, B, C and D are the GABA responses in the presence of respectively, 100, 300, and 1000µM. The corresponding dose-response curves are illustrated in Figure 60.

Figure 61 illustrates the dose-dependency of NCS247-90 antagonism, where A is the control GABA dose-response, B, C, and D, the GABA dose-response in the presence of respectively 100, 300, 1000µM NCS247-90. The corresponding dose-response curves are illustrated in Figure 62. As expected, GABA dose-response curves were displaced to the right in a parallel manner and without decrease in the maximal response: there was no variation of N or Δgmax (t-test, $p \leq 0.05$), only EC50 was significantly increased in the presence of 100, 300 and 1000µM NCS247-90 .

The classical Schild plots for SR95132 and SR42666: log(DR-1) against $\log X_B$, were linear but with a slope different from one: 0.73 for SR42666 (n=13), and 0.74 for SR95132 (n=9). However, the classical Schild plot does not account for a

Figure 51: Effects of 1mM SR42666, SR42640, SR95133 and SR95132 on the change in membrane input conductance produced by GABA 30μM.

A. Effect of SR42666 1mM: resting input conductance, g_o=2.2μS; GABA 30μM, Δg=5.0μS; SR95103 1mM + GABA 30μM, Δg=1.0μS; %antagonism=80%.

B. Effect of SR42640 1mM: resting input conductance, g_o=2.2μS; GABA 30μM, Δg=4.6μS; SR95133 1mM + GABA 30μM, Δg=3.9μS; %antagonism=15%.

C. Effect of SR95133 1mM: resting input conductance, g_o=2.0μS; GABA 30μM, Δg=3.7μS; SR95103 1mM + GABA 30μM, Δg=2.2μS; %antagonism=35%.

D. Effect of SR95132 1mM: resting input conductance, g_o=2.0μS; GABA 30μM, Δg=3.7μS; SR95103 1mM + GABA 30μM, Δg=0.7μS; %antagonism=81%.

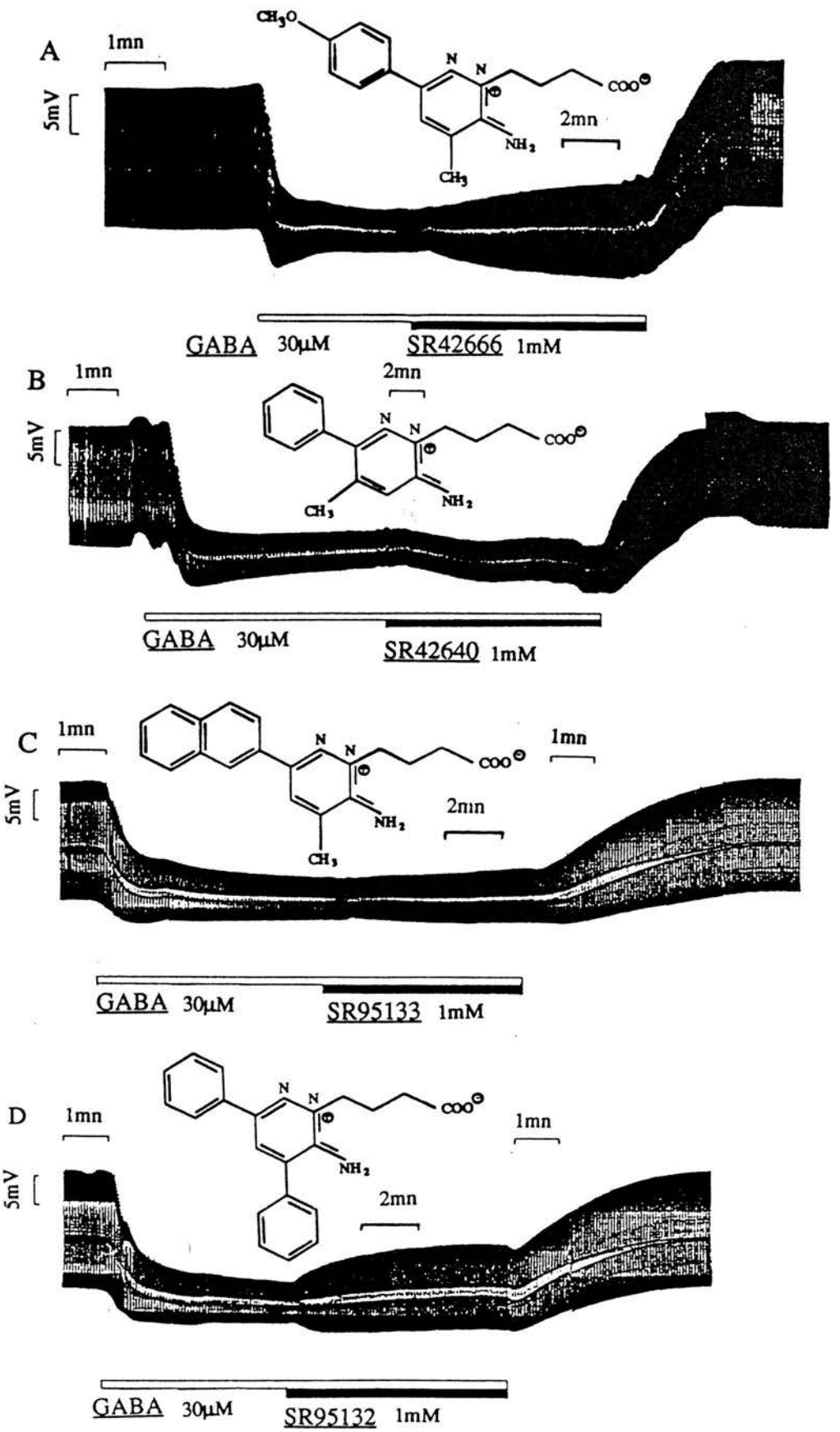

A
1mn
5mV
2mn
GABA 30µM
SR42666 1mM
B
1mn
2mn
5mV
GABA 30µM
SR42640 1mM
C
1mn
1mn
5mV
2mn
GABA 30µM
SR95133 1mM
D
1mn
1mn
5mV
2mn
GABA 30µM
SR95132 1mM

Figure 52: Effect of 1mM SR42627 on the change in membrane conductance produced by GABA 30μM.

Resting input membrane conductance, g_0=2.2μS; GABA 30μM, Δg=4.4μS; SR42627 1mM + GABA 30μM, Δg=3.1μS; %antagonism=29%.

1mn
5mV
GABA 30µM
SR42627 1mM
COO⊖
NH2
N
N
CH3
CH3

Figure 53: Effect of 1mM NCS247-90 on the change in membrane input conductance produced by 30μM GABA

Resting membrane conductance g_0=2.7μS; GABA 30μM, Δg=1.9μS; 1mM NCS247-90 + 30μM GABA, Δg=0μS; 100% antagonism.

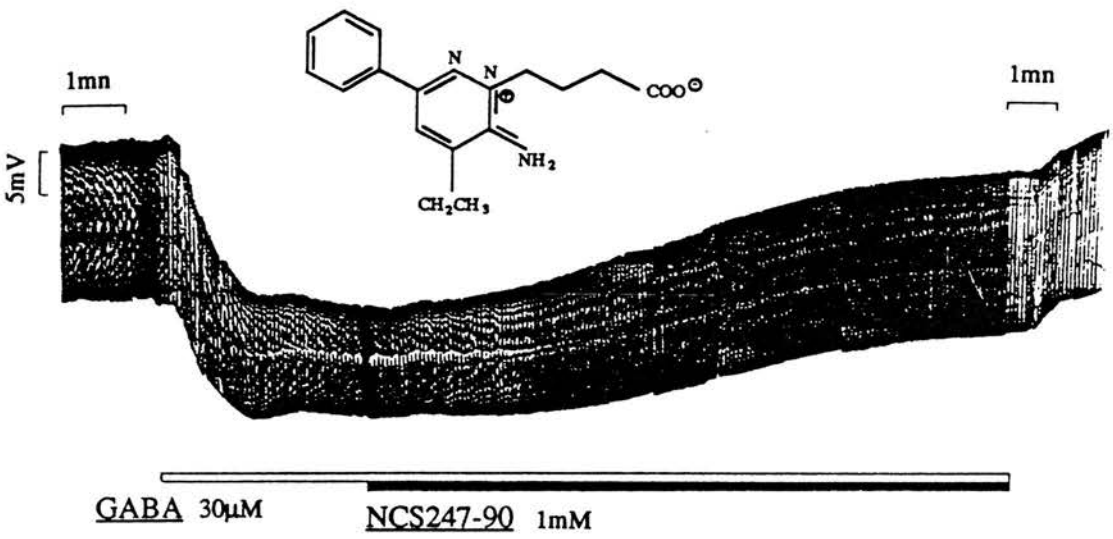

1mn
1mn
5mV
N
N
COO⊖
NH2
CH2CH3
GABA 30µM
NCS247-90 1mM

Figure 54: Effect of 1mM NCS248-90 and NCS194-83, on the change in membrane input conductance produced by 30μM GABA

A. NCS248-90: resting membrane conductance g_0=1.9μS; GABA 30μM, Δg=1.9μS; 1mM NCS248-90 + 30μM GABA, Δg=1.7μS; 10% antagonism.

B. NCS194-83: resting membrane conductance g_0=2.0μS; GABA 30μM, Δg=1.3μS; 1mM NCS194-83-90 + 30μM GABA, Δg=0.8μS; 38% antagonism.

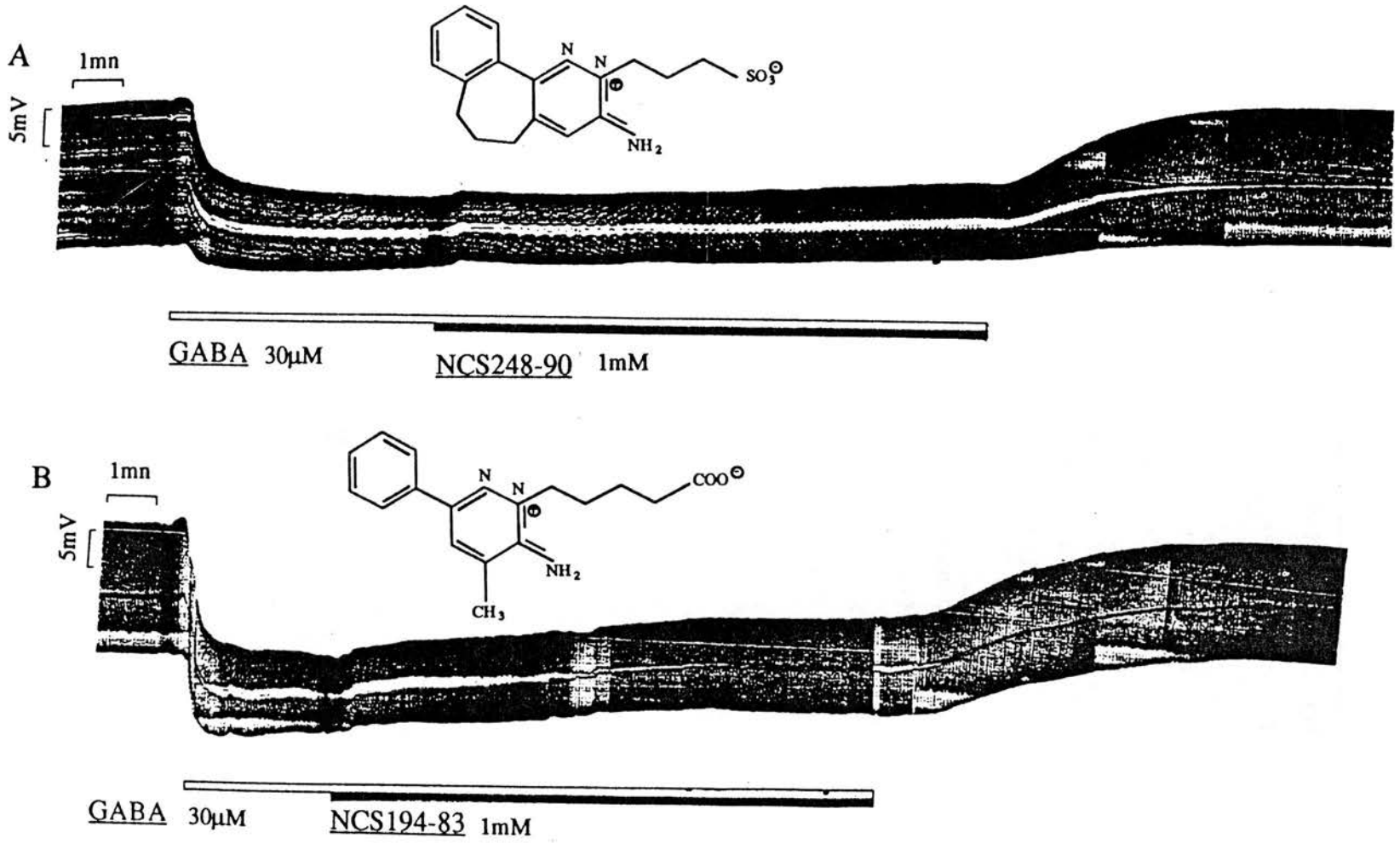

A
1mn
5mV
N
N
$SO_3^\ominus$
NH_2
GABA 30μM
NCS248-90 1mM
B
1mn
5mV
N
N
$COO^\ominus$
NH_2
CH_3
GABA 30μM
NCS194-83 1mM

Figure 55: Effect of 1mM NCS249-90, NCS250-90 and NCS252-90 on the change in membrane input conductance produced by 30μM GABA

A. NCS249-90: resting membrane conductance g_0=3.2μS; GABA 30μM, Δg=1.3μS; 1mM NCS249-90 + 30μM GABA, Δg=1.4μS; 0% antagonism.

B. NCS250-90: resting membrane conductance g_0=3.2μS; GABA 30μM, Δg=1.8μS; 1mM NCS250-90 + 30μM GABA, Δg=1.8μS; 0% antagonism.

C. NCS252-90: resting membrane conductance g_0=3.1μS; GABA 30μM, Δg=1.4μS; 1mM NCS252-90 + 30μM GABA, Δg=0.3μS; 78.6% antagonism.

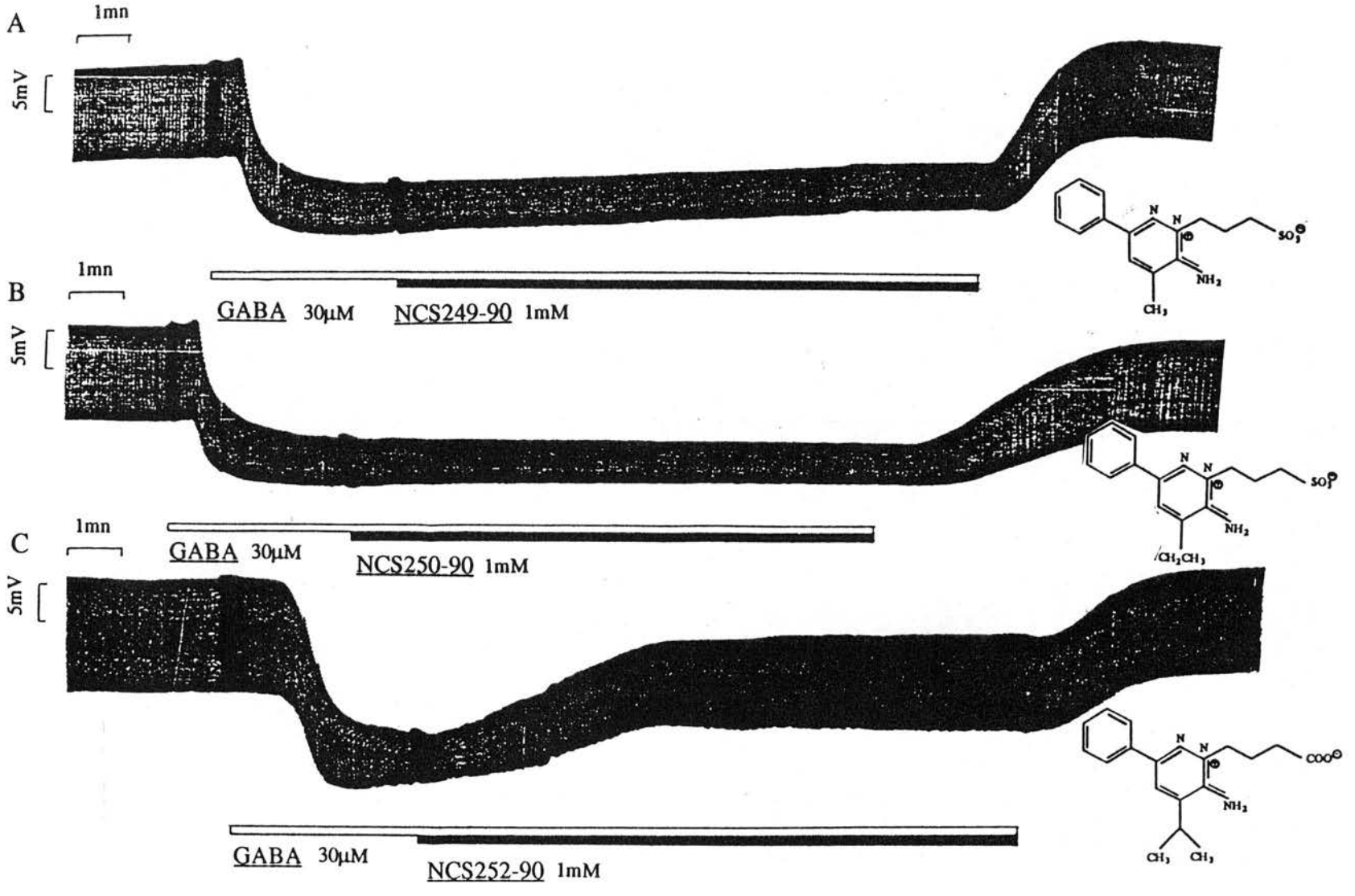
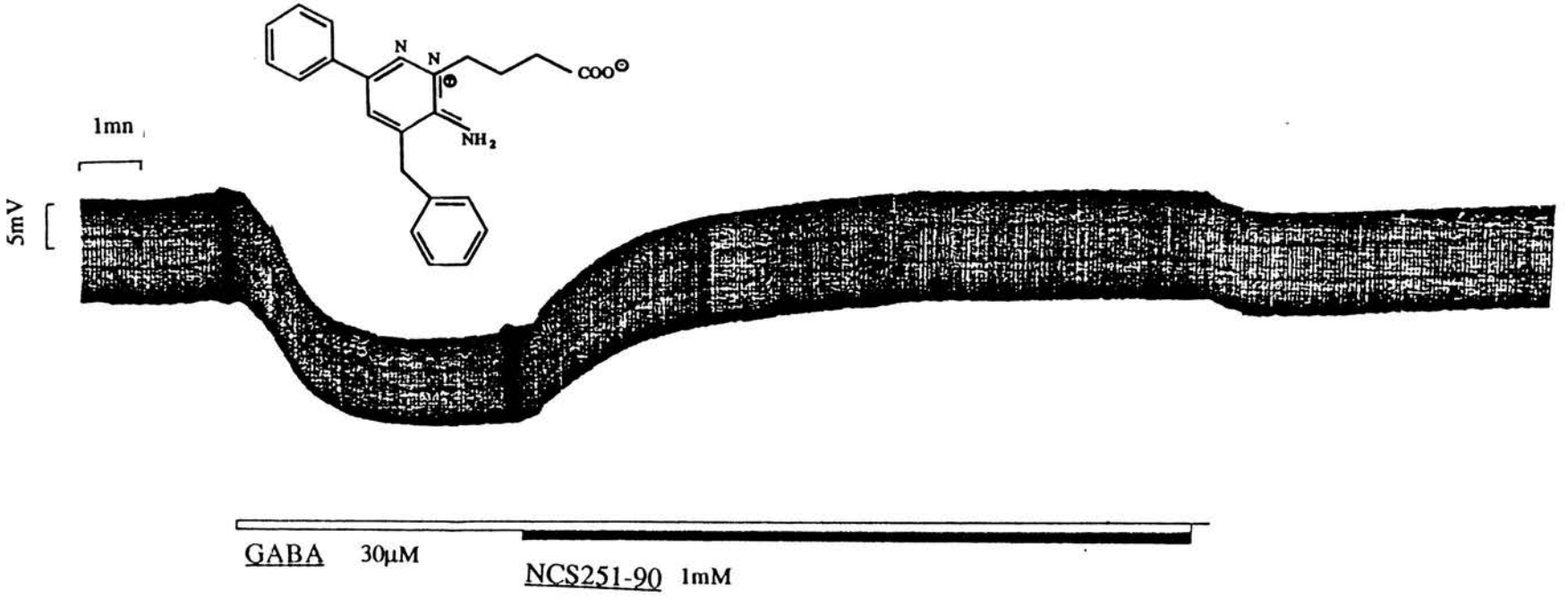

A
1mn
5mV
B
1mn
GABA 30µM
NCS249-90 1mM
5mV
C
1mn
GABA 30µM
NCS250-90 1mM
5mV
GABA 30µM
NCS252-90 1mM

Figure 56: Effect of 1mM NCS251-90 on the change in membrane input conductance produced by 30μM GABA

Resting membrane conductance g_0=3.2μS; GABA 30μM, Δg=0.8μS; 1mM NCS251-90 + 30μM GABA, Δg=0μS; 100% antagonism. Note that antagonism is stabilized rapidly within five minutes. The small hyperpolarization seen at the start of the washing phase may be an artefact due to the start of the perfusion.


1mn
5mV
N
N
COO⊖
NH2
GABA 30μM
NCS251-90 1mM

stoichiometry greater than one between GABA and its receptor (see Section 1.C and Section 5). The modified Schild equation was therefore used (Williams et al 1988), and $\log(DR^2-1)$ was plotted against $\log X_B$. The plot yielded a better fit for SR95132 ($R^{\wedge 2}=0.66$ against $R^{\wedge 2}=0.62$ for the Schild plot). The linear fit was not improved for SR42666 ($R^{\wedge 2}=0.61$), but for both compounds, the slopes of the modified Schild plots were close to one: 1.02 for SR42666 (n=13) and 1.06 for SR95132 (n=9).

Schild and modified Schild analysis were also used for NCS247-90. The best fit was obtained for the modified Schild plot with N=2, $R^{\wedge 2}=0.77$ (against $R^{\wedge 2}=0.74$, for the classical Schild plot). Unexpectedly, the slope of the classical Schild plot was, 1.00±0.1 (mean±SE, n=13), and the slope of the modified Schild plot was,1.50±0.23 (mean±SE, n=13). However the fit was better with N=2.

These results are consistent with a model which involves the activation of the receptor by two molecules of GABA, but only one molecule of SR42666, SR95132, and possibly NCS247-90, interact with the receptor to block this effect. This is in goodagreement with previous results obtained with SR95103 in Section 5. Although these results do not establish such mode of action, they provide a good description of the interaction antagonist-receptor, and allow the estimation of apparent dissociation constants. The dissociation constant, K_B, was estimated, assuming N=2 and M=1:

$$K_B = \frac{DR^2-1}{X_B},$$

SR42666: K_B=104.8±19.7μM (mean±SE, n=13), for SR95132: K_B=65.0±20.3μM (mean±SE, n=9), NCS247-90: K_B=54.6±16.2μM (mean±SE, n=13).

NCS247-90 appears therefore more potent than SR95103 and SR95132, but the difference is not statistically significant. Whereas SR42666 is definitely less potent than SR95103 (t-test, $p \leq 0.05$).

SR42666, SR95132 and SR95103 act in a competitive-like manner on the *Ascaris* GABA receptor. SR95132 is equipotent to SR95103 (K_B=63.8±12.9μM, mean±SE, n=14) and both are the most potent of the series, whereas SR42666 is less potent. Since arylaminopyridazine GABA derivatives seem to act as competitive antagonist at the *Ascaris* GABA receptor, as they do at the vertebrate GABA-A receptor, it is of interest to compare and discuss their respective structure-activity relationships.

Figure 57: Effects of 100 and 300μM SR95132 on GABA dose-response relationship.

A. Control responses to cumulative application of GABA. Note the small depolarization observed with 3μM GABA, there was no detectable change in membrane input conductance.

B. In the presence of SR95132 100μM.

C. In the presence of SR95132 300μM.
The corresponding dose-response curves are illustrated in Figure 58A.

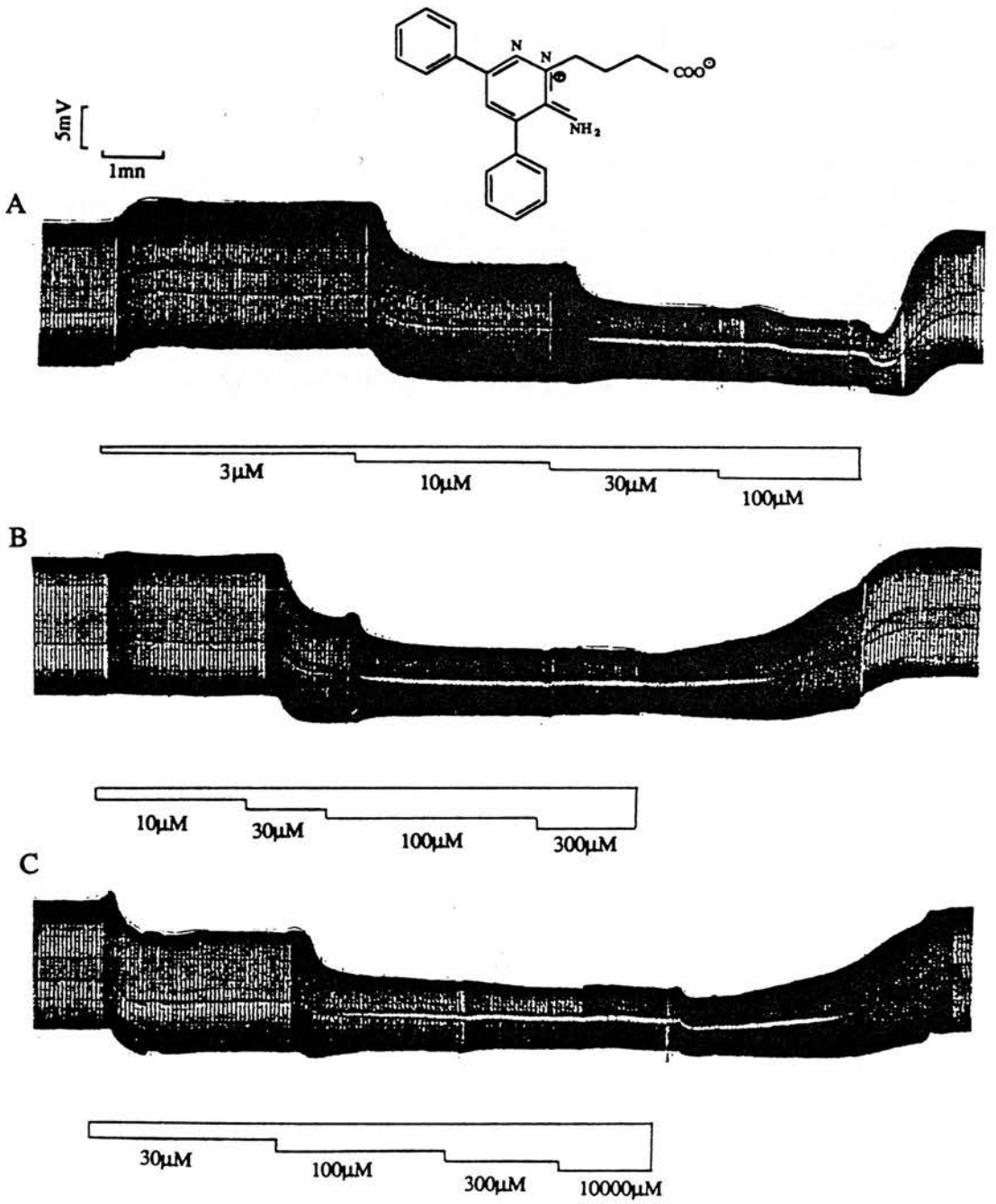

5mV
1mn
A
3µM
10µM
30µM
100µM
B
10µM
30µM
100µM
300µM
C
30µM
100µM
300µM
10000µM

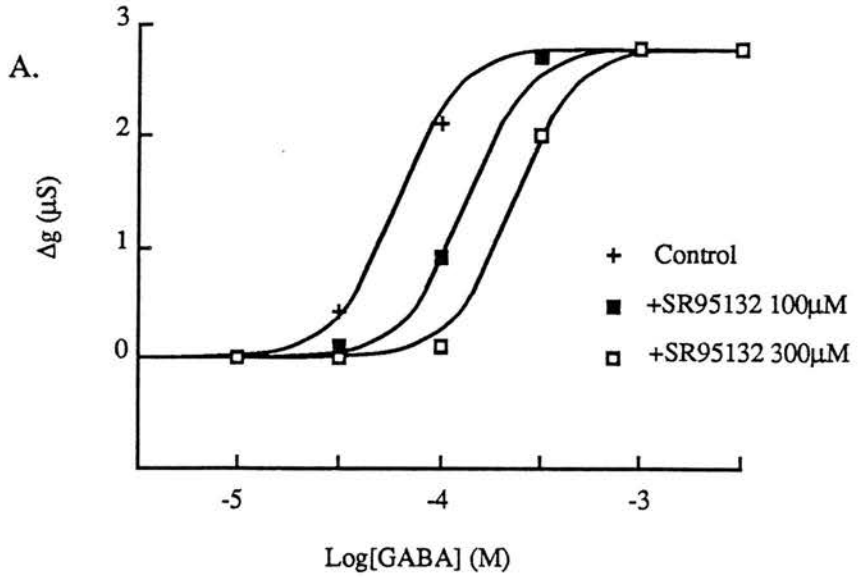
Figure 58: Dose-dependence of the antagonism produced by SR95132.

The lines correspond to the fitted modified Hill equation, whereas the points are the experimental data.

A. GABA dose-response curves
Control GABA dose-response curve, values estimated for the modified Hill equation are Δgmax=2.8μS, EC50=19.8μM, N=2.6.
GABA dose-response curve in the presence of 100μM SR95132, values estimated for the modified Hill equation are: Δgmax=2.8μS, EC50= 37.6μM and N=3.2.
GABA dose-response curve in the presence of 300μM SR95132, values estimated for the modified Hill equation are: Δgmax=2.8μS, EC50=77.2μM and N=3.5.

B. Schild and modified Schild plot;
(o) Schild plot, log(DR-1) versus logB, B is the concentration of antagonist. Correlation coefficient, r=0.71; slope=0.74±0.25, p=0.02 (F test); R^2=0.62.
(■) Modified Schild plot, log(DR^2-1) versus logB. Correlation coefficient r=0.75; slope=1.06±.32, p=0.01, (F test); R^2=0.66, suggests a better fit than with the classical Schild plot.

SR95132


A.
Δg (μS)
3
2
1
0
-5
-4
-3
Log[GABA] (M)
Control
+SR95132 100μM
+SR95132 300μM

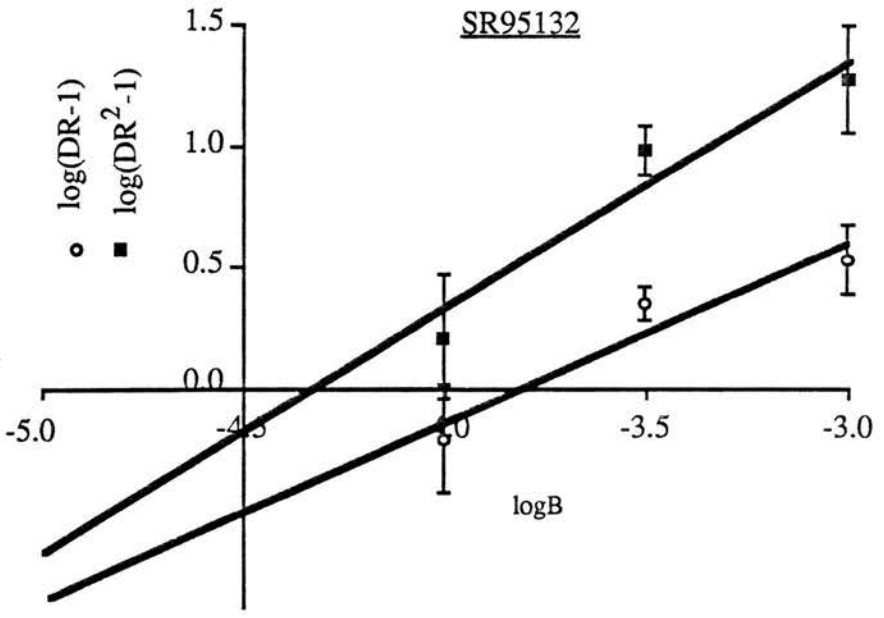

SR95132
log(DR-1)
log(DR²-1)
1.5
1.0
0.5
0.0
-5.0
-3.5
-3.0
logB

Figure 59: Effects of 100, 300 and 1000μM SR42666 on GABA dose-response relationship.

A. Control responses to cumulative application of GABA, g_0=2.2μS. Note the transient depolarizing response in the presence of 10μM GABA and the gain change in the presence of 300 and 1000μM (one unit is 2mV).

B. In the presence of 100μM SR42666. Not the gain change in the presence of 1000μM (one unit is 2mV).

C. In the presence of 300μM SR42666. Note the gain change in the presence of 300 and 1000μM (one unit is 2mV).

D. In the presence of 1000μM SR42666. Note the gain change in the presence of 300 and 1000μM (one unit is 2mV).
The corresponding dose-response curves are illustrated in Figure 60A.

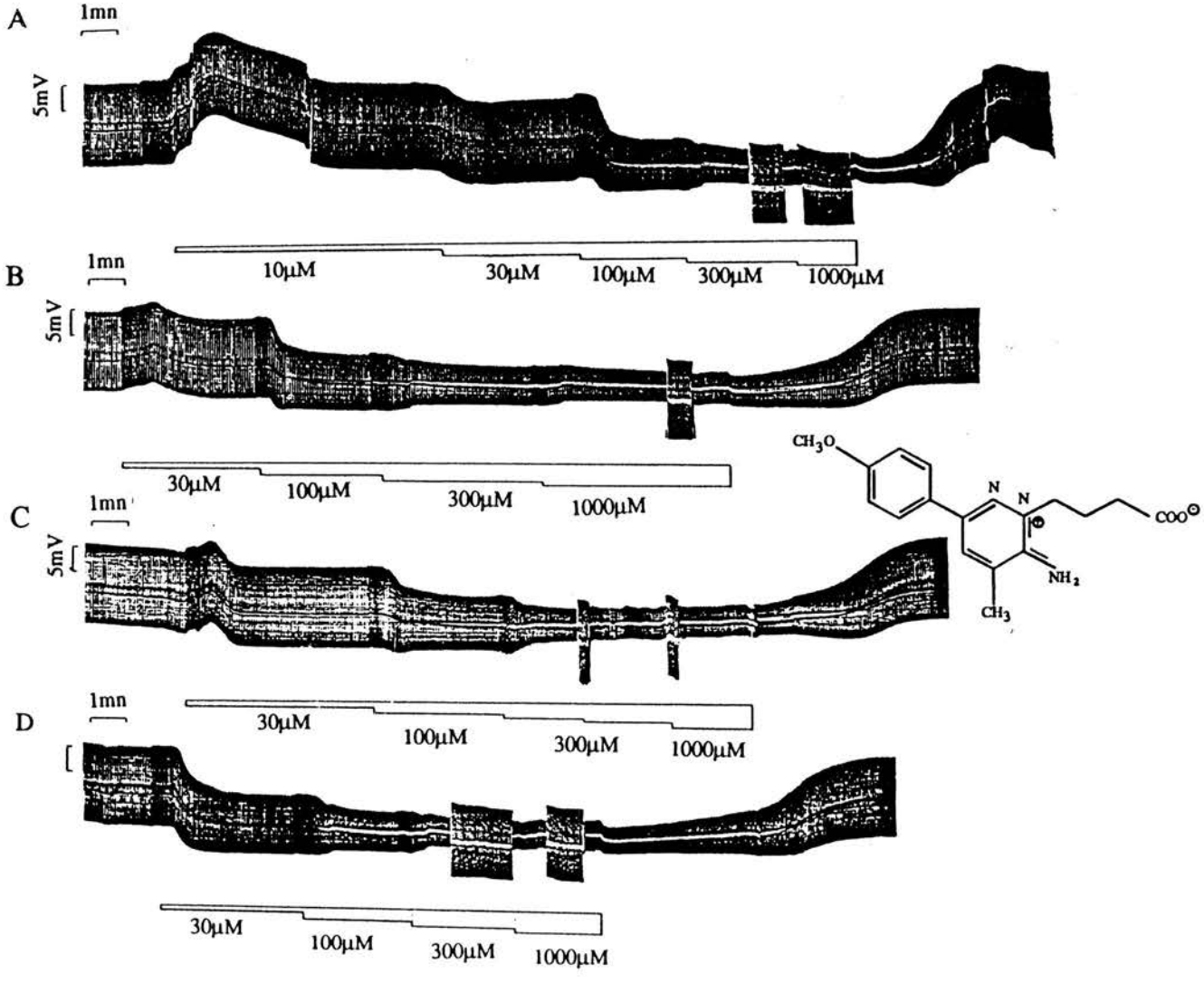

A
1mn
5mV
B
1mn
10µM
30µM
100µM
300µM
1000µM
5mV
30µM
100µM
300µM
1000µM
C
1mn
5mV
D
1mn
30µM
100µM
300µM
1000µM
30µM
100µM
300µM
1000µM
CH3O
N
N
COO⊖
NH2
CH3

Figure 60: Dose-dependence of the antagonism produced by SR42666.

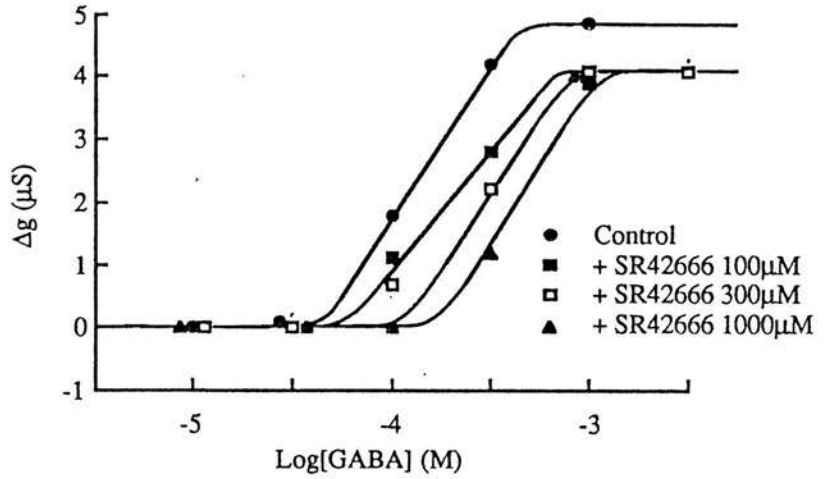
The lines correspond to the fitted modified Hill equation, whereas the points are the experimental data.

A. GABA dose-response curves
Control GABA dose-response curve, values estimated for the modified Hill equation are Δgmax=4.9μS, EC50=39.9μM, N=2.1.
GABA dose-response curve in the presence of 100μM SR42666, values estimated for the modified Hill equation are: Δgmax=4.1μS, EC50= 59.8μM and N=1.7.
GABA dose-response curve in the presence of 300μM SR42666, values estimated for the modified Hill equation are: Δgmax=4.3μS, EC50=89.7μM and N=1.8.
GABA dose-response curve in the presence of 1000μM SR42666, values estimated for the modified Hill equation are: Δgmax=4.3μS, EC50=134.4μM and N=3.2.
In this particular cell Δgmax appears to be smaller in the presence of SR42666. However, in other cells, Δgmax can be higher in the presence of SR42666, than for the control. This obviously illustrates the difficulty in measuring accurately high membrane conductance. SR42666 as the other arylaminopyridazine GABA derivatives tested quantitatively, did not affect Δgmax or N significantly.

B. Schild and modified Schild plot;
(○) Schild plot, log(DR-1)/logB, B is the concentration of antagonist. Correlation coefficient, r=0.78; slope=0.73±0.17, p=0.001 (F test); $R^{\wedge 2}$=0.62.
(■) Modified Schild plot, log(DR^2-1)/logB. Correlation coefficient r=0.78; slope=1.02±.25, p=0.001, (F test); $R^{\wedge 2}$=0.62.

SR42666

A.



SR42666

B.

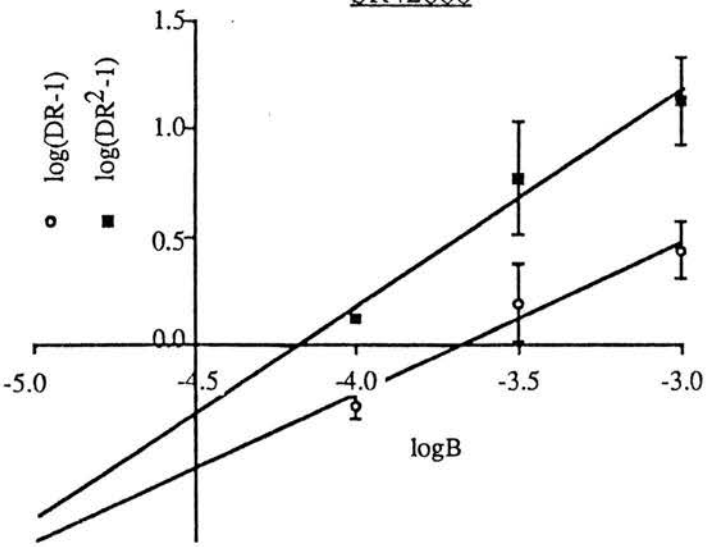


Figure 61: Effects of 100, 300 and 1000μM NCS247-90 on GABA dose-response relationship.

A. Control responses to cumulative application of GABA.

B. In the presence of 100μM NCS247-90.

C. In the presence of 300μM NCS247-90.

D. In the presence of 1000μM NCS247-90.

The corresponding dose-response curves are illustrated in Figure 62A.

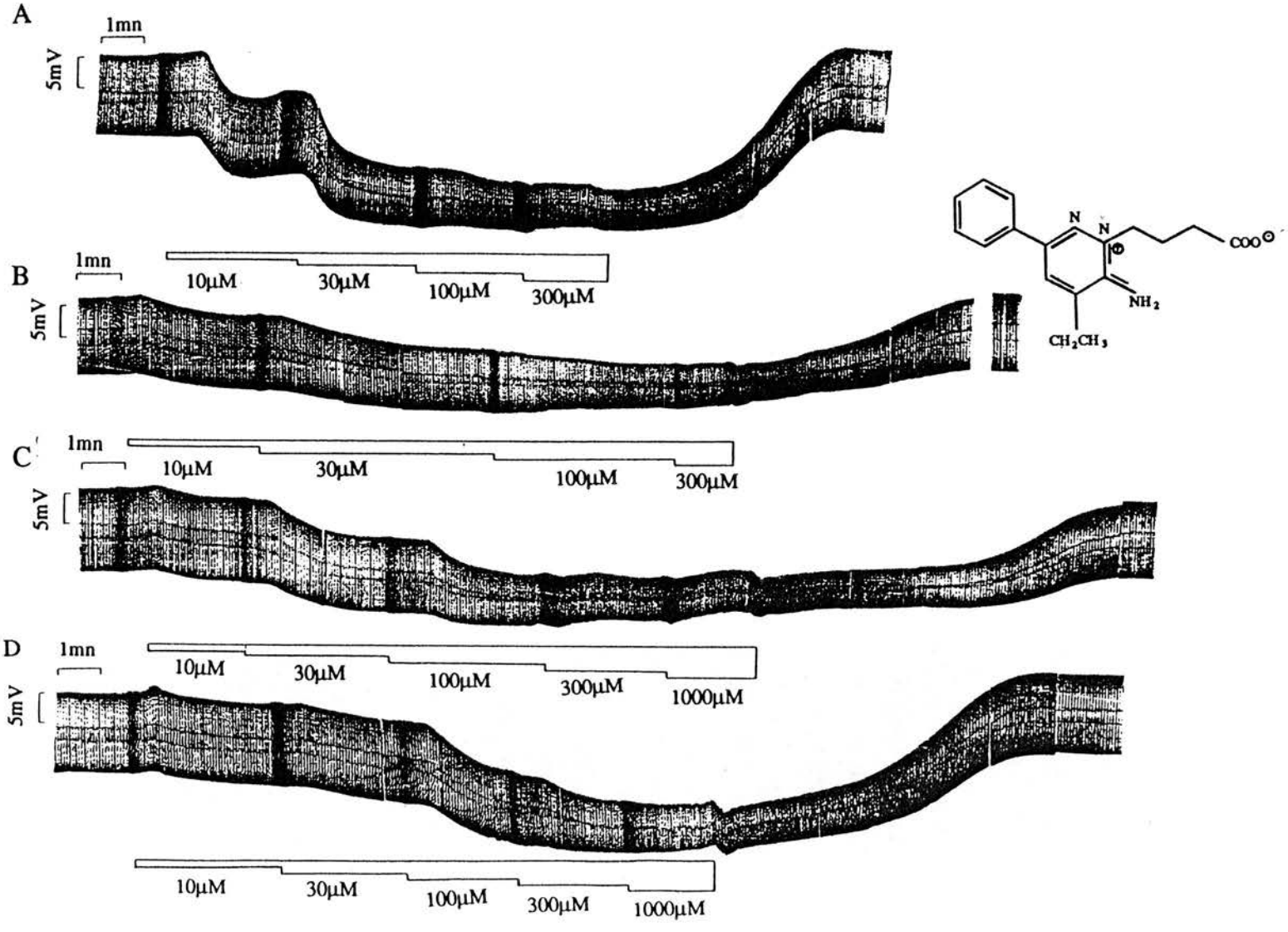

A
1mn
5mV
B
1mn
10μM
30μM
100μM
300μM
5mV
C
1mn
10μM
30μM
100μM
300μM
5mV
D
1mn
10μM
30μM
100μM
300μM
1000μM
5mV
10μM
30μM
100μM
300μM
1000μM
N
N
COO⊖
NH2
CH2CH3

Figure 62: Dose-dependence of the antagonism produced by NCS247-90.

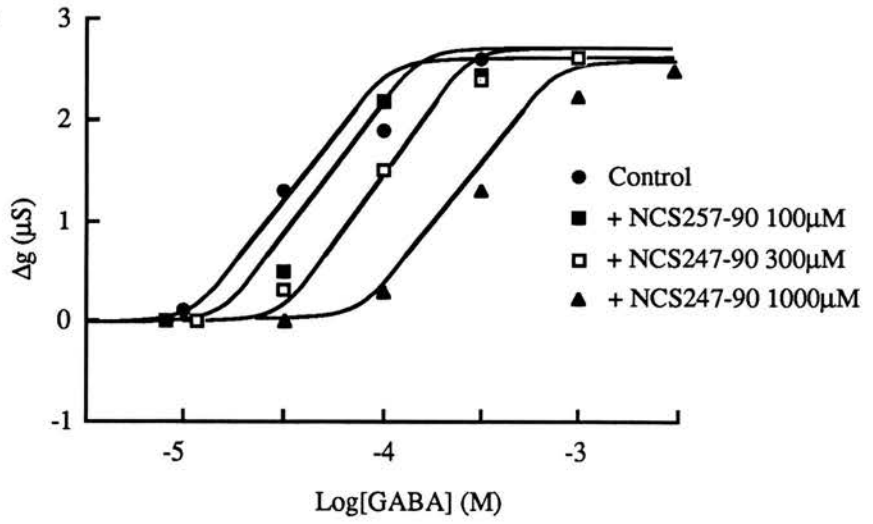
The lines correspond to the fitted modified Hill equation, whereas the points are the experimental data.

A. GABA dose-response curves
Control GABA dose-response curve,values estimated for the modified Hill equation are Δgmax=2.4μS, EC50=31.2μM, N=1.8.
GABA dose-response curve in the presence of 100μM NCS247-90, values estimated for the modified Hill equation are: Δgmax=2.6μS, EC50= 71.2μM and N=1.7.
GABA dose-response curve in the presence of 300μM NCS247-90, values estimated for the modified Hill equation are: Δgmax=2.6μS, EC50=85.6μM and N=2.0.
GABA dose-response curve in the presence of 1000μM NCS247-90, values estimated for the modified Hill equation are: Δgmax=2.8μS, EC50=265.0μM and N=1.9.

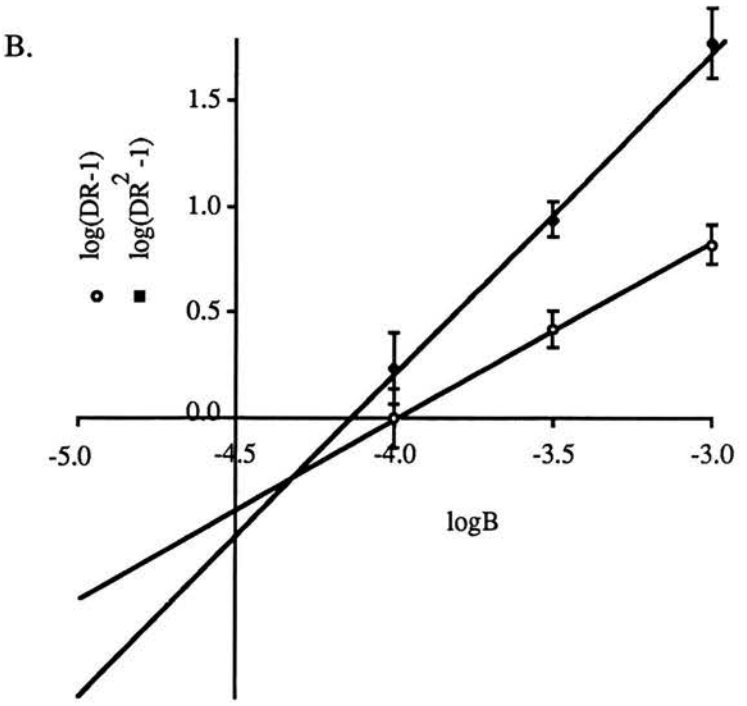
B. Schild and modified Schild plot;
(○) Schild plot, log(DR-1)/logB, B is the concentration of antagonist. Slope=1.00±0.17, $p<0.001$ (F test); $R^{\wedge 2}=0.74$.
(■) Modified Schild plot, $\log(DR^2-1)/\log B$. Slope=1.50±0.23, $p<0.001$, (F test); $R^{\wedge 2}=0.77$, suggests a better fit than with the classical Schild plot.

NCS247-90

A.


3
2
1
0
-1
Δg (μS)
-5
-4
-3
Log[GABA] (M)
Control
+ NCS257-90 100μM
+ NCS247-90 300μM
+ NCS247-90 1000μM

B.


log(DR-1)
log(DR2-1)
1.5
1.0
0.5
0.0
-5.0
-4.5
-4.0
-3.5
-3.0
logB

IIIDISCUSSION

1. Order of potency of the arylaminopyridazine GABA derivatives: comparison with the vertebrate GABA-A receptor

When applied at 1mM against 30μM GABA, SR42627, SR42640, SR95133, SR95132 and the two derivatives tested in Section 5, SR95103 and SR95531, present an order of potency different from what it is observed at the vertebrate GABA-A receptor (Wermuth et al., 1987):

at the *Ascaris* GABA receptor ,

SR95103≈SR95132>SR42666>SR95133>SR95531>SR42627>SR42640

at the mammalian GABA-A receptor:

SR95531>SR42666>SR42627>SR95103>SR95133>SR42640>SR95132.

SR95531, the most potent displacer of [^{3}H]GABA in the mammalian brain with a K_B of 0.15μM, is a weak antagonist on *Ascaris* muscle since only 44.2±6.1% of the GABA (30μM) response is antagonized by SR95531 1mM (mean±SE, n=5). Similarly, SR42627, a rather good displacer of [^{3}H]GABA in the mammalian brain with a K_B of 0.55μM, is one of the weakest antagonist in *Ascaris* muscle: 21.0±8.0% of the GABA (30μM) response is antagonized (mean±SE, n=2). In contrast SR95103 and SR95132, respectively a moderate (K_B=2.20μM) and a virtually inactive (K_B=100μM) antagonist at the vertebrate GABA-A receptor, are the most potent compounds in *Ascaris* muscle; SR95103 antagonized 93.4±2.7% (mean±SE, n=5) and SR95132, 89.0±4.4% (mean±SE, n=4) of the response to 30μM GABA.

The measurement of the percentage of antagonism can be compared to a snapshot of the antagonist-receptor interaction, since only one concentration of antagonist and agonist are tested together. For that reason, it does not provide any information about the type of antagonism involved or any real quantitative information. However, within a family of compounds supposed to act in a similar manner such as the arylaminopyridazine derivatives of GABA, the percentage of antagonism can be used to determine their potency sequence.

The method is nonetheless a sensitive technique for detecting antagonism. The GABA concentration used, 30μM, is near to the EC50 for GABA, and therefore in the middle of the linear part of the log dose-response curve. Because of positive cooperativity between GABA and its receptor, the slope is greater than one, and as a consequence the linear part of the curve is steep. For these reasons, any antagonism would be very easily detected. Compounds producing nearly 100% antagonism or on the contrary 0% antagonism, cannot be compared with each other since they are out of

Table 14: Comparison of some arylaminopyridazine GABA derivatives potencies in vertebrate with *Ascaris suum* muscle

(1) [^{3}H]GABA displacement from rat brain membranes, K_B = dissociation constant (Wermuth et al., 1987)
(2) %antagonism of the GABA 30μM response, produced by 1mM antagonist, in *Ascaris*
(3) K_B = dissociation constant estimated from the modified Schild analysis, in *Ascaris*

	VERTEBRATE	*ASCARIS* MUSCLE	
	K_B (μM) (1)	%antagonism (2)	K_B (μM) (3)
SR95531	0.15	44.2±6.1 (n=5)	—
SR42666	0.31	76.6±6.6 (n=5)	104.8±19.7 (n=13)
SR42627	0.55	21.0±8.0 (n=2)	—
SR95103	2.20	92.8±3.2 (n=5)	63.8±12.9 (n=14)
SR95133	2.60	58.0±8.0 (n=4)	—
SR42640	10.40	7.5±7.5 (n=2)	—
SR95132	100.00	89.0±3.4 (n=5)	65.0±20.3 (n=9)

the linear part of the antagonist dose-response curve. The only solution is to change the antagonist concentration and to use a lower concentration, in the case of two potent antagonists, or a higher concentration, in the case of two weak antagonists. For example, in the case of NCS251-90 which appeared to be a potent antagonist with an antagonism of 99.2±0.8% (mean±SE, n=5) when tested at 1mM, a lower concentration, 100μM, was tested in the same manner and the antagonism produced was only 28%. Compared with SR95103 100μM tested also in the same manner, where only 11% of the response was antagonized, NCS251-90 does not appear much more potent. It was decided that only compounds presenting a significant increase in potency will be quantitatively tested on GABA dose-response curves.

The use of the percentage of antagonism is therefore a good screening technique, providing the GABA concentration is chosen in the linear part of the log dose-response curve, but should not be used for quantitative purpose.

2. Arylaminopyridazine GABA derivatives are competitive antagonist at the *Ascaris* muscle GABA receptor

In *Ascaris*, the three most potent analogues: SR95103, SR95132 and SR42666, have been shown to displace GABA dose-response curves to the right without a decrease in the maximal response. The modified Schild plot, which accounts for the positive cooperativity between GABA and its receptor, was linear with a slope of one, suggesting a competitive mechanism in which only one molecule of the antagonist was required to block the effects produced by two molecules of GABA. Although plausible models for the drug-receptor interaction do not predict simple relationships as described by the Schild and modified Schild plots; under simplifying limitations they can be approximated by these plots and provide a reasonable description of the antagonist-receptor interaction. In the patch-clamp study, 30μM SR95103 was shown to antagonize GABA-activated channels, in a manner consistent with a competitive mechanism but with an additional non competitive component detectable at a higher concentrations (see Section 6). This non competitive component may represent only a small proportion of the total antagonism since detected only at high concentration, and may remain undetected with a less sensitive technique such as the current-clamp experiments. In conclusion, on the basis of the results obtained with SR95103, SR95132, SR42666, and NCS247-90, the mode of action of arylaminopyridazine derivatives of GABA in *Ascaris* is considered to be competitive. The difference in relative potencies for these compounds between the vertebrates and the *Ascaris* GABA receptors gives evidence for their different structural requirements. However these

structural differences do not involve the GABA binding site, since the flexible GABA-like moiety of the pyridazine derivatives supposed to interact with the GABA recognition site, is the same for all the analogues tested (except NCS248-90, NCS249-90, NCS250-90 and NCS194-83). In vertebrate preparations, antagonist properties rely on other parts of the molecule and may be interpreted in terms of Ariëns theory of accessory binding sites (Arïens et al., 1979). The differences in potency between mammalian preparations and *Ascaris* are in favour of the presence of different accessory binding sites.

3. Structure-activity relationship of a series of arylaminopyridazine GABA derivatives on *Ascaris* muscle

The structure-activity relationships of pyridazine derivatives have been investigated in great details in the vertebrate nervous system (Wermuth et al., 1987), and discussed in terms of Ariëns theory of accessory binding sites. The substituents on the pyridazine ring appear to be of prime importance. Refer to Figure 49 for the chemical structures.

3.1. 6-substituent

In vertebrates, a very important role is played by the substituent in the 6-position (Wermuth et al., 1987). In this study, all the analogues tested had a phenyl, substituted or not, in 6-position on the pyridazine ring except SR95133 and NCS248-90. The role played by the 6-substituent has been associated with the existence of additional binding interactions with the receptor, involving π electrons (Wermuth et al 1987); in other words, an accessory binding site as defined by Ariëns et al. (1979). The naphtyl 6-substituent found in SR95133, presents, as well, π electrons for interaction with the receptor, and may explain why SR95133 retains some antagonist properties. NCS248-90 presents π electrons, but is a very weak antagonist. However, this compound possesses a sulphonic acid group instead of a carboxylic group; and it has been shown by the inactivity of NCS249-90 and NCS250-90 that the presence of a sulphonic group instead of a carboxylic group, is strongly detrimental.

3.2. 6-phenyl substituent

The substituent effects on the 6-phenyl can influence the potency. For example substituents increasing the electron density (-CH_3 as in SR42627) or the aromatic system (-OCH_3 as in SR95531), increase the potency in mammalian preparation, in

contrast to electron-withdrawing substituents (Wermuth et al., 1987). In vertebrate, the order of potency for the 6-phenyl substituents is:

$$-OCH_3 > -CH_3 > -H$$

(SR42666 > SR42627 > SR95103)

and in *Ascaris* it is:

$$-H > -OCH_3 >> -CH_3$$

(SR95103 > SR42666 > SR42627)

suggesting an opposite effect.

However, in *Ascaris*, the 6-phenyl substituent is not the limiting factor for the antagonist properties, the presence of a 4-substituent on the pyridazine ring is more important.

3.3. 4-substituent

The role played by the 4-substituent in *Ascaris* is illustrated by the potent antagonists in *Ascaris*: SR95103, SR95132, NCS247-90, NCS251-90 and NCS 252-90, all substituted on the 4-position of the pyridazine ring, respectively with a methyl, a phenyl, an ethyl, a benzyl, and an isopropyl group. This contrasts with the vertebrate preparations where the substitution by a methyl or a phenyl group on 4-position on the pyridazine ring, was always detrimental to the antagonist potency (Wermuth et al., 1987). Among these analogues, only SR95132, SR95103, and SR95531, have been tested on mammalian preparation, and the order of potency for the 4-substituent is:

$$-H > -CH_3 >> -C_6H_5$$

(SR95531 > SR95103 > SR95132)

By comparison in *Ascaris*, it is:

$$-C_6H_5 \approx -CH_3 >> -H$$

(SR95132 ≈ SR95103 >> SR95531)

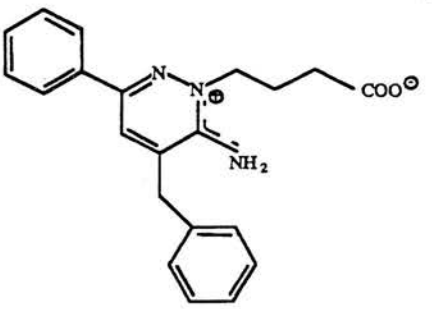
To illustrate the importance of the 4-substituent in Ascaris, one can compare the potency of SR42666 and SR95531: their chemical structure is identical except for the 4-methyl present on SR42666, but not on SR95531. SR42666 is more potent than SR95531 in *Ascaris*, than in mammalian preparations. The presence of a 4-substituent appears to be a prerequisite for the antagonist potency of pyridazine GABA derivatives in *Ascaris*, whereas the opposite situation is found in vertebrates (Figure 63).

Figure 63: Structure and activity of arylaminopyridazine GABA derivatives in *Ascaris*.

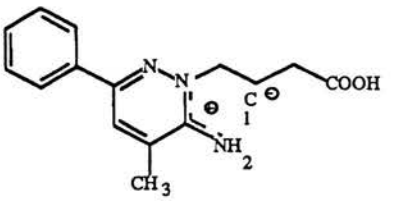
The percentage indicated besides each analogue is the percentage of antagonism of the response to GABA (30μM) produced by 1mM antagonist. All the potent GABA antagonist in *Ascaris* present a 4-substituent. The comparison between SR42666 and SR95132 illustrates the different structural requirements at the *Ascaris* GABA receptor: SR42666 and SR95531 are identical except for the 4-methyl present on SR42666, and SR42666 is more potent than SR95531 in *Ascaris*, in contrast to the mammalian preparations.

Active compounds

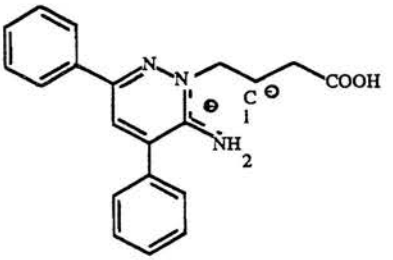
NCS251-90: 95%



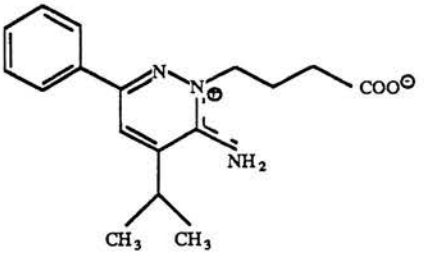
SR95103: 93%



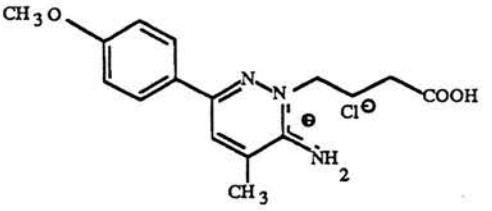
SR95132: 89%



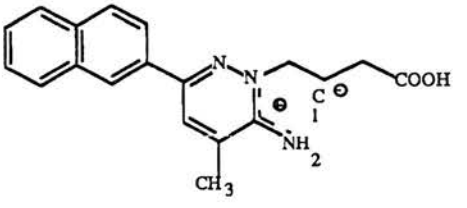
NCS252-90: 81%



SR42666: 76%

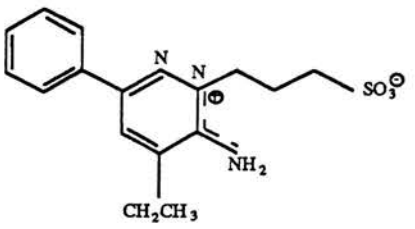


SR95133: 58%

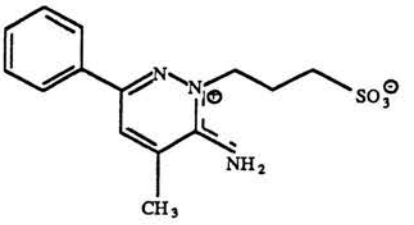


Inactive or weak compounds

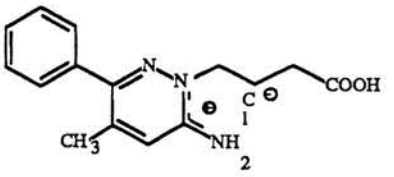
NCS250-90: 0%



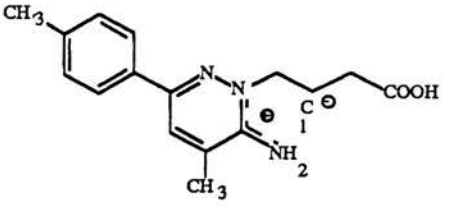
NCS249-90: 0%



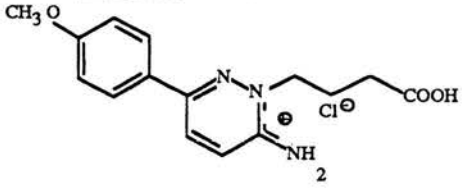
SR42640: 7%



SR42627: 27%



SR95531: 46%



3.4. Acid group

Section 3 has shown that sulphonic acid derivatives of GABA are inactive in *Ascaris*. The two sulphonic acid derivatives, NCS249-90 and NCS250-90, are identical respectively to SR95103 and NCS247-90, except for the sulphonic acid group. They are totally inactive at 1mM. NCS248-90 presents also, a sulphonic acid function on the side chain, and was found to be a very weak antagonist. This compound presents also a large substituent in the 6- and 5-positions on the pyridazine ring. 5-substituents (see compound SR42640) are usually detrimental in vertebrates (Wermuth et al., 1987) and in *Ascaris* .

The presence of a sulphonic acid group instead of a carboxylic acid group is therefore highly detrimental to the antagonistic properties of pyridazine GABA derivatives.

3.5. Side-chain length

As illustrated by NCS194-83, a longer side-chain (5C, like δ-aminovaleric acid) is detrimental to the potency of the compound (NCS194-83 is equivalent to SR95103 except for the side-chain length). Despite this some antagonistic activity remains, suggesting that the molecule can still recognize the GABA binding site. A similar situation is found in the vertebrate where the side chain length of arylaminopyridazine GABA derivatives can be altered without a complete loss of activity (Wermuth et al., 1987).

4. Computer graphics modelling

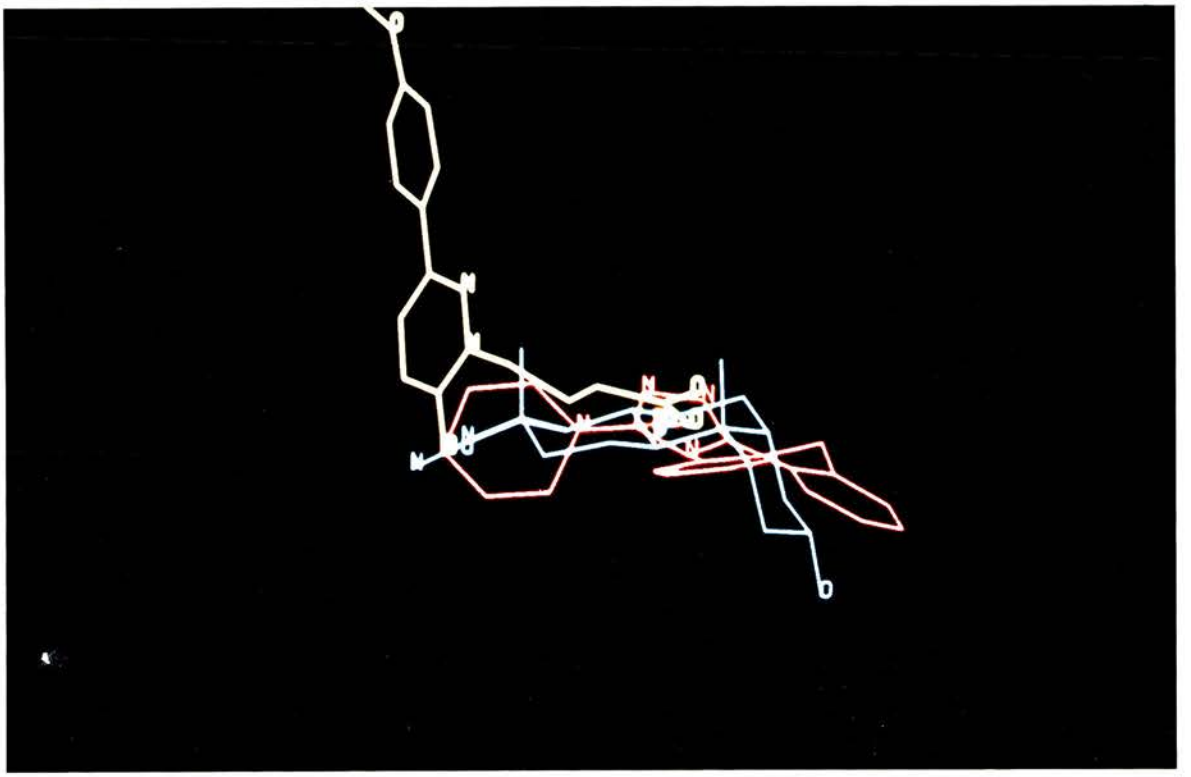
In vertebrates, computer graphic modelling of the pyridazinyl GABA-derivatives and other known GABA antagonists was used to illustrate a plausible model for the interaction of competitive antagonists with the GABA-A receptor (Figure 64, Wermuth and Rognan, 1987). The superimposition of (+)bicuculline, pitrazepine, iso-THAZ, SR95531, RU5135, securinine and tubocurarine shows the existence of a cationic and an anionic system separated by 4.7Å, which may mimic the GABA molecule and bind the GABA recognition site (Wermuth and Rognan, 1987). The antagonist properties can be explained by the presence of additional binding sites corresponding to the various aromatic or aliphatic rings of these compounds. The free access zone situated beneath the anionic and cationic systems is required for the interaction with the GABA-A binding site. The inactivity of 4-substituted aminopyridazine GABA derivatives and

Figure 64: Superimposition of some GABA-A antagonists using computer graphic modelling (photographs from Wermuth and Hoffmann, Centre de Neurochimie du CNRS).

A. Superimposition of potent GABA-A antagonists
Blue: RU5135
Red: pitrazepine
Yellow: SR95531

B. Superimposition of moderate GABA-A antagonists
Blue: Securinine
Red: Iso-THAZ
Purple: (+)Bicuculline

The superimposition of (+)bicuculline, pitrazepine, securinine, RU5135, SR95531, iso- and THAZ shows the existence of a cationic and an anionic systems separated by approximately 4.7Å, which may mimic the GABA molecule and bind the GABA recognition site. The antagonist properties can be explained by the presence of additional binding sites corresponding to the various aromatic or aliphatic rings of these compounds. The free access zone situated beneath the anionic and cationic systems is likely to correspond to the zone of interaction with the GABA-binding site.



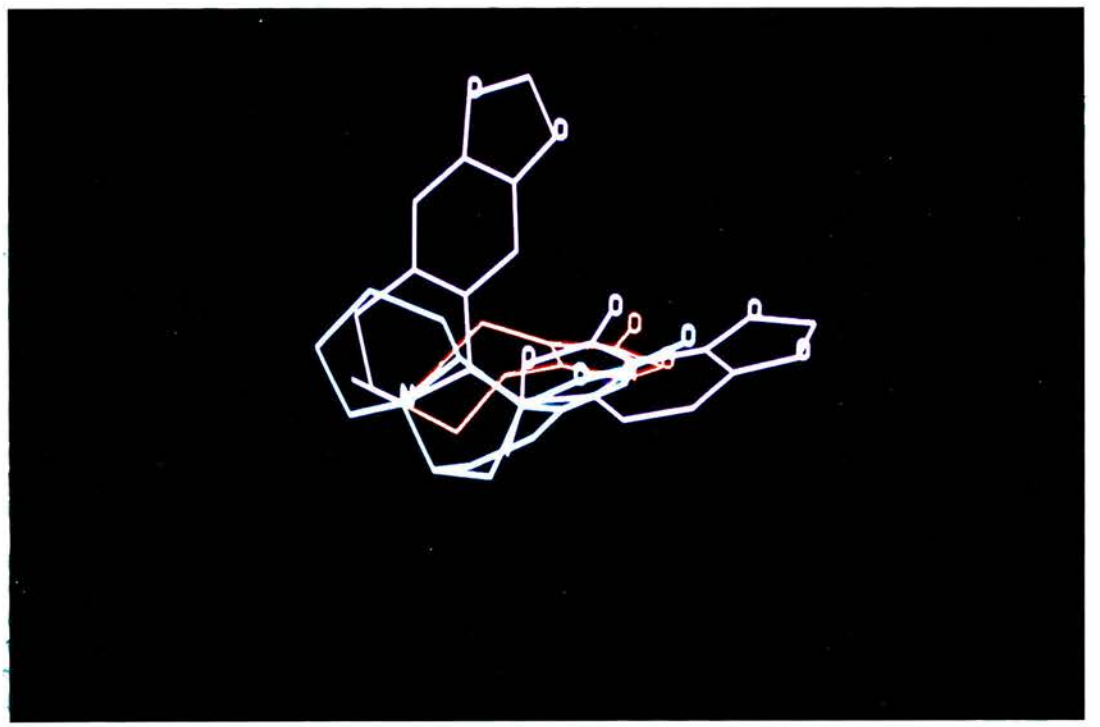
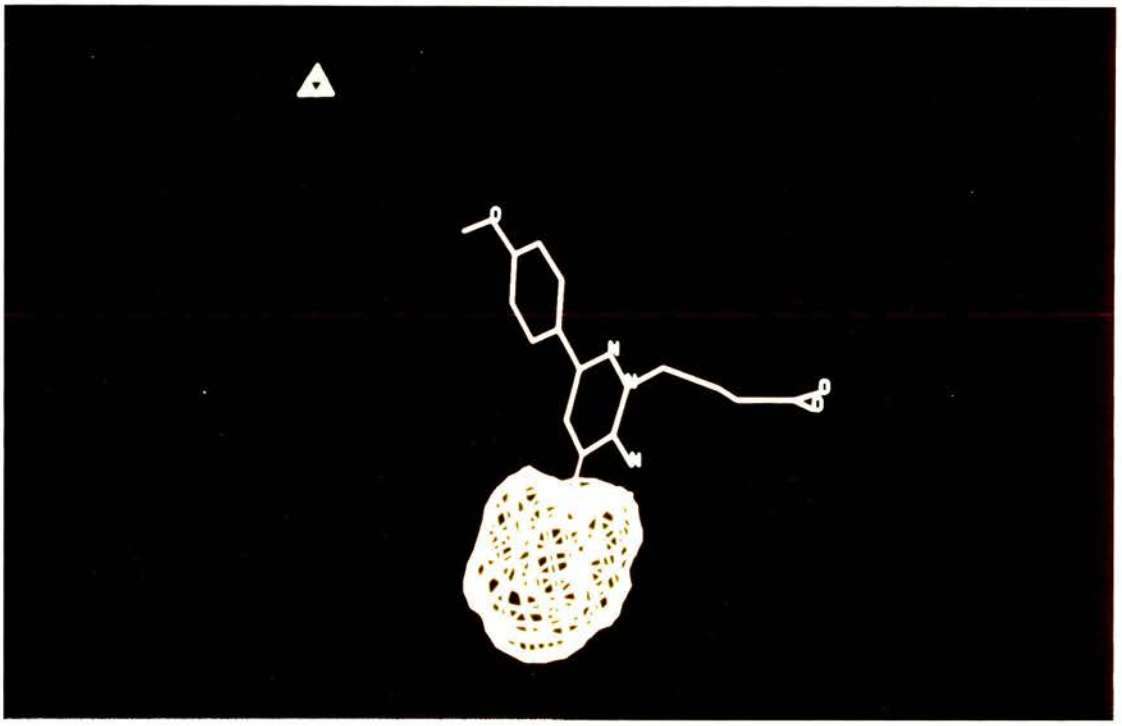


Figure 65: Superimposition of SR95132 and SR95531

The superimposition of these two analogues shows that the 4-substituent is found in proximity of the GABA recognition site. The inactivity of 4-substituted analogues at the vertebrate GABA-A receptor was explained in terms of restriction of the free access zone required for interaction with the receptor. The volume occupied by the phenyl group of SR95132 is visualized. (Photographs from Wermuth and Hoffmann, Centre de Neurochimie du CNRS).



some stereoisomers: (-)bicuculline, virosecurinine, allosecurinine, on the vertebrate GABA-A receptor is explained by a restriction of this free access zone due to the presence of substituents in this region (Wermuth and Rognan, 1987). Figure 65 shows the superimposition of SR95531 and SR95132; the lack of activity of SR95132 on the vertebrate GABA-A receptor, may be explained by the obstruction of the free access zone required for receptor recognition by the phenyl group in the 4- position (Wermuth and Rognan, 1987). Obviously this is not the case in *Ascaris*, and the 4-substituent is a requirement for the antagonistic activity.

CONCLUSION

Applied to other known GABA-A antagonists such as iso-THAZ, RU5135, pitrazepine, securinine, (+)bicuculline and its analogue (+)tubocurarine, the hypothesis of accessory binding sites also seems to hold. In all these compounds, a slight structural resemblance to GABA can be found: an electron rich area is separated by a distance of 4.7Å from a positively charged centre. All these antagonists also present several aromatic or alicyclic ring systems, which are either close to the electron rich side of the molecule (RU5135 and pitrazepine), or close to the cationic site (securinine), or even on both sides (bicuculline and tubocurarine).

The existence of different accessory binding sites responsible for antagonist properties in *Ascaris* can explain the inactivity of the classical competitive GABA-A antagonists: bicuculline, pitrazepine, securinine (Holden-Dye et al., 1988), tubocurarine and RU5135 (Section 4); in contrast, GABA-A agonists in *Ascaris* have relative potencies similar to that on the vertebrate GABA-A receptor.

These differences are obviously of great interest in the search for new anthelminthic drugs, and could lead to the synthesis of a potent GABA antagonists in *Ascaris*, which are inactive in mammals and therefore without toxicity or secondary effects in the host.

GENERAL DISCUSSION

In this study, the pharmacology of the *Ascaris* muscle GABA receptor has been studied using an electrophysiological approach. Despite some differences: inactivity of sulphonic acid derivatives, loss of relative potency of rigid analogues of GABA, the agonist profile of the *Ascaris* GABA receptor is correlated with the vertebrate GABA-A type. In contrast, the antagonist properties of the *Ascaris* GABA receptor appeared very different from those of the vertebrate GABA-A type. None of the classical GABA-A antagonists is potent, and the structure-activity relationships of the arylaminopyridazine derivatives is different in *Ascaris*. These differences may be explained by the existence of different accessory binding sites responsible for antagonist binding at the *Ascaris* GABA receptor. More generally, the GABA recognition site appears to have been well conserved throughout evolution (Walker and Holden-Dye, 1989); whereas the antagonist accessory binding sites show a greater variation across species. This seems to be valid for receptors of pharmacological interest, and more generally for "reactive proteins", as stated by Black (1987):

> *"...I expect that hormones will turn out to be very similar to other reactive proteins, enzymes, carriers and so on where only the reactive sites are highly conserved across species with respect to amino-acid composition. Therefore, while we may be entitled to expect the functionality of hormones and their conjugate receptors to be highly conserved across species, no such expectation is reliable when exploring receptors with competitive antagonists, because a reasonable interpretation of many structure-activity studies of agonists, antagonists is that the antagonists can explore wider molecular areas of the receptor than the native hormone can...".*

Two important implications can be drawn from this statement. Firstly, the study of comparative pharmacology gives some indications on where the selection pressure exerts. To this respect, antagonists do not seem to exert a strong selection pressure; and the main reason is because they are rarely naturally present in living organisms. When naturally present in an organism, antagonists must be inoffensive. They may be strictly localized to a specific organ, such as tetrodotoxin, which is highly concentrated in the ovaries of the puffer fish (*Sphareoides* sp.), or like α-bungarotoxin, an acetylcholine nicotinic blocker, which is localized in the venom glands of the Elapid snake, *Bungarus multicinctus*. Antagonists may be also found in organisms which are not sensitive to their action; this is the case of many plants which are obviously not sensitive to neurotoxins. For example, among the GABA-A antagonists, picrotoxin is found in the

seeds of a climbing shrub, *Anamirta cocculus* (Goodman and Gilman, 1985); securinine is also found in a schrub, *Securinega suffructicosa* (Goodmann and Gilman, 1985) and bicuculline can be extracted from plants of the gender *Corydalis* (sp.) (Curtis, Duggan, Felix et al., 1970). These examples also illustrate the role played by antagonists in living organisms: they are not aimed at the host, but are targeted towards potential predators or prey.

It is true that antagonists are rarely naturally occurring in an organism or in its biotope. However, it would be wrong to say that antagonists are not able to exert any kind of selection pressure. There are some cases where animals have developed resistance to antagonists. Although tetrodotoxin is highly concentrated in specific organs, it is also present in the whole organism and has access to nerves and muscle. This was demonstrated accidentally in the course of embryological experiments on interspecific grafts on salamanders, with species that make tetrodotoxin (*Taricha* and *Triturus*) and species that does not (*Ambystoma*) (see Hille, 1984b). When a piece of *Ambystoma* was grafted onto *Taricha*, the graft became paralysed, suggesting that the toxin is freely diffusible and that the two kinds of salamanders have a major difference in the toxin sensitivity of their Na channels. Saxitoxin, also a Na channel blocker, is another example; it is synthesized by marine dinoflagellates which do not have Na channels. In some conditions, these microorganism multiply and increase the toxin concentration in the sea. In warm waters of the Pacific Northwest, toxic concentrations in the sea can be found for major periods of the year. As a consequence, some animals in the food chain have evolved and show some resistance to saxitoxin (see Hille, 1984b).

Parasites, with special reference to internal parasitism, have been subject to a specific evolution. When compared to the free living species, they have evolved lately, and are under a very high selection pressure. One characteristic and general tendency in the evolution of internal parasites is that they tend to express the same phenotype than their host, in order to avoid any harmful interference with the host and its resultant rejection or destruction (Rogers, 1962):

> *"...The host-parasite relationship might be regarded as ultimately reaching a steady-state in which two genotypes reach to give a common phenotype on which selection might act. In response to selection a change in phenotype might react differently on the two genotypes, though in this state they could not react independently..."*

But parasites have also to deal with life conditions specific to their nature. The metabolism of intestinal parasites usually requires an adaptation to an anaerobic environment. The adaptation to parasitism is therefore characterized by two apparently

opposite tendencies; the necessity to evolve specific features related to its parasitic nature, and the necessity to mimic the environment, to avoid host reaction. This is not without implication on the strategies developed in antiparasitic drug research: successful drugs act on a metabolism or enzyme specific of the parasite (Urquhart et al., 1987):

> Benzimidazoles, such as thiabendazole, mebendazole, and probenzimidazoles, such as, fenbedazole, albendazole, oxibendazole etc... act by preventing the tubulin isomerization and desorganize the cell endoskeleton.
>
> Organophosphates, such as, dichlorvos, haloxon, trichlorfon, act as anticholinesterase agents.
>
> Salicylanilides, such as, nitroscanate, closantel interfere with enzymes specific to the parasites in the ATP production.
>
> Imidothiazoles and tetra-hydropyrimidines, such as, tetramisole, levamisole, pyrantel, morantel, are specific agonists at the acetylcholine receptor.

In the case of the GABA receptor, it appears that this receptor in *Ascaris* presents strong similarities, in terms of its agonist profile, with the host GABA-A receptor. It would seem therefore that GABA receptors in parasites are not a good target for anthelminthic drugs. However, Black's statement has a second important implication of therapeutical order: an antagonist is more likely than an agonist to be successful, especially since selectivity is an important factor in the therapeutic success of antiparasitic agents. In insects, the GABA receptor-complex is already considered as a target for insecticides: a primary target for toxaphen and cyclodiene, and a secondary target for lindane and type II pyrethroids (Eldefrawi and Eldefrawi, 1987). All these toxicants antagonize the GABA response by an action at the channel level. In *Ascaris* muscle, TBPS and the pyrethroid, dieldrin, were found without effect on GABA response (Colquhoun et al., 1989). This suggests that the GABA-gated Cl channel in *Ascaris*, differs from the vertebrate GABA-A-gated Cl channel and from the GABA-gated Cl channel in insects. The Cl channel associated with the GABA receptor in *Ascaris* may be also a potential target for anthelminthic drugs. Already a chloride transport blocker in renal tubules has been shown to antagonize GABA responses in *Ascaris* probably by an action on the channel site (Holden-Dye et al., 1989). In *Ascaris*, the GABA receptor itself is also a potential target for anthelminthic drugs, providing these are antagonists. In fact, among the antiparasitic drugs acting on the GABA-receptor complex in parasites, piperazine is a specific GABA agonist and even if ivermectin antagonizes GABA, (Holden-Dye et al., 1988), its therapeutic mechanism

of action is not clearly known. In conclusion, there is no GABA antagonist among the actual anthelminthic drugs available. One possible reason for the absence of antagonist in the actual anthelminthic pharmacopoeia, is that they are less frequently studied than agonists. Antagonists are rare, they are usually large and complex molecules, and also they are more difficult to study. Therefore structure-activity studies have not frequently been carried out for antagonists. It must be said also, that most anthelminthics were discovered well before that their mechanism of action was understood. The main strategy in drug research is to modify the chemical structure of a known anthelminthic, with the consequence of developing analogues with the same mechanism of action. To this extent, benzimidazoles represent a very successful family of anthelminthics. In contrast, to piperazines where piperazine itself is the only GABA agonist, its analogue diethylcarbamazine has a different mechanism of action despite a structural resemblance. This is in agreement with the fact that GABA agonists are not likely to be successful as anthelminthics.

The story of the arylaminopyridazine derivatives is very unusual. Aminopyridazine derivatives are known to interact with a great variety of receptors in the mammalian nervous system. Some will be selective agonist at the serotonin, dopamine, acetylcholine, noradrenaline, or benzodiazepine, receptors~~. (Wermuth, Bourguignon, Chambon et al., 1989).~~ Pyridizanyl GABA derivatives were developed with the objective of designing GABA-mimetic drugs able to penetrate the central nervous system, but quite surprisingly these compounds turned out to be specific and competitive GABA-A antagonists (Chambon et al., 1985; Heaulme et al., 1986a, 1986b; Michaud et al., 1986; Gynther and Curtis, 1986; Mienville and Vincini, 1987; Desarmenien et al., 1987; Hamann et al., 1988). When arylaminopyridazine GABA derivatives are compared to other competitive GABA-A antagonists, they appear among the most potent and specific GABA-A antagonists. The classical bicuculline for example is not such a potent GABA antagonist (Enna and Snyder, 1977), and is not highly specific to the GABA-A receptor either, since it has been shown to interfere with glycine receptors (Goldinger and Muller, 1980; Michaud et al., 1986). Some GABA-A antagonists are potent but not specific: pitrazepine (Gähwiler, Maurer and Wutrech, 1984), and the steroid amidine derivative RU5135 (Hunt and Clements-Jewery, 1981), both interfere strongly with the glycine receptor (Hunt and Clements-Jewery, 1981; Olsen, 1984; Simmonds and Turner, 1985; Curtis and Malik, 1985; Kemp, Marshall, Wong et al., 1985), and to a less extent with the benzodiazepine binding site (Hunt and Clements-Jewery, 1981). One is highly specific to GABA-A receptors, but has a low affinity: securinine (Beutler, Karbon, Brubaker et al., 1985). Arylaminopyridazine GABA derivatives are therefore potent and specific GABA-A antagonists in mammalian

preparations. However, when administered in vivo, by IV or IP, these compounds have a relatively low potency in eliciting clonico-tonic seizures suggesting that they do not readily cross the brain blood barrier (Chambon et al., 1985). For this reason, arylaminopyridazine GABA derivatives have no therapeutic application in mammals yet.

Arylaminopyridazine derivatives of GABA appear as good candidates for a new family of anthelminthic drugs. One of them, SR95132, has been shown in this study to act specifically at the parasite GABA receptor. The structure-activity study of these compounds has also shown that the structural requirements in mammals seem incompatible with the structural requirements in *Ascaris*: gain in antagonistic activity at the parasite site is associated with a parallel loss of activity at the mammalian site.

This study has shown that there are differences between the *Ascaris* and vertebrate GABA receptors which might be usefully exploited in antiparasitic therapeutic. But one question arise: is there really a need for new anthelminthic drugs? Another alternative is vaccination (see Gutteridge, 1989). Parasites vaccines are potentially safer, cheaper and more efficacious as prophylactics than drugs. Parasite vaccines already exist in two cases in veterinary medicine:against piroplasmid protozoa and dictyocaulid nematodes, however they are far from being developed for human purpose. The need of antigenically defined vaccine, and the complex immunological responses of both the host and the parasites, have introduced a delay in the discovery of acceptable human vaccines. Nevertheless drugs will still be required for people or animals already infected, for parasitic diseases for which vaccines have not been developed and for people or animals that became infected because of failure or contre-indication of the vaccine (Gutteridge, 1989).

Although the actual pharmacopoeia is rich in good antiparasitic drugs, there is a need for new anthelminthics. Earlier, it was stated that the main strategy in drug research is to modify the chemical structure of a known anthelminthic, with the consequence of developing analogues with the same mechanism of action. One detrimental consequence of this strategy, is the development of drug-resistance by selection of the resistant strains of parasites. Concerning nematodes, benzimidazoles are the chief compounds in that matter. Until now, antiparisitic drug-resistance was not considered seriously, mainly because it occurred only locally and remained confined to Australia and New-Zealand, where climatic conditions are favourable to the apparition of such resistances (Waller, 1985). However, benzimidazoles resistance has now spread in Europe, mainly in sheep and horses (Waller, 1985). No doubt that the compensatory use of other available anthelminthics such as ivermectin, will produce a

selection pressure and select resistant strains. Naturally occuring ivermectin-resistant strains of *Haemonchus contortus* have already been reported in Brazil (Echevarria and Trindade, 1989). There is therefore a constant need for drugs especially with new mechanisms of action.

The GABA receptor in *Ascaris*, with special reference to its antagonist properties, appears to be a potential target for anthelminthic drugs. To this respect, the exploration arylaminopyridazine GABA derivatives family may lead to the discovery of a new generation of anthelminthics.

REFERENCES

Abalis,I.M.; Eldefrawi,M.E.; Eldefrawi,A.T.; 1983; Biochemical identification of putative γ-aminobutyric acid /benzodiazepine receptors in house fly thorax muscle.; Pesticide Biochemistry and Physiology; 20; 39-48.

Adams,P.R.; Brown,D.A.; 1975; Actions of γ-aminobutyric acid on sympathetic ganglion cells.; Journal of Physiology (London); 250; 85-120.

Alger,B.E.; Nicoll,R.A.; 1982; Pharmacological evidence for two kinds of GABA receptors on rat hippocampal pyramidal cells studied in vitro.; Journal of Physiology (London); 328; 125-141.

Allan,R.D.; Dickenson,H.W.; Hiern,B.P.; Johnston,G.A.R.; Kazlauskas,I.; 1986a; Isothiouronium compounds as GABA agonists.; British Journal of Pharmacology; 88; 379-387.

Allan,R.D.; Dickenson,H.W.; Fong,J.; 1986b; Structure-activity studies on the activity of a series of cyclopentane GABA-analogues on GABA-A receptors and uptake.; European Journal of Pharmacology; 122; 339-348.

Apáthy,S; 1893; Ueber die muskelfasern von *Ascaris*, nebst Bemerkungen über die von *Lombricus* und *Hirudo*.; Zeitschrift für Wissenschaftliche Mikroskopie und Mikroscopie Technik; 10; - .

Apáthy,S.; 1894; Das leitende element in den muskelfasern von *Ascaris*.; Archiv für Mikroanatomie und Anatomie; 43; 886-911.

Ariëns,E.J.; 1954; Affinity and intrinsic activity in the theory of competitive inhibition. Part I: problems and theory.; Archives Internationales de Pharmacodynamie; 99; 32-49.

Ariëns,E.J.; Beld,A.D.; Miranda,J.F.R.; Simonis,A.M.; 1979; The pharmacon-receptor-effector concept.; In: The Receptors Vol 1; Plenum press, New York, pp33-91.

Arunlakshana,O.; Schild,O.; 1959; Some quantitative uses of drug antagonists.; British Journal of Pharmacology and Chemotherapy; 14; 48-558.

Ash,A.S.F.; Tucker,J.F.; 1966; Inhibition of *Ascaris* muscle by GABA, a possible new assay method.; Nature; 209; 306.

Ault,B.; Nadler,V.; 1983; Differenciation of two bicuculline-insensitive actions of GABA in the rat hippocampal slice.; Journal of Physiology (London); 345; 70P.

Baldwin,E.; Moyle,V.; 1947; An isolated nerve muscle preparation from *Ascaris suum*.; Journal of Experimental Biology; 23; 277-291.

Baldwin,E; Moyle,V.; 1949; A contribution to the physiology and pharmacology of *Ascaris suum*.; British Journal of Pharmacology; 4; 145-152.

Barker,J.L.; Nicoll,R.A.; 1973; The pharmacology and ionic dependancy of amino-acid responses in the frog spinal cord.; Journal of Physiology (London); 228; 259-277.

Barker,J.L.; Mac Burney,R.N.; 1979; GABA and glycine may share the same conductance on cultured mammalian neurones.; Nature; 277; 234-236.

Barker,J.L.; Harrison,N.L.; Lange,G.D.; Majewska,M.D.; Owen,D.G.; 1986; Voltage-clamp study of the potentiation of GABA activated chloride conductance by the steroid anaesthetic alphaxalone and a reduced metabolite of progesterone in cultured CNS neurons.; Journal of Physiology (London); 377; 83p.

Barnard,E.A.; Darlison,M.G.; Seeburg,P.; 1987; Molecular biology of the GABA-A receptor: the receptor channel superfamily.; TINS; 10; 502-509.

Barolet,A.W.; Kish,S.J.; Morris,M.E.; 1985; Identification of the extrasynaptic binding sites for [^{3}H]GABA in peripheral nerves.; Brain Research; 358; 104-109.

Bazemore,A.W.; Elliott,K.A.C.; Florey,E.; 1957; Isolation of factor I.; Journal of Neurochemistry; 1; 334-339.

Beer,B.; Klepner,C.A.; Lippa,A.S.; Squires,R.F.; 1978; Enhancement of [^{3}H]diazepam binding by SQ65-396 a novel antianxiety agent.; Pharmacology Biochemistry and Behaviour; 9; 849-851.

Benson,J.A.; 1988; Bicuculline blocks the response to acetylcholine and nicotine but not muscarine or GABA in isolated insect neuronal somata.; Brain Research; 458; 65-71.

Benson,J.A.; 1989; A novel GABA receptor in the heart of a primitive arthropod *Limulus polyphemus*.; Journal of Experimental Biology; 147; 421-438.

Beutler,J.A., Karbon,E.W., Brubaker,A.N., Malik,R., Curtis,D.R. & Enna,S.J., 1985, Securinine alkaloids: a new class of GABA receptor antagonist.; Brain Research, 330, 135-140.

Bilek,E.; 1909; Uber die fibrillären strukturen in den muskel und darmzellem der ascariden.; Zeitschrift für Wissenschafliche Zoologie; 93; 625-637.

Bilek,E.; 1910; Die muskelzellen der grossen *Ascaris* arten.; Anatomischer Azeiger; 37; 67-78.

Black,J.W.; 1987; Should we be concerned about the state of hormone receptor classification?; In: Perspectives on Receptor Classification; Alan.R.Liss, New York, pp11-15.

Blaxter,T.J.; Cottrell,G.A.; 1987; Actions of GABA and ethylenediamine on CA1 pyramidal neurones of the rat hippocampus.; Quaternaly Journal of Experimental Physiology; 70; 75-93.

Bormann,J.; Sakman,B.; Seifert,W.; 1983; Isolation of GABA activated single channel currents in the soma membrane of rat hippocampal neurones.; Journal of Physiology (London); 341;9P.

Bormann,J.; Clapham,D.E.; 1985; γ-aminobutyric acid receptor channels in adrenal chromaffin cells: a patch-clamp study.; Proceedings of the National Academy of Sciences USA; 82;2168-2172.

Bowery,N.G.; Brown,D.A.; 1974; Depolarizing actions of GABA and related compounds on rat cervical superior ganglion in vitro.; British Journal of Pharmacology; 50; 205-218.

Bowery,N.G.; Hudson,A.L.; 1979; GABA reduces the evoked release of [^{3}H]noradrenaline from sympathetic nerve terminals.; British Journal of Pharmacology; 66; 108p.

Bowery,N.G.; Doble,A.; Hill,D.R.; Hudson,A.L.; Turnbull,M.J.; 1980a; GABA facilitates or inhibits the evoked release of [^{3}H]noradrenaline from rat cerebellar cortex slices by an action at separate receptors.; British Journal of Pharmacology; 70; 77p.

Bowery,N.G.; Hill,D.R.; Hudson,A.L.; Doble,A.; Middlemiss,D.N.; Shaw,J.; Turnbull,M.; 1980b; (-)baclofen decreases neurotransmitter release in the mammalian central nervous sytem by an action at a novel GABA receptor.; Nature; 283; 92-94.

Bowery,N.G.; Hill,D.R.; Hudson,A.L.; 1983; Characteristics of GABA-B receptor binding sites on rat whole brain synaptic membranes.; British Journal of Pharmacology; 78; 191-206.

Bowery,N.G.; Price,G.W.; Hudson,A.L.; Hill,D.R.; Wilkin,G.P.; Turnbull,M.J.; 1984; GABA receptor multiplicity.; Neuropharmacology; 23; 219-231.

Bowery, N.G.; 1989; GABA-B receptors and their significance in mammalian pharmacology.; TIPS; 10; 401-407.

Bowman,W.C.; Rand,J.; 1980; Texbook of pharmacology.; Blackwell sientific publications, Oxford, pp37-3 -37-4.

Brading,A.F.; Caldwell,P.C.; 1964; The effects of ions on the resting potentials of muscle cells in *Ascaris lumbricoides*.; Journal of Physiology (London); 173; 36P.

Brading,A.F; Caldwell,P.C.; 1971; The resting membrane potential of the somatic muscle cells of *Ascaris lumbricoides*.; Journal of Physiology (London); 217; 605-624.

Braestrup,C.; Nielsen,M.;1981; GABA reduces binding of [^{3}H]methyl-β-carboline-3-carboxylate to brain benzodiazepine receptors.; Nature; 294; 472-474.

Braestrup,C.; Nielsen,M.; 1986; Benzodiazepine receptor binding in vivo and efficacy. In: Benzodiazepine/GABA Receptors and Chloride Channels; Structural and functionnal properties.; Alan R Liss New York, p167-184.

Brennan,M.J.W.; Cantrill,R.C.; 1979; δ-aminolaevulinic acid is a potent agonist for GABA autreceptors.; Nature; 280; 514-515.

Brown,D.A.; Marsh,S.; 1978; Axonal GABA receptors in mammalian peripheral nerve trunks.; Brain Research; 156; 187-191.

Bütschli,O.; 1874; Beiträge zur kenntniss des nervensystems der nematoden.; Archiv für Mikroanatomie und Anatomie; 10; 74-100.

Bütschli,O.; 1892; Ueber den feineren bau der contractilen substanz der muskelzellen von *Ascaris* nebst bemerkungen über die muskelzellen einiger andere Würmer.; Festschrift R.Leuckart, Leipzig, pp328-336.

Caldwell,P.C.; Ellory,J.C.; 1968; Ion mouvements in the somatic muscle cells of *Ascaris lumbricoides*.; Journal of Physiology (London); 197; 75-76P.

Callahan,H.; Cottrell,G.A.; Hather,N.Y.; Lambert,J.J.; Nooney,J.M.; Peters,J.A.; 1986; Modulation of the GABA-A receptor of bovine chromaffin cells in culture by some progesterone metabolites.; Journal of Physiology (London); 381; 117p.

Cappe de Baillon,P.; 1911; Etude sur les fibres musculaires d'*Ascaris*.; La Cellule; 27 (XXVII); 165-211.

Chambon,J.P.; Feltz,P.; Heaulme,M.; Restle,S.; Schliechter,R.; Biziere,K.; Wermuth,C.G.; 1985; Synthesis of SR95103, an arylaminopyridazine derivative of GABA.; Proceeding of the National Academy of Sciences USA; 82; 1832-1836.

Changeux,J.P.; Podleski,T.R.; 1968; On the excitability and cooperativity of the electroplax membrane.; Proceedings of the National Academy of Sciences; 59; 944-950.

Chaudhuri,J.; Martin,R.E.; Donahue,M.J.; 1988; Evidence for the absorption and synthesis of 5-hydroxytryptamine in perfused muscle and intestinal tissue and whole worms of adult *Ascaris suum.*; Parasitology; 96; 157-160.

Chaudhuri,J.; Donahue,M.J.; 1989; Serotonin receptors in the tissues of adult *Ascaris suum.*; Molecular and biochemical parasitology; 35; 191-198.

Clark,A.J.; 1926; The relation between acetylcholine and muscle cells.; Journal of Physiology London; 61; 530-546.

Cloquet,A.; 1824; Anatomie des vers intestinaux *Ascaride lumbricoides* et *Echinorhynque géant.*; Paris.

Colquhoun,D.; 1971; Non-linear curve fitting and the meaning of the best estimate. In: Lectures on biostatistics.; Clarendon Press; Oxford, pp257-272.

Colquhoun,D.; 1973; The relation between classical and cooperative models for drug action.; In: Drug Receptors, H.P.Rang, Macmillan Press, Oxford, pp149-182.

Colquhoun,D.; Hawkes,A.G.; 1977; Relaxation and fluctuations of membrane currents that flow through drug operated channels.; Proceeding of the Royal Society series B; 199; 231-262.

Colquhoun,D.; Hawkes,A.G.; 1982; On the stochastic properties of bursts of single ion channel openings.; Proceeding of the Royal Society series B; 211; 205-235.

Colquhoun,D.; Sakmann,B.; 1985; Fast events in single channel currents activated by acetylcholine and its analogues at the frog muscle end-plate.; Journal of Physiology (London); 369; 501-577.

Colquhoun,L.; Holden-Dye,L.; Walker,R.J.; 1989; 5-nitro-2-(3-phenylpropylamino) benzoic acid (5-NPB), is a non-competitive antagonist at the *Ascaris* GABA receptor.; British journal of pharmacology; 97; 369P.

Colquhoun,L; Holden-Dye,L.; Walker,R.J.; 1990; The pharmacology of cholinoceptors on the somatic muscle cells of the parasitic nematode *Ascaris suum.*; British Journal of Pharmacology; 99; 253P.

Constanti,A.; 1978; The mixed effect of picrotoxin on the GABA dose/conductance relation recorded from lobster muscle.; Neuropharmacology; 17; 159-167.

Cottrell,G.A.; Lambert,J.J.; Peters,J.A.; 1987; Modulation of GABA-A receptor activity by alphaxalone.; British Journal of Pharmacology; 90; 491-500.

Cowden,C.; Stretton,A.O.W.; Davis,R.E.; 1989; AF1 a sequenced bioactive neuropeptide isolated from the nematode *Ascaris suum.*; Neuron.; 2; 1465-1473.

Crompton,D.W.T.; Nesheim,N.C.; Pawlowsky,Z.S.;1989 ; Ascariasis and its control.; Taylor and Francis; London.

Curtis,D.R.; Duggan,A.W.; Felix,D.; Johnston,G.A.R.; 1970; Bicuculline and central inhibition.; Nature; 226; 1222-1224.

Curtis,D.R; Johnston,G.A.R.; 1974; Amino-acid transmitters in mammalian central nervous system.; Ergebnisse der Physiologie; 69; 97-188.

Curtis,D.R; Malik,R.; 1985; Glycine antagonism by RU5135.; European journal of pharmacology; 110; 383-384.

Curtis,D.R.; Watkins,J.C.; 1965; The pharmacology of amino-acids related to γ-aminobutyric acid.; Pharmacological Reviews; 17; 347-391.

Davenport,T.R.B.;Lee,D.L.;Isaac,R.E.; 1988; Immunocytochemical demonstration of a neuropeptide in *Ascaris suum* (nematode), using an antiserum to FMFRamide.; Parasitology; 97; 1-8.

Davis,R.E.; Stretton,A.O.W.; 1989a; Passive membrane properties of motorneurons and their role in long distance signaling in the nematode *Ascaris.*; Journal of Neuroscience; 9; 403-414.

Davis,R.E.; Stretton,A.O.W.; 1989b; Signaling propertied of *Ascaris* motorneurons: graded active responses and tonic transmitter release.; Journal of Neuroscience; 9; 415-425.

Dean,P.M.; 1987; The development of theories about drug-receptor interaction.; In: Molecular Fundations of Drug-Receptor Interaction; Cambridge; pp1-34.

DeBell,J.; Del Castillo,J.; Sanchez,V.; 1963; Electrophysiology of the somatic muscle cells of *Ascaris lumbricoides*.; Journal of Cellular and Comparative Physiology; 62; 159-177.

DeBell,J.; 1965; A long look at neuromuscular junctions in nematodes.; The Quaterly Review of Biology; 40; 233-251.

Deineka,D.; 1908; Das nervensystem von *Ascaris*.; Zeitschrift für wissenschaftliche Zoologie; 89; 242-307.

Del Castillo,J.; De Mello,J.; Morales,T.; 1963a; The physiologic role of acetylcholine in the neuromuscular system of *Ascaris lumbricoides*.; Archives Internationales de Physiologie; 71; 741-757.

Del Castillo,J.; Morales,T.; Sanchez,V.; 1963b; Action of piperazine on the neuromuscular system of *Ascaris*.; Nature; 200; 706.

Del Castillo,J.; De Mello,J.; Morales,T.; 1964a; Influence of some ions on the membrane potential of *Ascaris* muscle.; Journal of General Physiology; 48; 129-140.

Del Castillo,J.; De Mello,J.; Morales,T.; 1964b; Inhibitory action of γ-aminobutyric acid on *Ascaris* muscle.; Experientia; 20; 141-143.

Del Castillo,J.; De Mello,W.C.; Morales,T.; 1964c; Mechanism of the paralysing action of piperazine on *Ascaris* muscle.; British Journal of Pharmacology; 22; 463-477.

Del Castillo,J.; De Mello,W.C.; Morales,T.; 1967; Initiation of action potentials in the somatic musculature of *Ascaris lumbricoides*.; Journal of Experimental Biology; 46; 263-279.

Del Castillo,J; Riviera,J.; Solórzano,A.; Serrato,J.; 1989; Some aspects of the neuromuscular system of *Ascaris*.; Quaterly Journal of Experimental Physiology; 74; 1071-1088.

Desarmenien,M.; Feltz,P.; Loeffler,J.P.; Occhipinti,G.; Santangelo,F.; 1982; Multiple GABA receptors on Aδ and C primary neurones in the adult rat.; British Journal of Pharmacology; 76; 289p.

Desarmenien,M.; Desaulles,E.; Feltz,P.; Hamann,M.; 1987; Electrophysiological study of SR42641 a novel aminopyridazine derivative of GABA, antagonist properties and receptor selectivity of GABA-A versus GABA-B responses.; British Journal of Pharmacology; 90; 287-298.

Dickenson,H.W.; Allan,R.D.; Ong,J.; Johnston,G.A.R.; 1988; GABA-B receptor antagonists and GABA-A receptor agonists: properties of a δ-aminovaleric acid derivative Z-5-aminopent-2-enoic acid.; Neuroscience Letters; 86; 351-355.

Donahue,M.J.; Michnoff,C.A.; Masaracchia,R.A.; 1985; Calcium dependant muscle contraction in obliquely striated *Ascaris suum* muscle.; Comparative Biochemistry and Physiology; 82C; 395-403.

Donahue,M.J.; Yacoub,N.J.; Harris,B.G.; 1982; Correlation of muscle activity with glycogen metabolism in muscle of *Ascaris suum*.; American Journal of Physiology; 242; R514-521.

Donahue,M.J.; Yacoub,N.J.; Michnoff,C.A.; Masaracchia,R.A.; Harris B.G. ; 1981a; Serotonin a possible regulator of glycogenolysis in perfused segments of *Ascaris*.; Biochemical and Biophysical Research Communications; 105; 112-117

Donahue,M.J.; Yacoub,N.J.; Kaeine,M.R.; Harris,B.G.; 1981b; Activity of enzymes regulating glycogen in perfused muscle cuticule sections of *Ascaris suum*.; Journal of Parasitology; 67; 362-367.

Donahue,M.J.; Yacoub,N.J.; Kaeini,M.R.; Masaracchia,R.A.; Harris,B.G.; 1981c; Glycogen metabolism enzymes during starvation and feeding of *Ascaris suum*.; Journal of Parasitology; 67; 505-510.

Dunlap,K.; 1981; Two types of GABA receptors on embryonic sensory neurones.; British Journal of Pharmacology; 74; 579-585.

Dutar,P.; Nicoll,R.A.; 1988; A physiological role for GABA-B receptors in the central nervous system.; Nature; 332; 156-158.

Earl,J.; Large,W.A.; 1974; Electrophysiological investigation of GABA mediated inhibition at the hermit crab neuromuscular junction.; Journal of Physiology (London); 236; 113-127.

Ecchevaria,F.A.; Trindade,G.N.; 1989; Anthelminthic resistance by *Haemonchus contortus* to ivermectin in Brazil: a preliminary report.; Veterinary Record; 124; 147-148.

Ehlert,F.J.; Roeske,W.R.; Braestrup,C.; Yamamura,S.H.; Yamamura,H.I.; 1981; γ-aminobutyric acid regulation of the benzodiazepine receptor, biochemical evidence of pharmacologically different effects of benzodiazepine and propyl-β-carboline-3-carboxylate.; European Journal of Pharmacology; 70; 593-596.

Ehrlich,P.; Morgenroth,J.; 1900; On haemolysis.; In: The Collected Paper of P.Ehrlich, ed. F.Himmelweit, Vol 1, London Pergamon Press (published in translation 1956), pp205-215.

Eldefrawi,M.E.; Eldefrawi,A.T.; 1987; Receptors for γ-aminobutyric acid and voltage dependant chloride channels as targets for drugs and toxicants.; FASEB Journal; 1; 262-271.

Enna,S.J.; Snyder,S.H.; 1977; Influence of ions, enzymes and detergents on γ-aminobutyric acid binding in synaptic membranes of rat brain.; Molecular Pharmacology; 13; 442-453.

Falch,E.; Krogsgaard-Larsen,P.; 1982; The binding of the GABA-A agonist [^{3}H]THIP to rat brain synaptic membranes.; Journal of Neurochemistry; 38; 125-141.

Fayard,G.; 1949; Ascaridiose et piperazine.; Thèse de Doctorat; Paris.

Feltz,P.; Raminsky,P; 1974; A model for the mode of action of GABA on primary afferent terminals: depolarizing affects of GABA applied iontopheritically to neurones of mammalian dorsal root ganglion.; Neurophysiology; 13; 553-563.

Florey,E.; 1954; An inhibitory and an excitatory factor of mammalian nervous system and their action on a single sensory neuron.; Archives Internationales de Physiologie; 62; 33-53.

Gaddum,J.H.; 1936; The quantitative effects of antagonistic drugs.; Journal of Physiology (London); 89; 7P-9P.

Gähwiller,B.H.; Maurer,R.; Wutrech,H.J.; 1985; Pitrazepin a novel antagonist (GABA-A).; Neuroscience Letters; 45; 311-316.

Gee,K.W.; 1988; Steroid modulation of the GABA/benzodiazepine receptor-linked chloride ionophore.; Molecular Neurobiology; 2; 291-317.

Glane,H.; 1910; Beiträge zu einer monographie der nematoden species *Ascaris felis* und *Ascaris canis*.; Zeitschrift für Wissenschaftliche Zoologie; 95; 551-593.

Glasbey,C.; Martin,R.J.; 1986; Exploratory and confirmatory plots of single channel records.; Journal of Neuroscience Methods; 16; 239-249.

Goldinger,A.; Müller,W.E.; 1980; Stereospecific interaction of bicuculline with specific [^{3}H]strychnine binding to rat spinal cord synaptosomal membranes.; Neuroscience Letters; 16; 91-95.

Goldschmidt,D.T.R.; 1908; Das nervensystem von *Ascaris lumbricoides* und *megalocephala*. I; Zeitschrift für Wissenschaftliche Zoologie; 90; 73-196.

Goldschmidt,D.T.R; 1909; Das nervensystem von *Ascaris lumbricoides* und *megalocephala*. II.; Zeitschrift für Wissenschaftliche Zoologie; 92; 306-357.

Goldschmidt,D.T.R.; 1910; Das nervensystem von *Ascaris lumbricoides* und *megalocephala*. Ein versuch in den aufbau eines einfachen nervensystems einzudrigen III.; Fetschrift R.Hertwig, Jeva; 2; 253-354.

Goodman,A.; Gilman,L.S.; 1985; The pharmacological basis of therapeutics.; MacMillan, New York, pp1017-10118

Grundfest,H.; Reuben,J.P.; Rickles,W.H.; 1959; The electrophysiology and pharmacology of the lobster neuromuscular synapse.; Journal of General Physiology; 42; 1301-1323.

Guidotti,A.; Toffano,G.; Costa,E.; 1978; An endogenous protein modulates the affinity of GABA and benzodiazepine receptors in rat brain.; Nature; 275; 553-555.

Guidotti,A.; Gale,K.; Suria,.A; Toffano,G.; 1979; Biochemical evidence for two classes of GABA receptors in rat brain.; Brain Research; 172; 566-571.

Guidotti,A.; Konkel,D.R.; Ebstein,B; Gorda, M.G.; Wise, B.C.; Krutzch, H.; Meek, J.L.; Costa, E.; 1982; Isolation, characterisation and purification to homogeneity of a rat brain protein: GABA modulin.; Proceedings of the National Academy of Sciences USA; 79; 6084-6088.

Gutteridge,W.E.; 1989; Parasite vaccines versus anti-parasite drugs: rivals or running mates?; Parasitology; 98; S87-S97.

Gynther,B.D.; Curtis,D.R.; 1986; Pyridazinyl-GABA derivatives as GABA and glycine antagonists in the spinal cord of the cat.; Neuroscience Letters; 68; 211-215.

Hamann,M.; Desarmenien,M.; Desaulles,E.; Bader,M.F.; Feltz,P.; 1987; Quantitative evaluation of the properties of a pyridazinyl GABA derivative (SR95531), as a GABA-A competitive antagonist. An electrophysiological approach.; Brain Research; 442; 287-296.

Hamill,O.P.; Marty,A.; Neher,E.; Sakmann,B.; Sigworth,F.J.; 1981; Improved patch-clamp techniques for high resolution current recording from cells and cell-free membranes patches.; Pflugers Archiv für Gesante Physiologie des Menshen (European Journal of Physiology); 391; 85-100.

Harris,J.E.; Crofton,H.D.; 1957; Structure and function in the nematodes: internal pressure and cuticular structure in *Ascaris*.; Journal of Experimental Biology; 34; 116-130.

Harris,R.A.; Allan,A.M.; 1985; Science; Functional coupling of γ-aminobutyric acid receptors to chloride channels in brain membranes.; 228; 1108-1110.

Harrison,N.L.; Simmonds,M.A.; 1984; Modulation of the GABA receptor complex by a steroid anaesthetic.; Brain Research; 323; 287-292.

Harrow,I.D.; Gration,K.A.F.; 1985; Mode of action of the anthelmintics morantel, pyrantel and levamisole on muscle cell membrane at the nematode *Ascaris suum*.; Pesticide Science; 16; 662-672.

Heaulme,M.; Chambon,J.P.; Leyris,R.; Molimard,J.C.; Wermuth,C.G.; Biziere,K.; 1986a; Characterization of three arylaminopyridazine GABA derivatives with the GABA-A receptor site.; Brain Research; 384; 224-231.

Heaulme,M.; Chambon,J.P.; Leyris,R.; Wermuth,C.G.; Biziere,K.; 1986b; Specific binding of a phenylpyridazinium derivative endowed with GABA-A receptor antagonist activity to rat brain.; Neuropharmacology; 25; 1272-1283.

Hesse ,R.; 1892; Uber das nervensystem von *Ascaris megalocephala*.; Zeitschrift für Wissenschaftliche Zoologie; 92; 306-357.

Hewitt,G.M.; 1987; Electrophysiological studies on the *Ascaris* muscle γ-aminobutyric acid (GABA) receptor. PhD thesis. University of Southampton.

Hill,A.V.; 1909; The mode of action of nicotine and curari, determined by the form of contraction curve and the method of temperature coefficients.; Journal of Physiology (London); 39; 361-373.

Hill,A.V.; 1913; The combination of haemoglobin with oxygen and carbon monoxyde.; Biochemical Journal; 7; 471-480.

Hill,D.R.; Bowery,N.G.; 1981; [^{3}H]baclofen and [^{3}H]GABA bind to bicuculline insensitive GABA-B sites in rat brain.; Nature; 290; 149-152.

Hille,B.; 1984a; Selective permeability: independence.; In: Ionic Channels of Excitable Membranes.; Sinauer, Sunderland, Massachusetts; pp226-248.

Hille,B; 1984b; Evolution and diversity.; In: Ionic Channels of Excitable Membranes.; Sinauer, Sunderland, Massachusetts; pp371-383

Hitzemann,R.J.; Loh,H.H.; 1978; Effects of some conformationally restricted GABA analogues on GABA membrane binding and nerve ending transport.; Brain Research; 144; 63-73.

Hobson,A.D.; Stephenson,W.; Beadle,L.C.; 1952; Studies on the physiology of *Ascaris lumbricoides*.; Journal of Experimental Biology; 29; 22-29.

Holden-Dye,L.; Hewitt,G.M.; Wann,K.T.; Krogsgaard-Larsen,P.;Walker,R.J.; 1988; Studies involving Avermectin and the 4-aminobutyric acid (GABA) receptor in *Ascaris suum* muscle.; Pesticide Science; 24; 231-245

Holden-Dye,L.; Walker.R.J.; Nielsen,L.; Krogsgaard-Larsen,P.; 1989; Stereoselectivity of a bicuculline insensitive GABA receptor in *Ascaris suum* muscle.; British Journal of Pharmacology; 98; 841-849.

Hori,N.; Ikeda,K.; Roberts,E.; 1978; Muscimol, GABA and picrotoxin: effects on membrane conductance of a crustacean neuron.; Brain Research; 141; 364-370.

Hunt,P.; Clements-Jewery,S.; 1981; A steroid derivative R5135 antagonizes the GABA/benzodiazepine receptor interaction.; Neuropharmacology; 20; 357-361.

Iversen,L.L.; Kelly,J.S.; 1975; Uptake and metabolism of γ-aminobutyric acid by neurones and glial cells.; Biochemical Pharmacology; 24; 933-938.

Jackson,M.B.; Lecar,H.; Mathers,D.A.; Barker,J.L.; 1982; Single-channel currents activated by γ-aminobutyric acid, muscimol and (-)pentobarbital in cultured mouse spinal neurons.; Journal of Neuroscience; 2; 889-894.

Jarman,M; 1959; Electrical activity in the muscle cell of *Ascaris lumbricoides*.; Nature; 184; 1244.

Jenkinson,D.H.; 1960; The antagonism between tubocurarine and substances which depolarize the motor endplate.; Journal of Physiology; 152; 309-324

Jenkinson,D.H.; 1987; Heinz Schild's contribution to receptor classification.; In: Perspectives on Recetor Classification; Alan.R.Liss, New York; pp1-10.

Johnson,C.D.; Stretton,A.O.W.; 1987; γ-aminobutyric acid immunoreactivity in inhibitory motorneurons of the nematode *Ascaris*.; Journal of Neuroscience; 7; 223-235.

Johnston,G.A.R.; Willow,M.; 1982; GABA and barbiturate receptors.; TIPS; 3; 328-330.

Johnston, G.A.R.; Allan, D.R.; 1984; GABA agonists.; Neuropharmacology; 23; 831-832.

Johnston,G.A.R.; 1986; GABA receptor classification.; In: Benzodiazepine/GABA receptors and chloride channels: structural and functionnal properties, Alan.R.Liss, New York, pp41-56.

Karlin,A.; 1967; On the application of a "plausible model" of allosteric proteins to the receptor for acetylcholine.; Journal of Theoretical Biology; 16; 306-320.

Karobath,M.; Placketa,P.; Lippitsh,M.; 1979; Is stimulation of benzodiazepine binding mediated by a novel receptor ?; Nature; 278; 748-749.

Karobath,M.; Sperk,G.; 1979; Stimulation of benzodiazepine binding by γ-aminobutyric acid.; Proceeding of the National Academy of Sciences USA; 76; 1004-1006.

Kass,I.S.; Wang,C.C.; Walrond,J.P.; Stretton,A.O.W.; 1980; Avermectin B1a, a paralysing anthelminthic that affects interneurons and inhibitory motorneurons in Ascaris.; Proceedings of the National Academy of Sciences; 77; 6211-6215.

Kass,I.S.; Larsen,D.A.; Wang,C.C.; Stretton,A.O.W.; 1982; *Ascaris suum* differential effects of Avermectin B1a on the intact animal and neuromuscular strip preparations.; Experimental Parasitology; 54; 166-174.

Kataoka,Y.; Gutman,Y.; Guidotti,A.; Panula,P.; Wroblewsky,J.; Cosenza-Murphy,D.; Wu,J.Y.; Costa,E.; 1984; Intrinsic GABAergic system of adrenal chromaffin cells.; Proceedings of the National Academy of Sciences USA; 81; 3218-3222.

Katz,B.; 1969; Junctional transmission without impulses.; in: The release of neural transmitter, Sherington Lectures X, Liverpool Press, pp 30-35.

Kemp,J.A.; Marshall,G.R.; Wong,E.H.F.; Woodruff,G.N.; 1985; Pharmacological studies on pitrazepin, a GABA-A receptor antagonist.; Neuroscience Letters; 45; 311-316.

Kirkness,E.F.; Turner,A.J.; 1988; The stimulatory effects of secobarbital and pregnenolone on the GABA receptor can be blocked selectively.; European Journal of Pharmacology; 150; 385-388.

Kliks,M.M.; 1990; Helminths as heirlooms and souvenirs: a review of new world paleoparasitology.; Parasitology Today; 6; 93-100.

Krnjevíc,K.; 1974; Chemical nature of synaptic transmission in vertebrates.; Physiological Review; 54; 418-450.

Krogsgaard-Larsen,P.; Johnston,G.A.R.; 1975; Inhibition of GABA uptake in rat brain slices by nipecotic acid and various isoxazoles and related compounds.; Journal of Neurochemistry; 25; 797-802.

Krogsgaard-Larsen,P.; Johnston,G.A.R.; 1978; Structure-activity studies on the inhibition of GABA binding to rat brain membranes by muscimol and related compounds.;Journal of Neurochemistry; 30; 1377-1382.

Krogsgaard-Larsen,P.; Nielsen,L.; Falch,E.; 1986; The active site of the GABA receptor.; in: Benzodiazepine/GABA receptors and chloride channels: structural and functionnal properties, Alan.R.Liss, New York, pp73-95.

Krogsgaard-Larsen,P.; 1988; GABA synaptic mechanisms: stereochemical and conformational requirements.; Medicinal Research Review; 8; 27-56.

Lancet editorial; 6th May 1989; Ascariasis; 997-998.

Langley,J.N.; 1873; On the physiological action of jaborandi.; Proceedings of the Cambridge Philosophical Society; 402.

Langley,J.N.; 1878; On the physiology of salivary secretion II: on the mutual antagonism of atropin and pilocarpin having special reference to their relations in the salivary gland of the cat.; Journal of Physiology (London); 1; 339-369.

Langley,J.N.; 1905; On the reactions of cells and nerve endings to certain poisons chiefly as regards to the reaction of striated muscle to nicotinic and curari.; Journal of Physiology (London); 33; 374-413.

Leeb-Lundberg,F.; Snowman,A.; Olsen,R.W.; 1980; Barbiturate receptors are coupled with benzodiazepine receptors.; Proceedings of the National Academy of Sciences USA; 77; 7468-7472.

Lees,G.; Beadle,D.J.; Neumann,R.; Benson,J.A.; 1987; Responses to GABA by isolated insect neuronal somata: pharmacology and modulation by a benzodiazepine and barbiturate.; Brain Research; 401; 267-278.

Levitan,E.S.; Schofield,P.R.; Burt,D.R.; Rhee,L.M.; Wisden,W.; Kohler,M.; Fujita,N.; Rodriguez,H.F.; Stephenson,A; Darlison,M.G.; Barnard,E.A.; Seeburg,P.H.; 1989; Structural and functional basis for GABA-A receptor heterogeneity.; Nature; 335; 76-79.

Lummis,S.C.; Satelle,D.B.; 1985; Insect central nervous system γ-aminobutyric acid.; Neuroscience Letters; 60; 13-18.

MacDonald,R.; Barker,J.L.; 1978; Benzodiazepines specifically modulates GABA-mediated post-synaptic inhibition in cultured mammalian neurones.; Nature; 271; 563-564.

MacDonald,R.L; Rogers,C.J.; Twyman,R.E.; 1989; Kinetic properties of the GABA-A receptor main conductance state of mouse spinal cord neurones in culture.; Journal of Physiology (London) ; 410;479-499.

Majewska,M.D.; Bisserbe,J.C.; Eskay,R.L.; 1985; Glucocorticoids are modulators of GABA-A receptors in brain.; Brain Research; 339; 178-182.

Majewska,M.D.; Harrison,N.L.; Schwartz,R.D.; Barker,J.L.; Paul,S.M.; 1986; Steroid hormone metabolites are barbiturate-like modulators of the GABA receptors.; Science; 232; 1004-1007.

Martin,R.E.; Donahue,M.J.; 1987; Correlation of myosin light chain phosphorylation and γ-aminobutyric acid receptors in *Ascaris suum* muscle.; Comparative Biochemistry and Physiology; 87C; 23-29.

Martin,R.J; 1980; The effects of γ-aminobutyric acid (GABA) on the input conductance and membrane potential of *Ascaris suum*.; British Journal of Pharmacology; 71; 99-106.

Martin,R.J.; 1982; Electrophysiological effects of piperazine and diethyl carbamazine on *Ascaris suum* somatic muscle.; British Journal of Pharmacology; 77; 255-265.

Martin,R.J.; 1985; γ-aminobutyric acid and piperazine-activated single channel currents from *Ascaris* body muscle.; British Journal of Pharmacology; 84; 445-461.

Martin,R.J.; Pennington,A.J.; 1989; A patch-clamp study of effects of dihydroavermectin on *Ascaris* muscle.; British Journal of Pharmacology; 98; 747-756.

Mathers,D.A.; 1985; Spontaneous and γ-aminobutyric acid induced single-channel currents in cultured murine spinal cord neurons.; Canadian Journal of Physiology and Pharmacology; 63; 1228-1233.

Mathers,D.A.; 1987; The GABA-A receptor: new insights from single channel recordings.; Synapse; 1; 96-101.

Matsumoto,R.R.; 1989; GABA receptors: are cellular differences reflected in function?; Brain Research Review; 14; 203-225.

Meissner,G.; 1853; Beiträge zur anatomie und physiologie von Mermis Albicans.; Zeitschrift für Wissenschaftliche Zoologie; 5; 207-284.

Michaud,J.C.; Mienville,J.M.; Chambon,J.P.; 1986; Interaction of three arylaminopyridazine GABA derivatives with rat cortical GABA and glycine receptors: an in vivo microiontophoretic study.; Neuropharmacology; 25; 1197-1203.

Mienville,J.M.;Vincini,S.; 1987; Arylaminopyridazyl derivative of GABA; SR95531 is a potent antagonist of Cl channel openings regulated by GABA-A receptors.; Neuropharmacology; 26; 7A.

Mitchell,P.R.; Martin,I.L.; 1978; Is GABA release modulated by presynaptic receptors?; Nature; 274; 904-905.

Monod,J.; Wyman,J.; Changeux,J.P.; 1965; On the nature of allosteric transitions: a plausible model.; Journal of Molecular Biology; 12; 88-118.

Morris,M.E.; Di Costanzo,G.A.; Fox,S.; Werman,R.; 1983; Depolarizing action of GABA on myelinated fibers of peripheral nerves.; Brain Research; 278; 117-125.

Morrow,A.L.; Suzdack,P.D.; Paul,S.M.; 1987; Steroid hormone metabolites potentiate GABA receptor mediated chloride ion flux with nanomolar potency.; European Journal of Pharmacology; 142; 483-485.

Muhyaddin,M; Roberts,P.J.; Woodruff,G.N.; 1982; GABA reduces the evoked neuronal release of [^{3}H]noradrenaline from rat anococcygeus muscle, antagonism by 5-aminovalerate.; British Journal of Pharmacology; 77; 409p.

Natoff,I.L.; 1969; The pharmacology of the cholinoreceptor in *Ascaris* muscle preparation.; British Journal of Pharmacology.; 37; 251-257.

Nielsen,M; Braestrup,C.; Squires,R.F.; 1978; Evolution of the benzodiazepine receptor.; Brain Research; 141; 342-346.

Nistri,A.; Constanti,A.; 1979; Pharmacological characterization of different types of GABA and glutamate receptors in vertebrates and invertebrates.; Progress in Neurobiology; 13; 117-225.

Norton,S.; De Beer,E.J.; 1957; Investigation of the action of piperazine in *Ascaris lumbricoides*.; American Journal of Tropical Medecine and Hygiene.; 6; 899-905.

Ong,J.; Kerr,D.I.B.; Johnston,G.A.R.; 1987; Cortisol: a potent biphasic modulator of GABA-A receptor complexes in the guinea pig isolated ileum.; Neuroscience Letters; 82; 101-106.

Olsen,R.W.; 1982; Drug interactions at the GABA receptor-ionophore complex.; Annual Reviews in Pharmacology and Toxicology;22; 245-277.

Olsen,R.W.; 1984; γ-aminobutyric acid receptor binding antagonism by the amidine steroid RU5135.; European Journal of Pharmacology; 103; 333-337.

Olsen,R.W.; Tobin,A.J.; 1990; Molecular biology of GABA-A receptors.; FASEB Journal; 4; 1479-1480.

Paton,W.D.M.; 1961; A theory of drug action based on the rate of drug-receptor combination.; Proccedings of the Royal Society; B 154; 21-69.

Pennington,A.J.; Martin,R.J.; 1990; Acetylcholine activated single-channel current in *Ascaris suum* muscle.; Journal of Experimental Biology; in press.

Piggott,S.M.; Kerkut,G.A.; Walker,R.J.; 1977; The actions of picrotoxin, strychnine, bicuculline and other convulsants and antagonists on the response to acetylcholine, glutamate and GABA on *Helix* neurons.; Comparative Biochemistry and Physiology; 57C; 3107-116.

Pittaluga,A.; Asaro,D.; Pelligrini,G.; Raiteri,M.; 1987; Studies on [^{3}H]GABA and endogenous GABA release in rat cerebral cortex suggest the presence of autoreceptors GABA-B type.; European Journal of Pharmacology; 144; 45-52.

Polc,P.; Möhler,H.; Haefely,W.; 1974; The effects of diazepam on spinal cord activities: possible sites and mechanisms of action.; Naunyn Schmiederberg's Archiv für Pharmacologie; 284; 319-337.

Polc,P.; Haefely,W.; 1976; Effects of two benzodiazepines, phenobarbitone and baclofen on synaptic transmission in the rat cuneate nucleus.; Naunyn Schmiederberg's Archiv für Pharmacologie; 294; 121-131.

Praztor,V.M.; Bush,B.M.H.; 1982; Impulse coded and analog signaling in single mechanoreceptor neurons.; Science; 215; 1635-1637.

Price,D.A.; Greenberg,M.J.; 1977; The structure of a molluscan cardioexcitatory peptide.; Science; 197; 670-671.

Pritchett,D.B.; Luddens,H.; Seeburg,P.H.; 1989; Type I and type II GABA-A benzodiazepine receptors produced in transfected cells.; Science; 245; 1389-1392.

Raftery,M.A.; Conti-Tronconi,B.M.; Dunn,S.M.J.; Crawford,R.D.; Middlemas,D.; 1984; The nicotinic acetylcholine receptor: its structure multiple binding sites and cation transport properties.; In: Mechanisms of Receptor Regulation, Plenum Press, New York, pp255-278.

Rang,H.P.; 1971; Drug-receptor and their function.; Nature; 231; 91-96.

Roberts,C.J.; Krogsgaard-Larsen,P.; Walker,R.J.; 1981; Studies on the action of GABA, muscimol and related compounds on Periplaneta and Limulus central neurones.; Comparative Biochemistry and Physiology; 69C; 7-11.

Rogers,W.P; 1962;The evolution of parasitism.; In: The nature of parasitism; Academic press, New York, London, pp242-258.

Rohde,E.; 1885; Beiträge zur kenntniss der anatomie der nematoden.; Zoologische Beiträge; 1; 11-32.

Rosenbluth,J.; 1963; Fine structure of body muscle cells and neuromuscular junction in *Ascaris lumbricoides*.; Journal of Cell Biology; 19; 82A.

Rosenbluth,J.C.;1965a; Ultrastructural organization of obliquely striated muscle cells in *Ascaris suum*.; Journal of Cellular Biology; 25; 495-515.

Rosenbluth,J.C.; 1965b; Ultrastructure of somatic muscle cells in *Ascaris lumbricoides*.; Journal of cellular biology; 26; 579-591.

Rosenbluth,J.C.; 1973; Obliquely striated muscle.; In: The Structure and Function of the Muscle, Vol. I, Structure; Academic Press, New York and London; pp391-395.

Rozhkova,E.K.; Malyutina,T.A.; Shishov,B.A.; 1980; Pharmacological characteristics of cholinoreceptors in somatic muscles of the nematode *Ascaris suum.*; General Pharmacology; 11; 141-146.

Sakmann,B.; Hamill,O.P.; Bormann,J.; 1983; Patch-clamp measurements of elementary chloride currents activated by the putative inhibitory transmitters GABA and glycine in mammalian spinal neurons.; Journal of Neural Transmission and Supplements; 18; 88-95.

Satelle,D.B.; Pinnock,R.D.; Wafford,K.A.; David,J.A.; 1988; GABA receptors on the cell body of an identified insect motorneuron.; Proceedings of the Royal Society of London; B232; 443-456.

Scherer,R.W.; Ferkany,J.W.; Enna,S.J.; 1988; Evidence for pharmacologically distinct subsets of GABA-B receptors.; Brain Research Bulletin; 21; 439-443.

Schild,H.O.; 1947a; pA a new scale for the measurement of drug antagonism.; British Journal of Pharmacology; 2; 189-206.

Schild,H.O.; 1947b; The use of drug antagonists for the identification and classification of drugs.; British Journal of Pharmacology; 2; 251-258.

Schild,H.O.; 1949; pAx and competitive drug antagonism.; British Journal of Pharmacology; 4; 277-280.

Schild,H.O.; 1957; Drug antagonism and pAx.; Pharmacological Review; 9; 249-250.

Schneider,A.; 1866; Monographie der nematoden.; Berlin.

Schofield,P.A.; Darlison,M.G.; Fujita,N.; Burt,D.R.; Stephenson,F.A.; Rodriguez,H.; Rhee,L.M.; Ramachandron,J.; Reale,V.; Glencorse,T.A.; Seeburg,P.H.; Barnard,E.A.; 1987; Sequence and functional expression of the GABA-A receptor shows a ligand gated receptor superfamily.; Nature; 328; 221-227.

Schofield,P.A.; 1989; The GABA-A receptor: molecular biology reveals a complex picture.; TIPS; 10; 476-478.

Scott,R.H.; Duce,I.R.; 1987; Pharmacology of GABA receptors on skeletal muscle fibres of the locust *Schistocerca gregaria*.; Comparative Biochemistry and Physiology; 86C; 305-311.

Shank,R.P.; Pong,S.F.; Freeman,A.R.; Graham,L.T.; 1974; Bicuculline and picrotoxin as antagonists of GABA on neuromuscular inhibition in the lobster.; Brain Research; 72; 71-78.

Sieghart,W.; 1989; Multiplicity of GABA-A/Benzodiazepine receptors.; TIPS; 10; 407-411.

Simmonds,M.A.; 1982; Classification of some GABA antagonists with regard to site of action and potency in slices of rat cuneate nucleus.; European Journal of Pharmacology; 80; 347-358.

Simmonds,M.A.; 1983; Multiple GABA receptors and associated regulatory sites.; TINS; 6; 279-281.

Simmonds,M.A.; Turner,J.P.; Harrison,N.L.; 1984; Interaction of steroids with the GABA-A receptor complex.; Neuropharmacology; 23; 877-878.

Simmonds,M.A.; Turner,J.P.; 1985; Antagonism of inhibitory amino-acids by the steroid derivative RU5135.; British Journal of Pharmacology; 84; 631-635.

Soulsby,E.J.L.; 1965; Textbook of veterinary clinical parasitology.; Vol I Helminths, Blackwell scientific publications, Oxford, pp184-185.

Squires,R.F.; Casida,J.E.; Richarson,M.; Saederup,E.; 1983; [^{35}S]t-butylcyclophosphorothionate binds with high affinity to brain specific sites coupled to γ-aminobutyric acid and ion recognition sites.; Molecular Pharmacology; 23; 326-336.

Standen,O.D.; 1955; Activity of piperazine in vitro against *Ascaris lumbricoides*.; British Medical Journal; 2; 20-22.

Stephenson,,R.P.; 1956; A modification of receptor theory.; British Journal of Pharmacology; 11; 379-393.

Stephenson,F.A.; 1988; Understanding the GABA-A receptor: a chemically gated ion channel.; Biochemical Journal; 249; 21-32.

Steward,E.G.; Player,R.; Quillian,J.P.; Brown,D.A.; Pringle,M.J.; 1971; ; Naure New Biology; 233; 87-88

Stretton,A.O.W.; Fishpool,R.M.; Southgate,E; Donmoyer,J.E; Walrond,J.P; Moses,J.E.R; Kass,I.C; 1978; Structure and physiological activity of the motorneurons of *Ascaris*.; Proceedings of the National Academy of Sciences; USA.;75; 3493-3497.

Supavilai,P.; Karobath,M.; 1979; Stimulation of benzodiazepine receptor binding by SQ20009 is chloride dependent and picrotoxin sensitive.; European Journal of Pharmacology; 60; 111-113.

Supavilai,P.; Karobath,M.; 1984; [^{35}S]t-butylcyclophosphorothionate binding sites are constituants of the γ-aminobutyric acid benzodiazepine receptor complex.; Journal of Neuroscience; 4; 1193-1200.

Swagel,M.W.; Ikeda,K.; Roberts,E.; 1973; Effects of GABA and bicuculline on conductance of the crayfish abdominal stretch receptor.; Nature New Biology; 244; 180-181.

Takeuchi,A.; Onodera,K.; 1972; Effects of bicuculline on the GABA receptor of the crayfish neuromuscular junction.; Nature New Biology; 236; 55-56.

Takeuchi,A.; Takeuchi,N; 1975; The structure-activity relationship for GABA and related compounds in the crayfish muscle.; Neuropharmacology; 14; 627-634.

Tallman,J.F; Thomas,J.W.; Gallager,D.W.; 1978; GABA-ergic modulation of benzodiazepine site sensitivity.; Nature; 274; 383-385.

Thorn,P.; Martin,R.J.; 1987; A high-conductance calcium dependent chloride channel in *Ascaris suum* muscle.; Quaterly Journal of Experimental Physiology; 72; 31-49.

Ticku,M.K.; Olsen,R.W.; 1978; Interaction of barbiturates with dihydropicrotoxinin binding site related to GABA receptor ionophore system.; Life Sciences; 22; 1643-1651.

Toscano-Rico,J.; 1926; Sur la sensibilité de l'*Ascaris* à l'action de quelques drogues.; Comptes Rendus de la Société de Biologie; 94; 921-923.

Trapido-Rosenthal,H.G.; Morse,D.E.; 1986; Avaibility of chemosensory receptors is down regulated by habituation of larvae to morphogenetic signals.; Proceedings of National Academy of Sciences USA; 20; 7658-7662.

Turner,J.P.; 1986; Potentiation of GABA analogue muscimol by physiologically occuring steroids.; British Journal of Pharmacology; 87;148p.

Twyman,R.E.; Rogers,C.J.; Mac Donald,R.L.; 1989; Differential regulation of γ-aminobutyric acid receptor channels by diazepam and phenobarbital.; Annals of Neurology; 25; 213-220.

Urquhart,G.M.; Armour,J.A.; Duncan,J.L.; Dunn,A.M.; 1987; In: Veterinary Parasitology, Longman Scientific and Technical, F.W.Jennings.

Vehovszky,A.; Bokisch,A.J.; Krogsgaard-Larsen,P.; Walker,R.J.; 1989; Pharmacological profile of γ-aminobutyric acid (GABA) receptor of identified central neurones from *Helix aspersa*.; Comparative Biochemistry and Physiology; 92C; 391-399

Wafford,K.A.; Satelle,D.B.; Abalis,I.; Eldefrawi,M.E.; 1987; γ-aminobutyric acid activated ^{36}Cl influx: a functional in vitro assay for CNS γ-aminobutyric acid receptors of insects.; Journal of Neurochemistry; 48; 177-180.

Walker,R.J.; Holden-Dye,L.; 1989; Commentary on the evolution of transmitter receptors and ion channels in invertebrates.; Comparative Biochemistry and Physiology; 93A;25-39.

Waller,P.J.; 1985; Resistance to Anthelminthics and the Implication for Animal Production. In : Resistance in Nematodes to Anthelminthic Drugs. Ed. N.Anderson, CSIRO, Australia,pp 1-12

Walrond,J.P.; Kass,I.C.; Stretton,A.O.W.; Donmoyer,J.E.; 1985; Reciprocal inhibition in the motorneurons system of the nematode *Ascaris*.; Journal of Neuroscience; 5; 1-9.

Walrond,J.P.; Stretton,A.O.W.; 1985a; Reciprocal inhibition in the motor nervous system of the nematode *Ascaris*: direct control of ventral inhibitory motorneurons by dorsal excitatory motorneurons.; Journal of Neuroscience; 5; 9-15.

Walrond,J.P.; Stretton,A.O.W.; 1985b; Excitatory and inhibitory activity in the dorsal musculature of the nematode *Ascaris* evoked by single dorsal excitatory motorneurons.; Journal of Neuroscience; 5; 16-22.

Wann,K.T.; 1987; The electrophysiology of the somatic muscle cells of *Ascaris suum* and *Ascaridia galli*.; Parasitology; 94; 555-566.

Waud,D.R.; Parker,R.B.; 1971; Pharmacological estimation of drug-receptor dissociation constants. Statistical evaluation. II Competitive antagonists.; Journal of Pharmacology and Experimental Therapeutics; 177; 13-24.

Weisblat,D.A.; Byerly,L.; Russel,R.L.; 1976; Ionic mechanisms of electrical activity in somatic muscle of the nematode *Ascaris lumbricoides*.; Journal of Comparative Physiology; 111; 93-113.

Weiss,D.S.; Magleby,K.L. 1989; Gating schemes for single γ-aminobutyric acid activated chloride channels determined from stability plots, dwell time distribution and adjacent interval deviations.; Journal of Neuroscience; 9; 1314-1324.

Wermuth,C.G.; Bourguignon,J.J.; Schlewer,G.; Gies,J.P.; Schoenfelder,A.; Melikian,A.; Bouchet,M.J.; Chantreux,D.; Molimard,J.C.; Heaulme,M.; Chambon,J.P.; Biziere,K.; 1987; Synthesis and structure activity relationships of a series of aminopyridazine derivatives of GABA acting as selective GABA-A antagonists.; Journal of Medical Chemistry; 30; 239-249.

Wermuth,C.G.; Rognan,D.;1987; Modélisation d'antagonistes des récepteurs GABA-A; Actualités Chimiques et Thérapeutiques; 14; 215-229.

Williams,M.; Yarbrough,G.; 1979; Enhancement of in vitro binding and some pharmacological properties of diazepam by a novel anthelmintic agent, avermectinB1a.; European Journal of Pharmacology; 56; 273-276.

Williams,M.; Risley,E.A.; 1979; Enhancement of binding of [^{3}H]flunitrazepam to rat brain membranes in vitro by SQ20009 a novel anxiolytic, GABA and muscimol.; Life Sciences; 24; 833-841.

Williams,T.L.; Smith,D.A.S.; Burton,N.R.; Stone,T.W.; 1988; Amino-acid pharmacology in neocortical slices: evidence for bimolecular actions from an extension of the Hill and Gaddum-Schild equations.; British Journal of Pharmacology; 95; 805-810.

Wong,E.H.F.; Leeb-Lundberg,L.M.F.; Teichberg,V.I.; Olsen,R.W.; 1984; γ-aminobutyric acid activation of [^{36}Cl] flux in rat hippocampal slices and its potentiation by barbiturates.; Brain Research; 303; 267-275.

Yarowsky,P.J.; Carpenter,D.O.; 1978; Receptors for GABA in *Aplysia* neurones.; Brain Research; 144; 75-94.

APPENDIX:

Some quantitative aspects of competitive antagonism are developed in this appendix. The relationship between the dose-ratio and the antagonist concentrationin particular will be considered.

Schild (1947a; 1947b; 1957) applied the notion of Null hypothesis and dose-ratio to the analysis of competitive antagonism (see Section 1.C). This work led Arunlakshana and Schild (1959), to formulate a relationship between the dose-ratio and the antagonist concentration. Their analysis is based on a very simple and non-cooperative model for drug-receptor interaction, but is still widely used to determine whether an antagonist is competitive or not. Although cooperativity between some drugs and their receptors, such as GABA or acetylcholine, is now well established (see Colqhoun, 1973); it is only recently that the modified Schild analysis, which account for cooperativity, has been proposed as an alternative for the classical Schild analysis (Williams et al., 1988). The emergence of new techniques, such as single channel recording, has led to further complication of the models for drug-receptor interaction.

Here, the existence of the channel in two states: open and closed, and the existence of intermediate complex, will be also considered. The relationship between the dose-ratio and the antagonist concentration will be examined in these cases, and it will be seen that the Schild plot is not always linear with a slope of one.

I Classical Schild equation

The classical Schild analysis is based on a simple model of drug-receptor interaction:

$$RB \underset{}{\overset{K_B}{\rightleftharpoons}} R \overset{K_D}{\rightleftharpoons} RA^* \qquad \text{(M1)}$$

where, R is the receptor not occupied; RA* is the receptor occupied by one molecule of agonist; RB is the receptor occupied by one molecule of antagonist. (*) signals that the receptor is in an activated form; for example, in the case of GABA-A receptors, the channel is open. K_D is the dissociation constant for the agonist, and K_B is the dissociation constant for the antagonist. At equilibrium, by applying the law of mass action, the proportion of channels open in can be expressed in function of the agonist concentration, X_A, and in function of the antagonist concentration, X_B.

In the absence of antagonist, the proportion of channels open is:

$$Po = \frac{[RA^*]}{[R]_0},$$

where $[R]_0$ is the total concentration in receptor: $[R]_0 = [R]+[RA^*]$

$[RA^*]$ can be expressed in function of X_A:

$$[RA^*] = \frac{[R].X_A}{K_D}, \text{ and } [R]_0 = [R].[1+\frac{X_A}{K_D}] .$$

Po becomes: $Po = \frac{X_A/K_D}{1+X_A/K_D}$,

$$Po = \frac{1}{1+K_D/X_A} \quad \textbf{(A1)}.$$

In the presence of the antagonist, the proportion of channels open is:

$$Po' = \frac{[RA^*]}{[R]+[RA^*]+[RB]},$$

and [RB] can be expressed as a function of the antagonist concentration X_B, since at equilibrium and by applying the law of mass action:

$$[RB] = \frac{[R].X_B}{K_B} .$$

Po' becomes: $Po' = \frac{X_A/K_D}{1+X_A/K_D+X_B/K_B}$

$$Po = \frac{1}{1+K_D/X_A.(1+X_B/K_B)} \quad \textbf{(A2)}.$$

In the presence of the antagonist, the proportion of channels open is smaller than in the absence of antagonist. To obtain the same Po in the presence of the antagonist, the concentration of agonist must be increased to a value X_A'. For the same proportion of channels open, the ratio of the agonist concentration in the presence, X_A', and in the absence, X_A, of antagonist, X_B, is the dose-ratio, $DR = \frac{X_A'}{X_A}$.

DR can be expressed in function of X_B and K_B only, since:

$$Po = \frac{1}{1+K_D/X_A} = \frac{1}{1+K_D/X_A'.(1+X_B/K_B)}$$

$$\text{then, } \frac{K_D}{X_A} = \frac{K_D}{X_A'}.(1+\frac{X_B}{K_B})$$

$$\text{and} \quad X_A'.K_D = K_D.X_A+\frac{K_D.X_A.X_B}{K_B} \quad \textbf{(A3)}.$$

If **(A3)** is divided by X_A and K_D, $DR = 1+\frac{X_B}{K_B}$, which can be written under the form of the classical Schild equation,

$$\log(DR-1) = \log X_B - \log K_B \quad \textbf{(A4)}.$$

The same logical steps will be followed in the subsequent developments, but for the sake of simplicity, the demonstrations are not presented.

II <u>The modified Schild equation</u>

The modified Schild equation is based on a similar model, but several molecules of agonist or/and antagonists are allowed to bind the receptor. For example in the case of two molecules of agonist and one molecule of antagonist:

$$RB \overset{K_B}{\rightleftharpoons} R \overset{K_D}{\rightleftharpoons} RA_2^* \qquad \textbf{(M2)},$$

where RA_2^* is the receptor activated and occupied by two molecules of agonist; K_D is the dissociation constant of RA_2^*, and K_B is the dissociation constant of RB.

The relationship between dose-ratio and X_B becomes,

$DR^2 = 1+\frac{X_B}{K_B}$, which can be written:

$$\log(DR^2-1) = \log X_B - \log K_B \qquad \textbf{(A5)}.$$

If there were two molecules of antagonist, the relationship would be:

$DR^2 = 1+\frac{X_B^2}{K_B}$, which can be written:

$$\log(DR^2-1) = 2\log X_B - \log K_B \qquad \textbf{(A6)}.$$

And more generally, if N molecules of agonist and M molecules of antagonist, bind the receptor:

$$RB_M \overset{K_B}{\rightleftharpoons} R \overset{K_D}{\rightleftharpoons} RA_N^* \qquad \textbf{(M3)},$$

the relationship would be: $DR^N = 1+ \frac{X_B^M}{K_B}$, which can be written under the general form of the modified Schild equation (Williams et al., 1988):

$$\log(DR^N - 1) = M.\log X_B - \log K_B \qquad \textbf{(A7)}.$$

N is the number of agonist molecules bound to the receptor, and M the number of antagonist molecules bound to the receptor; K_B is the dissociation constant of RB_M; and K_D is the dissociation constant of RA_N.

III Two-state model

The receptor-channel can exist in two states: open or closed. It is assumed that if the receptor is not occupied by the agonist, or is occupied by the antagonist, the channel is closed, and that if the receptor is occupied by the agonist, the channel may be open or closed:

$$RB \overset{K_B}{\rightleftharpoons} R \overset{K_D}{\rightleftharpoons} RA \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} RA^* \qquad \textbf{(M4)},$$

where RA is the receptor occupied by one molecule of agonist, but is not activated and the channel is closed; RA*, is the receptor occupied by one molecule of agonist and activated, the channel is therefore open; α is the channel closing rate, β is the channel opening rate constant.

In the absence of antagonist, Po becomes:

$$Po = \frac{1}{1+\frac{\alpha}{\beta}\cdot\left\{\frac{K_D+X_A}{X_A}\right\}} \qquad \textbf{(A8)},$$

and in the presence of the antagonist the proportion of channels open is:

$$Po = \frac{1}{1 + \frac{\alpha}{\beta}\left\{\frac{K_B.X_A'+K_D.X_B+K_B.K_D}{X_A'.K_B}\right\}} \qquad \textbf{(A9)},$$

so that $\frac{K_B.X_A'+K_D.X_B+K_B.K_D}{X_A'.K_B} = \frac{K_D+X_A}{X_A}$ **(A10)**,

and the dose-ratio, DR, can still be expressed in function of X_B and K_B: $DR = 1+\frac{X_B}{K_B}$, see the Schild equation **(A4)**.

The addition of a closed state did not modify the relationship between the dose-ratio and the antagonist concentration, and the Schild equation is still valid.

IV Intermediate complex

In the case of cooperative interaction, intermediate complex can be formed, RA and RB may exist:

$$RB_2 \overset{K_{B1}}{\rightleftharpoons} RB \overset{K_{B2}}{\rightleftharpoons} R \overset{K_{D1}}{\rightleftharpoons} RA \overset{K_{D2}}{\rightleftharpoons} RA_2 \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} RA_2^* \qquad \textbf{(M5)},$$

where K_{D1} is the dissociation constant for RA; K_{D2} is the dissociation constant for RA_2*; K_{B1} is the dissociation constant of RB; K_{B2} is the dissociation constant of RB_2; other symbols as before.

In the absence of antagonist, Po is:

$$Po = \frac{1}{1+\frac{\alpha}{\beta}.\left\{1+\frac{K_{D2}}{X_A}+\frac{K_{D1}.K_{D2}}{X_A^2}\right\}} \qquad \textbf{(A11)},$$

In the presence of antagonist, Po is:

$$Po = \frac{1}{1+\frac{\alpha}{\beta}.\left\{1+\frac{K_{D2}}{X_A'}+\frac{K_{D1}.K_{D2}.X_B^2}{K_{B1}.K_{B2}.X_A'^2}+\frac{K_{D1}.K_{D2}.X_B}{K_{B1}.X_A'^2}+\frac{K_{D1}.K_{D2}}{X_A'^2}\right\}} \qquad \textbf{(A12)}.$$

The relationship between the dose-ratio and X_B becomes:

$$A.X_A'.DR.(DR-1)+B.(DR^2-1) = (C.X_B+D).X_B \qquad \textbf{(A13)},$$

where $A = K_{D2}.K_{B1}.K_{B2}$

$B = K_{D1}.K_{D2}.K_{B1}.K_{B2}$

$C = K_{D1}.K_{D2}.K_{B1}$

$D = K_{D1}.K_{D2}.K_{B2}$

In this caseDR is also dependent on the agonist concentration, X_A', and by definition this means that the agonist dose-response curves, in the presence or absence of the antagonist, are not parallel any more. If (RA) is very small compared to (RA_2), K_{D1} is very large compared to K_{D2}.

If (A13) is divided by K_{D1},

$$\frac{K_{B1}.K_{B2}.X_A'}{K_{D1}}.DR.(DR-1)+K_{D2}.K_{B1}.K_{B2}.(DR^2-1)=(K_{D2}.K_{B1}.X_B+K_{D2}.K_{B2}).X_B \qquad \textbf{(A14)},$$

$\frac{K_{B1}.K_{B2}.X_A'}{K_{D1}}$ tends towards 0, so that **(A14)** can be simplified to,

$$K_{D2}.K_{B1}.K_{B2}.(DR^2-1) = (K_{D2}.K_{B1}.X_B+K_{D2}.K_{B2}).X_B \qquad \textbf{(A15)}.$$

If the intermediate complex (RB) is very small, K_{B1} must be very large. When (A15) is divided by K_{B1}, simplification can be made since $\frac{K_{D2}.K_{B2}}{K_{B1}}$ tends towards 0, and:

$K_{B2}.(DR^2-1) = X_B^2$, which can be written in the form of the modified Schild equation **(A6)**,

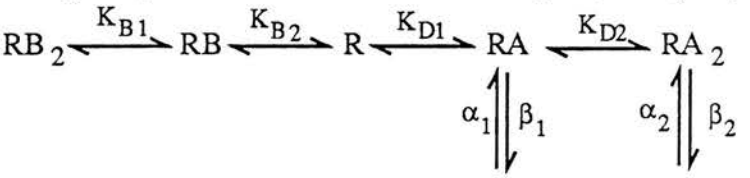
$$\log(DR^2-1) = 2.\log X_B - \log K_{B2}\,.$$

Reciprocally, if (RB) predominates and (RB_2) is very small, K_{B2} must be very large and the expression can be simplified and written in the form (A5),

$$\log(DR^2-1) = \log X_B - \log K_{B1}\,.$$

V Intermediate complex can exist in two states

Further complexity arises if the intermediate complex, RA opens, RA*:



(M6),

where α_1 is the channel closing rate constant and β_1, the channel opening rate constant of RA*; α_2 is the channel closing rate constant and β_2 the channel opening rate constant of RA_2*.

The proportion of channel open in the absence of antagonist is:

$$Po = \frac{1}{1+\alpha_1.\alpha_2.\left\{\frac{X_A^2+K_{D2}.X_A+K_{D1}.K_{D2}}{\alpha_1.\beta_2.X_A^2+\alpha_2.\beta_1 X_A}\right\}} \qquad \textbf{(A16)}\,,$$

and in the presence of the antagonist: **(A17)**

$$Po = \frac{1}{(1+\alpha_1.\alpha_2)\left\{\frac{X_A'^2+K_{D2}.X_A'+\frac{K_{D1}.K_{D2}}{K_{B1}.K_{B2}}.X_B^2+\frac{K_{D1}.K_{D2}}{K_{B1}}.X_B+K_{D1}.K_{D2}}{\alpha_1.\beta_2.X_A'^2+\alpha_2.\beta_1 X_A'}\right\}}$$

The dose-ratio can be expressed as:

$$A.X_A'.DR.(DR-1)+B.X_A'.DR.(1-DR)+C.(DR^2-1)+\frac{D}{X_A'}.(DR-1) = (E.X_B^2+F.X_B).(G+\frac{H}{X_A'}) \qquad \textbf{(A18)}$$

where $A = K_{B1}.K_{B2}.K_{D2}.\alpha_1.\beta_2$ $\qquad$ $E = K_{D1}.K_{D2}$

$B = K_{B1}.K_{B2}.K_{D2}.\alpha_2.\beta_1$ $\qquad$ $F = K_{D1}.K_{D2}.K_{B2}$

$C = K_{D1}.K_{D2}.K_{B1}.K_{B2}.\alpha_1.\beta_2$ $\quad G = \alpha_1.\beta_2$

$D = K_{D1}.K_{D2}^2.K_{B1}.K_{B2}.\alpha_2.\beta_1$ $\quad H = \alpha_2.\beta_1.K_{D2}$

If [RA*] very small, α_1 must be very large, and the expression simplifies to:

$$A'.X_A'.DR.(DR-1)+C'.(DR^2-1) = (E.X_B^2+F.X_B).G' \quad \textbf{(A19)},$$

where $A' = K_{B1}.K_{B2}.K_{D2}.\beta_2$

$C' = K_{D1}.K_{D2}.K_{B1}.K_{B2}.\beta_2$

$G' = \beta_2$

E and F as before. **(A19)** can be divided by β_2, and the expression is the same as **(A13)**. Further simplifications can be made, see **(A13)**, **(A14)** and **(A15)**.

CONCLUSION

Using the general model **(M6)**, and by applying the law of mass action, the dose-ratio is shown not to be a linear function of the antagonist concentration; moreover DR is also dependent on the agonist concentration. These results differ from those obtained when a Monod-Wyman-Changeux allosteric model is used, where even in the general case, the dose-ratio will still be a linear function of the antagonist concentration (see Colqhoun, 1973). The purpose of this appendix is not to decide which one of the occupancy model or the allosteric model, is valid, but to illustrate the basis for the analytical approach used in this thesis, although still a simplification. There are certainly some uncertainties on whether the law of mass action can apply to drug-receptor interaction, but the allosteric alternative is not fully satisfactory either. Both are probably wrong in the fact that they are still oversimplifications. The results presented in this thesis were analysed on the basis of an occupancy model. The reasons are that occupancy models are still widely used, and here, it provided a good description of the data even if some complications had to be introduced in the analysis of the antagonism. Waud (1987) has prviously commented on using a simplified approach:

> *"...I do not blush when caught using a competitive kinetic ananlysis heuristically when the underlying model may in fact be more along allosteric lines..."*

and,

> *"...A value for a Km can be useful even if the mechanism is not really that formulated by Michaelis-Menten. Similarly if you tell me the KB of a new antagonist, I know a lot about the drug..."*

DUITTOZ, A.H.
PR.D. 1991

2

EFFECTS OF THE ARYLAMINOPYRIDAZINE-GABA DERIVATIVES, SR95103 AND SR95531 ON THE *ASCARIS* MUSCLE GABA RECEPTOR: THE RELARIVE POTENCY OF THE ANTAGONISTS IN *ASCARIS*, IS DIFFERENT TO THAT AT VERTEBRATE GABA-A RECEPTORS.

A.H.Duittoz & R.J.Martin

Department of Preclinical Veterinary Sciences, R.(D).S.V.S, University of Edinburgh, Summerhall, EH1 9QH Edinburgh.

GABA; antagonists, arylaminopyridazines; SR95103; SR95531; Ascaris suum.

SUMMARY

1. GABA-induced changes in input conductance in the bag region of Ascaris suum muscle were monitored using a two-microelectrode current-clamp technique.
2. Effects of the arylaminopyridazine GABA derivatives SR95103 and SR95531 on GABA conductance responses were observed. SR95103 was more potent than SR95531 as an antagonist; this potency order contrasts with vertebrate GABA-A receptors.
3. The anatgonism of SR95103 was associated with a parallel shift to the right in the GABA dose-response relationship. A modified Schild plot was used to describe the action of SR95103: the data was concistent with two molecules of GABA but one molecule of antagonist interacting with the receptor. The K_B for SR95103 was 64±13μM (mean±SE, n=14).

INTRODUCTION

A variety of antinemartodal drugs, including piperazine and ivermectin may interact with the parasite g-aminobutyric acid (GABA) receptors, (Martin, 1987), so it is of interest to compare the pharmacology of [arasite and host receptors in an attempt to identify and exploit differences for therapeutic purposes.

In mammals, GABA receptors have been divided into two main classes: GABA-A receptors and GABA-B receptors (Bowery et al., 1983). Isoguvacine and piperidine-4-sulphonic acid (P4S) are selective GABA-A agonists and bicuculline is a selective GABA-A antagonist (Bowery et al., 1983); baclofen is a specific GABA-B angonist and phaclofen is a selective GABA-B antagonist (Kerr et al., 1987). The GABA-A receptor is directly linked to a Cl channel; the GABA-B receptor acts via a second messenger on a K or Ca conductance (Dunlap, 1981; Desarmenien et al., 1982; Dutar and Nicoll, 1988). This classification is useful although a simplification, since both classes appear to be heterogeneous (Krogsgaard-Larsen, 1988; Levitan et al., 1988; Pritchett et al., 1989). Bicuculline and baclofen insensitive GABA receptors have also been described (Ault and Nadler, 1983; Johnston and Allan, 1984) and tentativelly named GABA-C receptors (Johnston, 1986).

A nematode GABA receptor, amenable to electrophysiological study is found on the extrasynaptic region of large muscle cells of *Ascaris suum* (Martin, 1980); this receptor is linked to a Cl channel (Martin, 1980, 1985). Prvious pharmacological studies indicate that most GABA-A agonists, except the sulphonic acid derivatives 3APS and P4S are active at the Ascaris receptor; but the GABA-A antagonists picrotoxin, bicuculline, pitrazepin, securinine and TBPS are inactive (Holden-Dye et al., 1988, 1989).

The actions of arylaminopyridazine derivatives have been investigated in the mammalian central nervous system, where they are potent and selective competitive antagonists of GABA-A receptors (Chambon et al., 1985); SR95531 is the most potent of the series. However, the action of this

series of compounds remains to be fully explored in *Ascaris* and more generally in invertebrates. In these two papers, we examine and describe the actions of SR95103 and SR95531 (Figure 1) on the Ascaris GABA receptor: the first paper describes effects on GABA conductance dose-response relationships and shows that in *Ascaris* SR95103 is more potent than SR95531; the second paper describes effects of SR95103 at the single-channel level. The action of these antagonists suggests that a knowledge of the pharmacology of arylaminopyridazine-GABA derivatives in Ascaris may be useful for the design of new antiparasitic agents. A preliminary account of this work has appeared (Duittoz and Martin, 1989).

MATERIAL AND METHODS

- The preparation

Specimens of *Ascaris suum* were collected from the local slaughter house and maintained in Locke's solution t 37°C for up to four days. A two cm flap preparation (Martin, 1980) was mounted in the experimental chamber which had a volume of 2.5ml. The preparation was bathed in high-CL, low-Ca solution containing (mM): NaCl 135; KCl 3; $MgCl_2$ 15.7; glucose 3; TRIS 5; pH adjusted to 7.6 with maleic acid.

- Current-clamp technique

Two glass microelectrodes (K acetate 2M filled) were introduced into the bag region of a muscle cell; one pipette (resistance 10-20MΩ) was used to inject hyperpolarizing current pulses (40nA, 2s, 0.2Hz), the other pipette (resistance 20-30MΩ) was used to record membrane potential. Signals were recorded with a laboratory made amplifier (Martin, 1982) in the current-clamp mode and monitored on a Tectronix 2210 oscilloscope and a Lectromed two-channel chart-recorder.

- Voltage-clamp technique

The voltage-sensitivity of the action of SR95103 was examined by applying GABA iontophoretically to the bag membrane with a third microelectrode; the two intracellular micropopettes, prviously placed for current-clamp, were used for voltage-clamp under the control of an Axoclamp 2A (set in the voltage-clamp mode). Details of this technique have been described previously (Martin, 19820. The holding-ppotential was 0 or -10mV and 80mV voltage-steps to -80mV or -90mV were made for 500ms at 1Hz. GABA was applied iontophoretically as a cation from a fine micropipette (20MΩ) using a standard charge of 150nC to obtain each response. GABA 2M at pH 2.5 filled the iontophoretic pipette.

- Data analysis

The input conductance of the bag was calculated directly from the amplitude of the plateau of the voltage-reaponse to the injected current pulse; the I/V plot of the bag is linear during the injection of hyperpolarizing current and input conductance is unaltered by hyperpolarization over the range of membrane potentials observed during these experiments (Martin, 1980). The effects of GABA was then determined by measuring the change in input conductance. The bag region of the muscle cell was approximated to a sphere, consequently, the input conductance was directly related to the change in membrane conductance. Dose-response relationships were described by the modified Hill equation (Nistri and Constanti, 1979):

$$\Delta g = \frac{\Delta gmax}{1+(EC50/X_A)^N}$$

where Δg is the change in input conductance, Δgmax is the maximum response, EC50 is the concentration of GABA producing 50% of the maximum response, XA is the agonist concentration and N is the Hill coefficient. A non-linear regression programme (patterensearch Colquhoun, 1971) was used to obtain the least-square estimates for Δgmax, EC50, and N. Dose-response curves were considered parallel if the Hill coefficient and Δgmax for the control GABA dose-response curve and the response in the presence of antagonsit were not significantly different. Dose-ratios were measured for the effect of SR95103 on GABA dose-response curves and initially examined using a Schild plot (Arunlakshana and Schild, (1959). Since the slope of this plot was

significantly less than unity, the dose-ratios were further examined using a modified Schild plot (Williams et al., 1988) which has the advantage of taking account of the observed cooperativity seen at the GABA receptor. It is pointed out that the relationship, between receptor occupancy and fractionnal resposne, is in general not known and that physically plausible models for the interaction of agonist and antagonist do not predict simple or integral values for these plots. However it is considered that the plots provide a useful empirical approach for describing agonist and antagonist action.

- Bath-application of drugs
Initially, GABA was bath-applied using a non-cumulative dose method with washing of the preparation between applications. GABA produced, as reported previously (Martin, 1980; Holden-Dye et al., 1988) a dose dependent hyperpolarization and an increase in input conductance. However, one limitation of this technique was that the recovery of the resting input conductance was often incomplete after application of high concentrations of GABA; this may have been due to slight movement of the muscle flap preparation producing a decrease in the leak resistance around the micropopettes. It was found that the recovery after application of high GABA was more often complete if cumulative bath-application of GABA, without intermediate washing were used, Figure 2. The method involved gentle draining of the bath (volume 2.5ml), and then flushing the bath with 5ml of the next drug solution. This method of application has the advantage of a more certain drug concentration over the cell surface than the "local perfusion" technique using a micropipette. The bath was not perfused continuously because of the limited quantities of antagonist available. When the dose-response relationships, obtained by non-cumulative and by cumulative application of GABA, were described by the modified Hill equation, it was found that the EC50s and Hill coefficients were not significantly different, Table1. In contrast Δgmax was on average smaller when GABA was applied cumulatively. One possible explanation for the reduced maximum response seen with cumulative application is desensitization. Despite this, Δgmax was remarkably constant for the smae cell, varying only between cells. Cumulative dose-response relationships were therefore used to examine the effects of SR95103 on EC50, N and Δgmax.

- Statistical tests
Means and standard errors (SE) were calculated for EC50, N and Δgmax values for GABA alone and in the presence of different concentrations of antagonist. Statistical significance was assessed using a two-tailed independent t-test.

- Drugs
GABA was obtained from Sigma; SR95531 (2-(3-carboxypropyl)-3-amino-6-(p-methoxy)-phenylpyridazium chloride) and SR95103 (2-(3-carboxypropyl)-3-amino-4-methyl-6-phenylpyridazium chloride), Figure 1, were obtained as gifts from SANOFI (France).

RESULTS

The results reported here are based on an analysis of experiments conducted on 18 muscle cells from 13 preparations. Cells selected for recording and analysis had resting membrane potentials greater than 20mV and resting input conductances between 1.9-4.0μS (2.5±0.1μS, mean±SE, n=18). The recordings were also rejected if resting input conductances failed to return to at least 80% of the control after washing GABA from the preparation.

- SR95531 and SR95103 as GABA antagonists
In mammalian preparations, SR95531 is the most potent arylaminopyridazine GABA-A antagonist and will antagonize electrophysiologucal responses to GABA (Chambon et al., 1985; Desarmenien et al., 1987; Michaud et al., 1986; Hamann et al., 1988). In *Ascaris*, the application of 1mM SR95531 had a small effect on

conductance changes produced by 30μM GABA, Figure 3B. SR95103 is less potent than SR95531 at the GABA-A receptor in rat brain membranes (Heaulme et al., 1986a, 1986b). This contrasts with effects seen in *Ascaris*, Figure 3A, where 1mM SR95103, unlike SR95531, produces nearly complete antagonism. The antagonism reverses after washing.

The percentage of antagonism of the response to 30μM GABA produced by 1mM concentrations of SR95103 and SR95531 was measured in a series of experiments; it was found that SR95103 reduced the response by 92.3±3.2% (mean±SE, n=5) and that SR95531 reduced the response by 46.2±6.7% (mean±SE, n=5). These observations illustrate the contrast between the pharmacology of a GABA-A receptor and the *Ascaris* GABA receptor.

- Dose-dependent nature of the SR95103 antagonism

The dose-dependent nature of the antagonism was tested by examining the effect of 100, 300 and 1000μM SR95103 on cumulative GABA dose-response curves. Figure 4 shows, from a single cell, the typical effects of 100μM antagonist where there is a parallel shift to the right with no reduction in the maximum response. Table 2 shows pooled results with the mean and SE values for the parameters of the modified Hill equation used to describe the dose-response curves. It can be seen that SR95103 produces increases in the EC50s without a significant change in the Hill coefficient or Δgmax.

- Schild plots

Dose-ratios were determined from the change in EC50 values and a classical Schild plot obtained. This was characterized by an apparently linear relationship between log(DR-1) and $\log X_B$ and an intersect on the abcissa giving a pA2 of 4.0. However this plot was characterized by a slope (0.45±0.15, mean±SE, n=14) significantly less than one ($p \leq 0.05$, t-test). Thus the dose-ratios were smaller at higher antagonist concentrations, than predicted by the Schild equation.

One interpretation of this result is that SR95103 might not be acting on a single receptor in a competitive manner. However, the classical Schild analysis does not account for some cooperative models of agonist action involving two agonist binding sites (see for example Rang, 1971; Williams et al., 1988). We therefore carried out a modified Schild analysis, where it was assumed that two agonist molecules were required to bind to activate the receptor and the slope of the plot is the number of antagonist molecules required to inhibit the receptor. Figure 5 shows the modified Schild plot of $\log(DR^2-1)$ against $\log X_B$. The slope was 0.62±0.17 (mean±SE, n=14) which is not significantly less than one ($p \leq 0.05$, t-test). These results then, are consistent with a model which involves activation of the receptor by two agonist molecules but only one antagonist molecule is required. Although these results do not exclusively establish such a mode of action for SR95103 it does permit the estimation of an apparent dissociation constant. The arithmetic mean of the dissociation constant K_B as calculated from the modified Schild equation using N=2 and M=1, i.e.:

$$K_B = \frac{(DR^2-1)}{X_B}$$

K_B was 64±13μM (mean±SE, n=14).

- Voltage-sensitivity of the antagonism

The two-micoelectrode voltage-clamp technique with the iontophoretic application of GABA (Martin, 1982) was used to examine the voltage-sensitivity of the antagonism produced by bath-application of 0.1-1mM SR95103. A holding potential of 0 or -10mV was used and voltage steps to -80mV or -90mV were made for 500ms at 1Hz. A standard charge of 150nC was used to eject the GABA as a cation from the iontophoretic pipette in the absence and then in the presence of the antagonist. The peak responses at the holding potential and at the step potential were measured from the envelope of the current trace. The voltage-sensitivity (V_S) of the antagonism was expressed as:

V_S=1-\f(%antagonism at 0 or -10mV, % antagonism at -80 or -90mV)

If SR95103 were acting predominently as an anion to produce a channel block, then it would be expected that the degree of channel block would decrease at more negative potentials. However the antagonism showed little evidence of voltage-sensitivity: V_S=0.05±0.17 (mean±SE, n=4).

DISCUSSION

The main finding of this paper was that SR95103 but not SR95531 acts as a moderatly potent antagonist in *Ascaris* with an apparent KB of 64μM; the study also shows that the potency of arylaminopyridazine GABA analogues can vary at the *Ascaris* GABA receptor. In a previous study with SR95531, (Holden-Dye et al., 1989), it was found that SR95531 was not a potent antagonist in *Ascaris*. However, it was suggested that this class of compounds does not recognise the *Ascaris* GABA receptor, although no other arylaminopyridazine GABA analogues were examined.

It has already been pointed out that GABA-A receptors may be heterogeneous (Levitan et al., 1988; Pritchett et al., 1989; for a review see Krogsgaard-Larsen, 1988), but that it is still useful to compare the *Ascaris* receptor to a typical GABA-A receptor. The greater relative potency of SR95103 compared to SR95531 in *Ascaris* contrasts with the relative potency at mammalian GABA-A receptors: SR95531 displaces [^{3}H]GABA from rat brain membranes with aglobal K_I=0.15μM, (Heaulme et al., 1986a) and SR95103 has a K_I of 2.2μM (Chambon et al., 1985); SR95103 has a pA2 of 5 and SR95531 has a pA2 of 7 in electrophysiological experiments on rat dorsal root ganglion cells (pA2 values calculated from data presented by Chambon et al., 1985 and Hamann et al., 1988).

SR95531 is similar in structure to SR95103 (Figure 1) but has a methoxy group on the 6-phenyl substituent of the pyridazine ring and no substituent on the 4-position; SR95103 has no substituent on the 6-phenyl ring but has a methyl group in the 4-position on the pyridazine ring. This difference, and the greater relative potency of SR95103 on the *Ascaris* GABA receptor, indicates that its structural requirements for antagonists are different to a typical GABA-A receptor. The lack of effect of bicuculline on Ascaris, (Wann, 1987; Holden-Dye et al., 1988), also supports this view. However, the agonist profiles of GABA-A and *Ascaris* receptors show a similar stereo-selectivity (Holden-Dye et al., 1989) so that the structure-activity relationships of agonists are apparently correlated for the two receptor types.

Ariëns et al., (1979), have argued that hydrophobic regions of a competitive antagonist molecule binding to "accessory" sites on the receptor, are responsible for the molecule behaving as a competitive antagonist; and that the structure of the moiety binding to the agonist-recognition site is less critical than for the agonists. The mode of action and structure-activity relationships of arylaminopyridazine-GABA antagonists at the vertebrate GABA-A receptor has also been explained in terms of Ariëns theory (Wermuth et al., 1987). The fact that potencies of vertebrate GABA-A agonists correlate with the potencies of agonists in *Ascaris*, but the potencies of antagonists do not, suggests that there are differences between the "accessory" sites of the GABA-A receptor and the *Ascaris* GABA receptor. The differences in accessory binding sites revealed by the actions of arylaminopyridazine-GABA antagonists, suggests that further exploration of the pharmacology of these compounds on the *Ascaris* GABA receptor may be useful for the design of new anthelminthic agents.

The examination of the antagonism shown by SR95103 was not described by a classical Schild plot with a slope of one. However, this observation cannot be used to exclude a competitive mode of action, since even simple plausible models of agonist and antagonist action predict that the classical Schild plot will not have a slope of unity (Williams et al., 1988). We used a modified Schild plot to describe the data and obtained evidence, which was consistent with two molecules of agonist but one molecule of antagonist interacting with the receptor. Intuititively, this is reasonible, if binding of two molecules

of GABA to different sites are required to open the channel and binding of a single molecule of antagonist at either site would be sufficient to prevent opening.
In the subsequent paper the actions of SR95103 were further explored at the single-channel level.

ACKNOWLEDGEMENTS

This work was supported by the Wellcome trust, SERC and the Harriet Thompson trust.

REFERENCES

Alger B.E. and Nicoll R.A. (1982) Pharmacological evidence for two kinds of GABA receptors on rat hippocampal pyramidal cells studied in vitro. J.Physiol. (London) **328**, 125-141.

Ariëns E.J., Beld A.D., Miranda J.F.R. and Simonis A.M. (1979) The pharmacon-receptor-effector concept. In: The Receptors Vol 1, Plenum press, New York, pp33-91.

Arunlakshana O. and Schild O. (1959) Some quantitative uses of drug antagonists. Br.J.Pharmacol.Chem. **14**, 48-558.

Ault B. and Nadler V. (1983) Differenciation of two bicuculline-insensitive actions of GABA in the rat hippocampal slice. J.Physiol. (London) **345**, 70P.

Bowery N.G., Hill D.R. and Hudson A.L. (1983) Characteristics of GABA-B receptor binding sites on rat whole brain synaptic membranes. Br.J.Pharmacol. **78**, 191-206.

Chambon J.P., Feltz P., Heaulme M., Restle S., Schliechter R., Biziere K. and Wermuth C.G. (1985) Synthesis of SR95103, an arylaminopyridazine derivative of GABA. Proc.Nat.Acad.Sci. USA **82** 1832-1836.

Colquhoun D. (1971) Non-linear curve fitting and the meaning of the best estimate. In: Lectures on biostatistics. Clarendon Press Oxford, pp257-272.

Desarmenien M., Feltz P., Loeffler J.P., Occhipinti G. and Santangelo F. (1982) Multiple GABA receptors on Aδ and C primary neurones in the adult rat. Br.J.Pharmacol. **76**, 289p.

Desarmenien M., Desaulles E., Feltz P. and Hamann M. (1987) Electrophysiological study of SR42641 a novel aminopyridazine derivative of GABA, antagonist properties and receptor selectivity of GABA-A versus GABA-B responses. Br.J.Pharmacol. **90**, 287-298.

Duittoz A.H. and Martin R.J. (1989) SR95103 acts as a GABA antagonist in Ascaris suum muscle. Br.J.Pharmacol. **97**, 490P.

Dunlap K. (1981) Two types of GABA receptors on embryonic sensory neurones. Br.J.Pharmacol. **74**, 579-585.

Dutar P. and Nicoll R.A. (1988) A physiological role for GABA-B receptors in the central nervous system. Nature **332**, 156-158.

Hamann M., Desarmenien M., Desaulles E., Bader M.F. and Feltz P. (1987) Quantitative evaluation of the properties of a pyridazinyl GABA derivative (SR95531), as a GABA-A competitive antagonist. An electrophysiological approach. Brain Res. **442**, 287-296.

Heaulme M., Chambon J.P., Leyris R., Wermuth C.G. and Biziere K. (1986) Specific binding of a phenylpyridazinium derivative endowed with GABA-A receptor antagonist activity to rat brain. Neuropharmacol. **25**, 1272-1283.

Holden-Dye L., Hewitt G.M., Wann K.T., Krogsgaard-Larsen P. and Walker R.J. (1988), Studies involving Avermectin and the 4-aminobutyric acid (GABA) receptor in *Ascaris suum* muscle. Pest.Sci. **24**, 231-245

Holden-Dye L., Walker R.J., Nielsen L. and Krogsgaard-Larsen P. (1989) Stereoselectivity of a bicuculline insensitive GABA receptor in *Ascaris suum* muscle. Br.J.Pharmacol. **98**, 841-849.

Johnston G.A.R. and Allan D.R. (1984) GABA agonists. Neuropharmacol. **23**, 831-832.

Johnston G.A.R. (1986) GABA receptor classification. In: Benzodiazepine/GABA receptors and chloride channels: structural

and functionnal properties, Alan.R.Liss, New York, pp41-56.

Kerr D.I.B., Ong J., Prager R.H., Gythner B.D. and Curtis D.R. (1987) Phaclofen: a peripheral and central baclofen antagonist. Brain Res. **405**, 150-154.

Krogsgaard-Larsen P. (1988) GABA synaptic mechanisms: stereochemical and conformational requirements. Med.Res.Rev. **8**, 27-56.

Levitan E.S., Schofield P.R., Burt D.R., Rhee L.M., Wisden W., Kohler M., Fujita N., Rodriguez H.F., Stephenson A, Darlison M.G., Barnard E.A. and Seeburg P.H. (1989) Structural and functional basis for GABA-A receptor heterogeneity. Nature **335**, 76-79.

Martin R.J. (1980) The effects of γ-aminobutyric acid (GABA) on the input conductance and membrane potential of *Ascaris suum*. Brit.J.Pharmacol. **71**, 99-106.

Martin R.J. (1982) Electrophysiological effects of piperazine and diethyl carbamazine on *Ascaris suum* somatic muscle. Br.J.Pharmacol. **77**, 255-265.

Martin R.J. (1985) γ-aminobutyric acid and piperazine-activated single channel currents from *Ascaris* body muscle. Br.J.Pharmacol. **84**, 445-461.

Martin R.J. (1987), The γ-aminobutyric acid receptor of Ascaris as a target for anthelminthics. Biochem.Soc.Trans. **15**, 61-65.

Michaud J.C., Mienville J.M. and Chambon J.P. (1986) Interaction of three arylaminopyridazine GABA derivatives with rat cortical GABA and glycine receptors: an in vivo microiontophoretic study. Neuropharmacol. **25**, 1197-1203.

Pritchett D.B., Luddens H. and Seeburg P.H. (1989) Type I and type II GABA-A benzodiazepine receptors produced in transfected cells. Science **245**, 1389-1392.

Rang H.P. (1971) Drug-receptor and their function. Nature **231**, 91-96.

Wann K.T. (1987) The electrophysiology of the somatic muscle cells of *Ascaris suum* and *Ascaridia galli*. Parasitol. **94**, 555-566.

Wermuth C.G., Bourguignon J.J., Schlewer G., Gies J.P., Schoenfelder A., Melikian A., Bouchet M.J., Chantreux D., Molimard J.C., Heaulme M., Chambon J.P. and Biziere,K. (1987) Synthesis and structure activity relationships of a series of aminopyridazine derivatives of GABA acting as selective GABA-A antagonists. Journal of Med.Chem. **30**, 239-249.

Williams T.L., Smith D.A.S., Burton N.R. and Stone T.W. (1988) Amino-acid pharmacology in neocortical slices: evidence for bimolecular actions from an extension of the Hill and Gaddum-Schild equations.; Br.J.Pharmacol. **95**, 805-810.

FIGURE LEGENDS

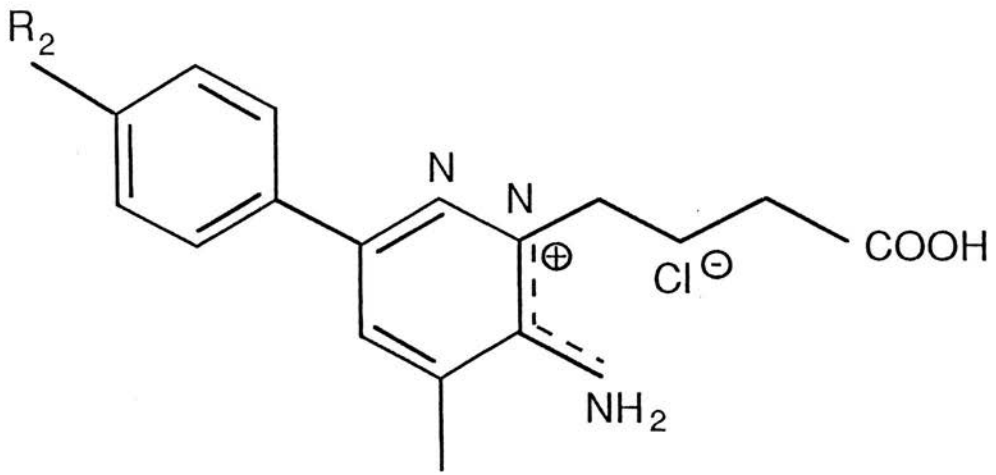
Figure 1: Chemical structure of SR95103 and SR95531
SR95103: R_1=-CH_3 R_2=-H
SR95531: R_1=-H R_2=-OCH_3

Figure 2: GABA cumulative dose-response relationship
The upper trace illustrates the change in input conductance during cumulative application of GABA. The width of the trace represents the change in membrane potential during the injection of hyperpolarizing current pulses (see methods). In this experiment, the resting input conductance was 3.0μS; in the presence of GABA 10μM Δg=0.2μS; in GABA 30μM Δg=1.4μS; in GABA 100μM Δg=3.1μS; in GABA 300μM Δg=3.1μS. the conductance returned to the resting level after washing. The chart speed was altered at the marked intervals during the experiment. The lower diagram shows the dose-response relationship. The experimental values are represented by filled circles ( ); the solid line represents the fitted modified Hill equation (Δgmax=3.5μS; EC50=32.6μM; N=2.6).

Figure 3: SR95103 is a more potent GABA antagonist than SR95531.
A: SR95103. The resting input conductance is 2.2μS. In the presence of GABA 30μM Δg=4.4μS. SR95103 1mM antagonized 86% of the GABA resposne. The antagonism was reversed when the preparation was washed in 30μM GABA.
B: SR95531. SR95531 was tested in the same cell. The resting input conductance was 2.2μS. In the presence of 3μM GABA Δg=4.4μS. SR95531 1mM antagonized only 36% of the GABA response. The antagonism was completely reversed when the preparation was washed in 30μM GABA.

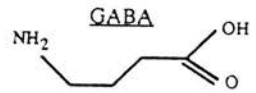
Figure 4: Effect of SR95103 on the cumulative GABA dose-response relationship.
( ): control GABA response and modified Hill fit; Δgmax=5.8μS; EC50=24.4μM; N=1.7.
( ): GABA response in the presence of SR95103 100μM; Δgmax=5.8μS; EC50=66.2μM; N=1.5. Ordinate: change in input conductance. Abscissa: GABA concentration.

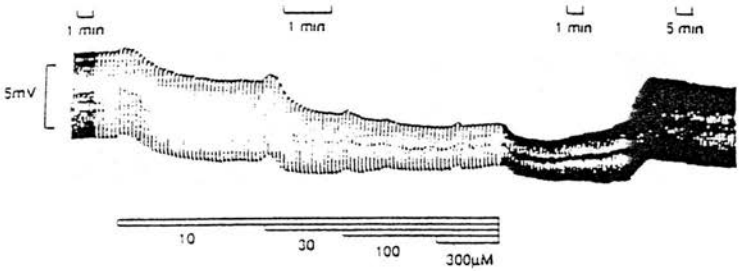
Figure 5: Modified Schild plot: agonist: GABA, antagonist: SR95103.
The plot of log(DR2-1) against logXB appears linear with a slope of 0.62±0.17 (mean±SE, n=14). The linear regression had a F value of 0.0041, indicating a better fit. The slope was not significantly less than 1.0 (t-test, $p \leq 0.05$).

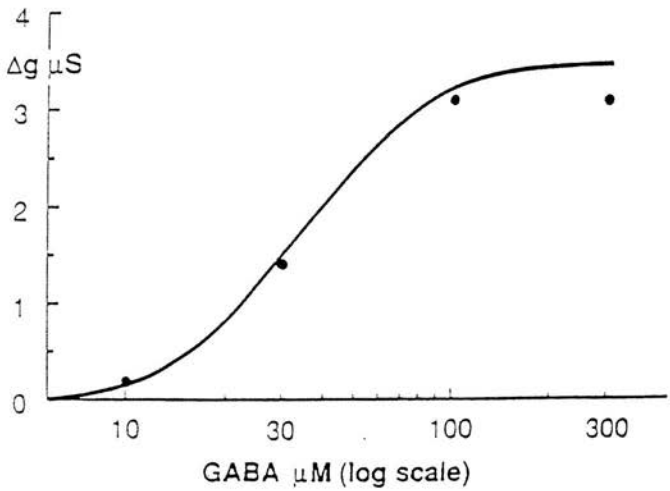


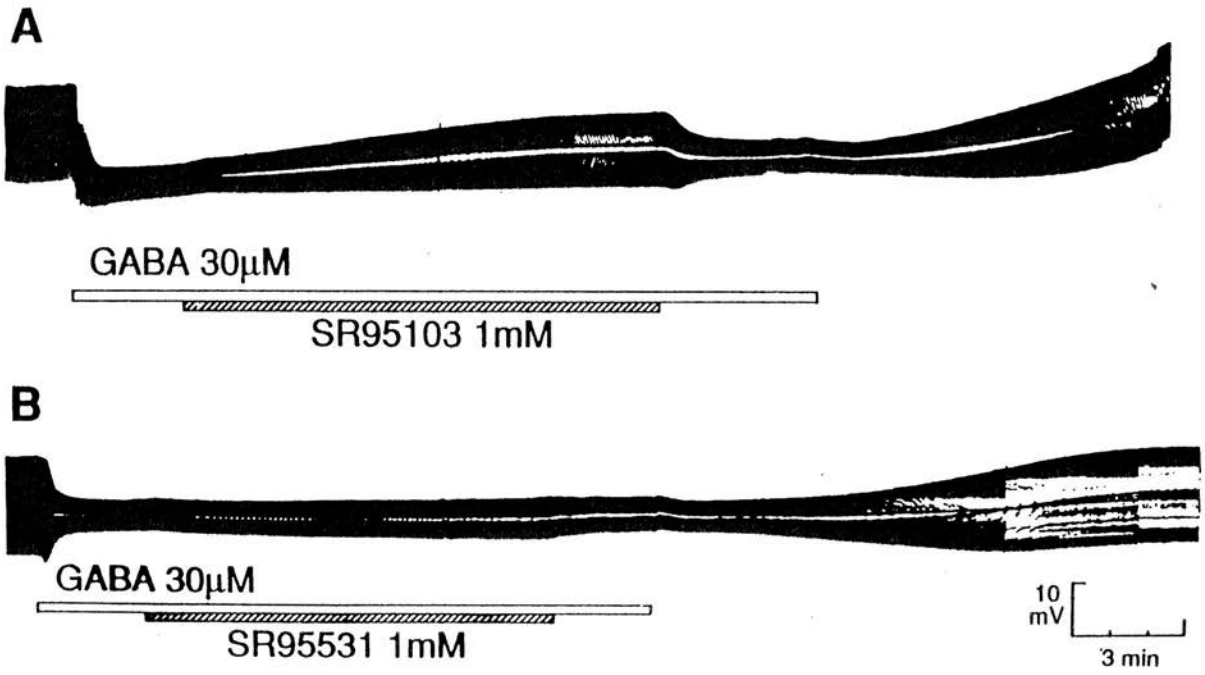
SR95103: $R_1 = CH_3$
$R_2 = H$

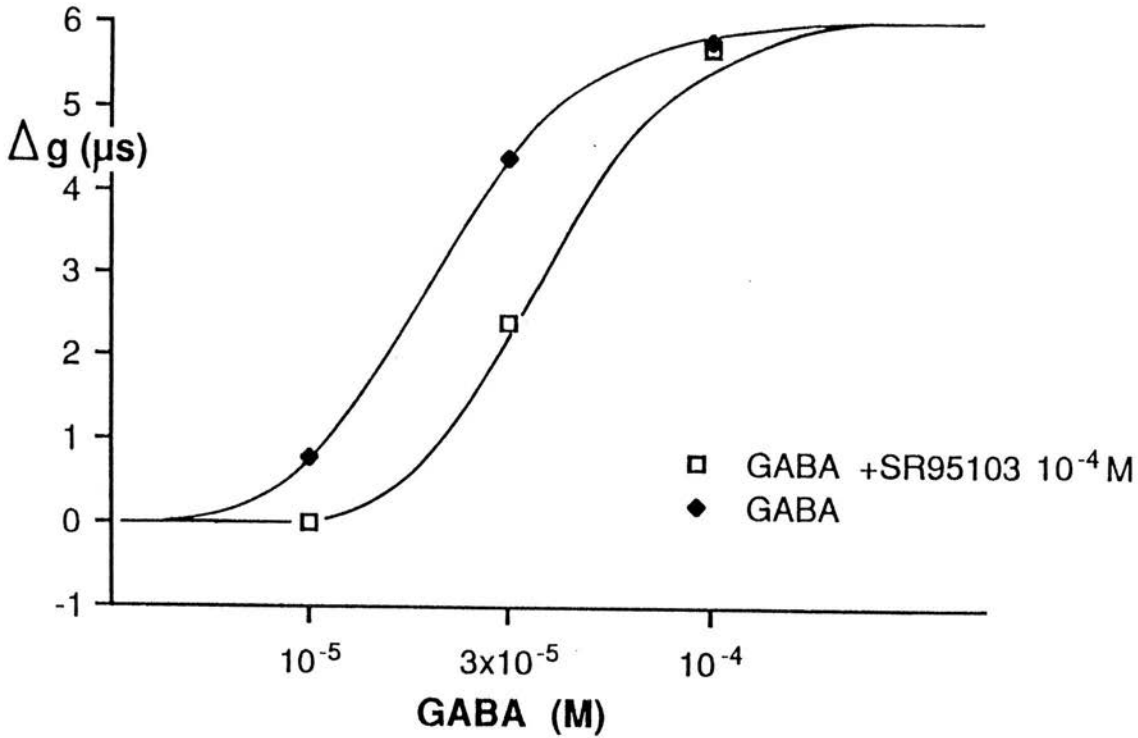
SR95531: $R_1 = H$
$R_2 = CH_3O$


GABA
NH2
OH
O


1 min
1 min
1 min
5 min
5mV
10
30
100
300µM


4
Δg µS
3
2
1
0
10
30
100
300
GABA µM (log scale)


A
GABA 30μM
SR95103 1mM
B
GABA 30μM
SR95531 1mM
10
mV
3 min


6
5
Δg (μs)
4
3
2
1
0
-1
10-5
3x10-5
10-4
GABA (M)
GABA +SR95103 10-4 M
GABA

Modified Schild analysis

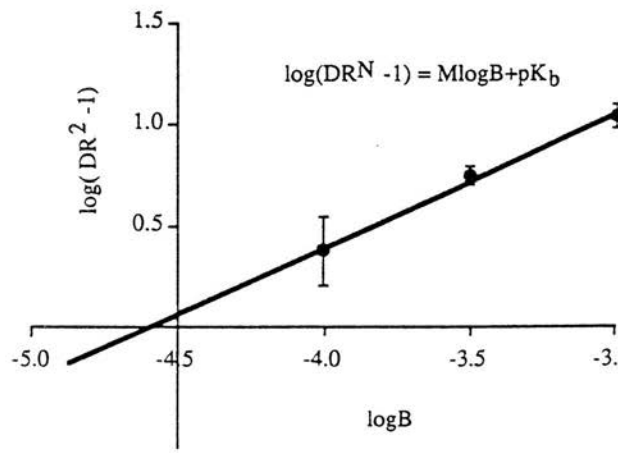


Table 1: SR95103: Effects on GABA dose-response curve parameters

Parameters for the modified Hill equation used to describe cumulative GABA dose-response curves in the presence of SR95103. ** indicates $p \leq 0.05$, T-test

	EC50 (μM)	Δgmax (μS)	N
GABA control (n=8)	24.9±3.3	6.1±0.1	2.6±0.3
GABA + 100μM SR95103 (n=6)	46.5±4.2**	5.1±0.6	2.3±0.3
GABA + 300μM SR95103 (n=3)	81.2±15.3**	5.5±0.6	1.7±0.7
GABA + 1000μM SR95103 (n=4)	119±5.7**	4.5±0.7	2.7±0.6

Duittoz, A.H.
Ph.D. 1991

EFFECTS OF SR95103 ON GABA-ACTIVATED SINGLE-CHANNEL CURRENTS FROM *ASCARIS SUUM* MUSCLE

A.H.Duittoz & R.J.Martin

Department of Preclinical Veterinary Sciences, R.(D).S.V.S, University of Edinburgh, Summerhall, EH1 9QH Edinburgh.

SR95103; Ascaris suum; competitive antagonist; single-channel currents; channel block.

ABSTRACT

1. An outside-out patch-clamp technique was used to examine the actions of SR95103 (30μM to 2mM) on GABA-activated single-channel currents recorded from the bag region of somatic muscle of Ascaris suum.
2. Effects of SR95103 on open-probability and the distribution of open-, closed- and burst durations were examine quantitatively.
3. The mode of action of SR95103 is discussed; it is suggested that most of the antagonism is competitive and produced by a single molecule of SR95103 combining with the GABA-receptor-channel complex; there is an additional small non-competitive component, possibly a channel-blocking action.

INTRODUCTION

In the previous paper, we have described the action of SR95103 on the GABA receptor of *Ascaris suum* muscle under current-clamp. We have shown that SR95103 produces a parallel shift to the right in conductance dose-response curves, but that the classical Schild plot (Arunlakshana and Schild, 1959) had a slope less than one. This obsevation however, did not exclude competitive antagonism since it was possible to use a modified Schild plot (Williams et al., 1988) to describe the observations and which suggested that only one molecule of antagonist interacts with the receptor.
In order to investigate further the action of SR95103 on the Ascaris GABA receptor, a patch-clamp study was carried out to look at effects at the single-channel level. The paper describes the results of those experiments. It shows that most of the antagonism appears to be competitive but evidence for a small non-competitive component is seen at high antagonist concentrations.

MATERIALS AND METHODS

The preparation was maintained in a solution containing (mM): NaCl 35; Na acetate 105; KCl 2; HEPES 10; glucose 3; ascorbic acid 2; pH 7.2 with NaOH. Electrodes were filled with a solution containing (mM): CsCl 145; MgCl2 3; CaCl2 1; EGTA 11; HEPES 10; pH 7.2 with CsOH.

- Recording technique
Standard patch-clamp techniques were used to obtain outside-out patches (Hamill et al., 1981). Patch electrodes were made from micro-haematocrit capillary tubes (Garner glass 7052) and had a resistance of 1-5MΩ. Sylgard™ was used to coat electrodes, to improve the frequency responses (typically greater than 2kHz). Currents were recorded with a List EPC-7 and a modified Sony Betamax video system. An agar-plug electrode made with Agar 150mM KCl set in a polythene tube, was used to minimize junction potentials. Records on tape were played back into a CED 1401 interface, connected to a DCS 286 PC computer and under the control of PAT software (Dr J.Dempster, Strathclyde University).

- Data processing
Records on tape were played back into a CED 1401 interface, connected to a

DCS 286 PC computer and under the control of PAT software (Dr J.Dempster, Strathclyde university).
Records were filtered by a laboratory made 8-pole Bessel filter, -3dB at 1kHz, and sampled at an interval of 100µs. The PAT software was used to obtain current amplitude histograms which were used to measure proportions of the time when 1,2,3,...N channels were open. Records with less than 5% of all openings appearing as simultaneous openings were selected and used to measure channel open- and closed-times; the double openings were edited and not used for analysis. The threshold for detection was set at 50% of the unit channel current. This method of detecting the transition between open- and closed- times is different to that described previously on the same preparation (Martin, 1985). The "effective mean open times" described previously (Martin, 1985) approximates to the mean "long" bursts in this study. The PAT software produced files of open and closed durations, which were transferred to a mainframe computer for further analysis.

- Exponential curve fitting
In these experiments, individual channel activations were interrupted by short closed-times. Bursts of openings were therefore defined as groups of openings separated by closed times shorter than a specified time, T_{crit}. T_{crit} was chosen to make the proportion of long closing misclassified (as short) equal to the proportion of short intervals misclassified (as long), by solving numerically the equation:

$$1-e^{(Tcrit/Ts)} = e^{(-Tcrit/Tm)}$$

(Colquhoun and Sakmann, 1985), where T_s and T_m are short and intermediate time constants in the distribution of all closed times. An interval of 1-2ms was used so that only brief closings were classified as gaps in bursts. After T_{crit} had been determined, the files of open and closed times were rearranged to give files of burst durations.
The next stage in the analysis was the fitting of exponentials to the distributions of closed and burst durations. Only events greater than 0.5ms (twice the dead time) were used to fit 1,2,3 or 4 exponentials to each data file to see which was the best fit. A maximum likelihood procedure was used to estimate the parameters of the probability density function (p.d.f.) which best describes the distributions:

$$\text{p.d.f.} = \sum_{i=1}^{k} \frac{ai}{Ti.e^{(-t/Ti)}}$$

where a_i represents the area of the i^{th} component ($\sum a=1$), T_i is the fitted time constant, t is the time in milliseconds and k is the number of exponentials fitted. A decrease of three log units with each additional component fitted was taken as a significant improvement in the fit.

- Correction for missed events
After the exponentials had been fitted to the burst and closed times, it was possible to correct for missing events, using the method described below (Colquhoun and Sakmann, 1985). It was assumed that very short closings and openings will be missed in the usual analysis of open and closed times because of the limited resolution of the recording technique. The number of short events was estimated by extrapolation from the data which had been recorded.
Firstly, the total number of closed-times and burst-times was estimated from the observed number of events; this was done separatly for the closed-times and burst-times. Secondly, the total time spent in gaps within bursts i.e., gaps shorter than Tcrit, including those which are undetected, was calculated. Thirdly, the corrected total open-time was calculated. The corrected mean open-time estimated in this way is shorter than the mean open-time estimated in another way because it allows for missed short events and reduces the mean value. The corrected mean lenght of an individual opening in a "long" burst was then calculated assuming that there were few gaps in the "short" bursts.

- Stationarity
It was possible to examine open-times and closed-times in sequence and, to test for changes in their properties with time. Desensitization for example, may

produce after a delay, an increase in the mean closed-time and a reduction in the probability of the channel being open. CUSUM plots (see Glasbey and Martin, 1986) were used to examine for this and test for time-dependent changes in channel durations (non-stationarity). An example of these plots is shown in Figure 1; it can be seen that there is no evidence of change in channel properties throughout the record. This and similar observations on other patches, showed no evidence of changes in channel properties throughout the records.

- Binomial distribution

If channels in a patch have the same probability of being open and are independent, then the distribution of the proportion of time, P_r, that 0,1,2,...N, channels are open will be described by the binomial distribution:

$$P_r = \frac{N!}{r!(N-r)!p^r(1-p)^{N-r}}$$

where p is the probability that an individual channel is open and N is the number of channels present. The probability (p) of an individual channel being open was calculated from the areas under the amplitude histograms. If the proportions of total recording time spent with 1,2,....N, channels open were $T_1, T_2, ...T_N$ then:

$$p = \frac{T_1+2T_2+...+NT_N}{N}$$

The fraction of time which r channels and only r channels were open was calculated from the T values. The binomial distribution was the used with the value of p to predict the proportion of time with only 0,1,2,...N channels open.

- Percent antagonism

In order to compare the level of antagonism produced by different concentrations of SR95103, we defined the percentage of antagonism as:

$$\text{percent antagonism} = 100x(1-\frac{p_{SR}}{p_C})$$

where p_{SR} was the probability of an individual channel being open in the presence of SR95103 and p_C is the control probability in the absence of antagonist. The relationship between percent antagonism and SR95103 concentration was described by the modified Hill equation and a least squares estimate of the parameters made as described in the preceeding paper.

- t-test

The mean and standard errors for these observations were calculated and examined with a two-tailed unpaired t-test, to determine if effects reached statistical significance.

RESULTS

The following report is based on the analysis of experiments on 11 outside-out patches prepared from separate parasites.

- Single-channel relationship

Non-symmetrical Cl concentrations across the patch (41mM outside, 153mM inside) were used throughout this series of experiments to increase the amplitude of the single channel currents (at -50mV) and to further test the identity of the ions flowing through the channels. Figure 2 shows a representative I/V plot of the main (most frequent) conductance state seen in a particular patch. The plot shows inward rectification and a reversal potential of +27mV; the rectification and a reversal potential close to this is predicted by the Goldman constant field equation (Hille, 1984) for a Cl ion-channel under these conditions. Figure 2 also shows (solid line) the I/V relationship predicted by the constant field equation for a Cl channel with a permeability of 6.3 $10^{-14}cm^3s^{-1}$.

- Main-state conductance

GABA channels in *Ascaris* are characterized by the presence of more than one conductance state (Martin, 1985). The main state at -50mV had an amplitude of 1.7pA, which corresponds to a chord conductance of 22pS, if a driving force of 77mV is assumed. The two subconductance states had amplitude of 1.0 and 0.5pA at -50mV (conductances of respectively 13 and 6.5pS). In the following analysis, the main state conductance was used because it occurred most frequently; the channel current spent only a very small proportion of the open-time at the subconductance levels. The inclusion of subconductance states in the analysis

was further minimized by threshold setting and editing record manually.

- Channels have the same P_{open} and operate independently

The application of 3μM GABA to outside-out patches produced the simultaneous opening of up to three channels. If GABA channels in a patch have the same probability of being open and are independent, then the distribution of the proportion of time, P_r, that 0,1,2,...N, channels are open will be described by the binomial distribution. Figure 3 shows predicted and observed values of P_r from a typical patch record with three channels present, and p=0.082. It can be seen that observed and predicted values are close; this supports the hypothesis that the channels behave independently and have the same open probability. Similar results were obtained from all the patches.

As a consequence of these observations, the GABA channels were assumed to be homogenous; effects of SR95103 were not subsequently explained by a selective action on a group of GABA channels which have long open-times and a different open probability.

- SR95103 reduces GABA channel opening frequency but not amplitude

Figure 4 shows the inhibitory effect of SR95103 on the opening frequency of GABA-activated channels at -50mV. Figure 4A shows the effect of 3μM GABA alone, where channel opening is seen, at this time scale, as clusters of inward currents spikes. When 30μM SR95103 was added, the frequency of opening reduced from $0.7s^{-1}$ to $0.35s^{-1}$. Figure 4B. The rate of channel opening was further reduced to less than $0.05s^{-1}$, when SR95103 was increased to 100μM, Figure 4C. When the antagonist was washed from the preparation, the opening frequency returned to $0.5s^{-1}$, Figure 4D.

The lack of effect of SR95103 on the amplitude of GABA single-channel currents is illustrated in Figure 5 which shows the current-amplitude histograms for the traces shown in Figure 4. The current-amplitude histograms were fitted and described by Gaussian distributions. The control amplitude (Figure 5A) was -1.74±0.02pA (mean±SE); inthe presence of 30μM antagonist (Figure 5B) it was 1.67±0.04pA (mean±SE); in the presence of 100μM antagonist (Figure 5C) it was -1.72±0.03pA (mean±I‰). Similar results were obtained from all the other experiments; it was concluded that SR95103 does not affect single-channel conductance.

- Modified Hill equation and percent inhibition

The relative area under the open-channel-current histogram allows estimation of the proportion (p) of time an individual channel is open in the patch (see above). In Figure 5A for example, the control p was 0.036; in 5B it was 0.025; in 5C it was 0.001. Figure 6 shows the dose-response relationship for the percent antagonism and the fitted modified Hill equation. With 3μM GABA as the agonist, the Hill coefficient was 0.98, suggesting that only one molecule of SR95103 combines with the GABA receptor; the concentration producing half saturation (IC50) was 24μM and the estimated percent inhibition at saturating concentrations of antagonist was 97%. The fact that the maximum antagonism is near 100% suggests that most, but perhaps not all of the antagonism is produced by an action at a single binding site.

- Effects of SR95103 on burst-distributions

Figure 7 illustrates representative channel currents at a high time-resolution and shows the effect of SR95103 on these currents: Figure 7A shows example of currents in the absence of antagonist; Figure 7B shows bursts in the presence of 100μM SR95103; Figure 7C shows the shorter bursts seen in the presence of 100μM SR95103; Figure 7D shows the channel bursts after washing. The main apparent effect was a reduction in the mean burst length at the 100μM concentration of antagonist. The effect reversed after SR95103 was washed off.

The effects of SR95103 on burst-times were further explored by examining the burst-time distributions. Figure 8

shows burst-time frequency histograms, from a representative experiment where the effect of 30μM, then 100μM SR95103, on the distribution of channel bursts produced by GABA 3μM was examined. Inspection of these histograms shows that the effect of SR95103 is to produce an incrase in the proportion of "brief" bursts. This effect was described quantitatively by fitting exponential distributions.The control distribution (Figure 8A) was best described by the sum of two exponentials:

$$pdf_{burst} = \frac{0.57}{3.1} e^{(-t/3.1)} + \frac{0.43}{33} e^{(-t/33)}$$

where pdf_{burst} is the burst probability density function and t is time in milliseconds. Thus 57% of the bursts were of the "short" type with a mean duration of 3.1,s; the "long" type of burst had a mean duration of 33.2ms. The short bursts appeared to be mostly single short openings, whereas the long burstsa were mostly multiple openings. The effect of SR95103 30μM on this distribution was small (Figure 8B), in contrast to the effect of SR95103 100μM (Figure 8C), where the burst distribution was best fitted by the equation:

$$pdf_{burst} = \frac{0.82}{3.1} e^{(-t/3.1)} + \frac{0.18}{41.9} e^{(-t/41.9)}$$

The main effect was to increase the proportion of short bursts, in this experiment from 57 to 82%. Similar results were obtained in other experiments and are summarized in Table 1. The The table shows that there is a statistically significant increase in the proportion of short bursts with 100μM but not 30μM SR95103, and that there is no significant effect on the mean duration of the short or long bursts at either concentrations. It is evident that the increase in the proportion of short bursts in presence of 100μM SR95103, is responsible for the reduction in mean burst duration.

It has been shown (above), that a high concentration of SR95103 increase the proportion of short bursts; it was of interest to determine if an increase in agonist concentration could reverse this effect. In one patch 60μM SR95103 was used to produce an increase in the proportion of short bursts from 25% to 83%; an increase in GABA concentration from 3 to 6μM then, partially reversed the effect of the antagonist and decreased the proportion of short bursts from 83 to 60%. Further details of these results are shown in Table 2.

- Effects of SR95103 on closed-time distributions

Inspections of Figure 4 shows that the antagonism of SR95103 was associated with an increase in long closed-times. The effects of SR95103 on closed-times distributions were examined by fitting exponentials to describe the p.d.f. Control GABA 3μM closed-time distributions were best described by three exponentials. In one representative patch the result was:

$$p.d.f._{closed} = \frac{0.29}{0.7} e^{(-t/0.7)} + \frac{0.16}{6.0} e^{(-t/6.0)} + \frac{0.55}{63} e^{(-t/63)}$$

where $p.d.f._{closed}$ is the closed time probability density function and t the time in ms. This result is comparable to that reported previously (Martin, 1985), and shows that there are at least three closed states giving rise to the "brief" (mean duration 0.7ms), "intermediate" (mean duration 6.0ms) and "long" (mean duration 63ms) types of closings. the effect of 30 and 100μM SR95103 on the distribution of closed times of this patch was to increase the duration of the intermediate and long closings. The closed time distribution for the same patch with 30μM SR95103 was best described by:

$$p.d.f._{closed} = \frac{0.2}{1.7} e^{(-t/1.7)} + \frac{0.36}{15.4} e^{(-t/15.4)} + \frac{0.54}{133} e^{(-t/133)}$$

The mean closed time increased from 35.8ms to 77.7ms; the mean duration of the intermediate closings increased from 6 to 15.4ms and the mean duration of the long closings increased from 63 to 133ms. At 100μM, the mean closed time increased to 99.6ms; the mean intermediate closed time to 21.8ms and the mean long closed time to 215ms. The closed time distribution was best described by:

$$p.d.f._{closed} = \frac{0.34}{0.7} e^{(-t/0.7)} + \frac{0.22}{21.8} e^{(-t/21.8)} + \frac{0.44}{215} e^{(-t/215)}$$

No increase in the number of components was required to describe the closed times distributions in this or other patches; therefore no increase in the number of closed states in presence of the antagonist was detected. The effects of 30μM and 100μM SR95103 on closed-time distributions, from the other patches, was qualitatively similar, and was characterized by an increas in the duration of intermediate and/or long closings. However, there was a large variation in mean estimates for the intermediate closings and long closings, possibly due to the variation in the number of channel present in the patch. In contrast, SR95103 had no consistent effect on the duration of the brief closings; Table 1 shows mean values for the brief closings seen in the different patches.

- Effects of SR95103 on corrected mean open-times

One difficulty in measuring open times directly is that unresolved brief closings lead to bursts appearing as long single openings (see Colquhoun and Hawkes, 1982). This artefact increases the observed open times. However it is possible to compensate for these unresolved closings by estimating the true number of closings by extrapolating from the closed time distributions. The estimated total open time is divided by the true number of closings to obtain the mean open time (see methods). The control mean open time with GABA 3μM for one typical patch was 11.9ms; this was unchanged in presence of SR95103 30μM : 12.0ms; and reduced to 5.8ms in presence of SR95103 100μM. Table 1 shows the mean±SE values obtained from the different patches and shows that there is a significant decrease in the corrected mean open time in presence of 100μM antagonist but not at 30μM.

DISCUSSION

This patch-clamp study of SR95103 was carried out to further investigate the mode of antagonism. The percent inhibition dose-response curve was described by the modified Hill equation with a coefficient close to one. A lower antagonist concentration (30μM), reduced the probability of the channel being open without significantly affecting the burst distribution, corrected mean open-time or channel conductance; only the mean duration of the two slower closed-time components was increased. A higher (100μM) antagonist concentration, further decreased the probability of channel opening, but significantly increased the proportion of short bursts. In the subsequent discussion, it is suggested that SR95103 acts mostly as a simple competitive antagonist; but at higher concentrations there is an additional small non-competitive action, possibly channel block.

- A possible kinetic scheme for the antagonism

A variety of patch-clamp studies in different preparations have been carried out on GABA-activated channels where the open-time distributions were described by two exponentials and the closed-times distributions described by three exponentials (Ascaris, Martin 1985; mouse spinal neurones, Jackson et al. 1982, Sackmann et al. 1983, Mathers 1985; chick cerebral neurones, Weiss and Magleby 1989; and bovine chromaffin cells, Bormann and Clapham 1985). More recently, however, MacDonald et al. (1989) have described an additional open-time component, which suggests a greater complexity. Molecular biology studies on the GABA channel structure (Schofield et al., 1987) have shown that the receptor-channel complex is formed by two α and two β protein transmembrane subunits. The β subunits were shown to bind GABA (Barnard et al., 1986) so it was concluded that two agonist molecules combine with the receptor-channel complex to bring about opening. However, in contrast to GABA the results of the current-clamp experiments presented in the previous paper, and the percent inhibition dose-response curve presented here, suggest that one molecule of SR95103 is responsible for most of the antagonism.

Figure 9 shows a minimal kinetic scheme which may explain these observations. It predicts (Colquhoun and Hawkes, 1977; 1982) in the

absence of antagonist, three components to the closed-time distribution and two components to the open- and burst-time distributions. However in the presence of the antagonist, an extra closed state should give rise to an extra component in the closed-time distribution. Our experiments did not reveal an increase in the number of components in the closed-time distributions. But the long duration of many closed-times, seen in the presence of the antagonist, reduced the number of events available for analysis and the ability to separate components. There was also the further complication of different numbers of channels present in each patch; this complication gave rise to considerable variation in estimates of the mean duration of "intermediate" and "long" closed-times. Both of these factors would make the detection of any extra long closed-states in the presence of the antagonist difficult.

- Low but not high concentrations of SR95103 appear to be competitive

Simple competitive antagonism predicts no change in the channel mean open-time or burst distribution. In our experiments there was no significant effect of 30μM SR95103 on corrected mean open-times or on the burst distribution, so it is argued that this concentration of antagonist behaves like a competitive antagonist. The effects of 100μM however, were not compatible with just simple competitive antagonism, because they were associated with reductions in corrected mean open-times and a significant increase in the proportion of short bursts.

- Possible mode of action for SR95103

A variety of experimental preparations have been used in to look at the action of arylaminopyridazine GABA derivatives on the vertebrate GABA-A receptor. Binding studies (Chambon et al., 1985; Heaulme et al., 1986a; 1986b) and electrophysiological studies (Chambon et al., 1985; Michaud et al., 1986; Desarmenien et al., 1987) have indicated that arylaminopyridazine derivatives of GABA can act as simple competitive antagonists.

SR95531 (a close derivative of SR95103) has been examined at the single-channel level, in bovine chromaffin cells (Hamann et al., 1988); SR95531 reduced the open propbability of GABA channels without affecting open and burst kinetics, an effect compatible with competitive mechanism. However, it was suggested that an additional channel-block might occur at higher concentrations, but the potent competitive action of the analogue did not allow further analysis. A small inhibitory effect of SR95103 on the glycine receptors, as well as the GABA receptors, of rat cortical neurones has been described by Michaud et al. (1986), but not for other pyridazinyl GABA derivatives. The GABA- and glycine-activated channels both conduct Cl so that the pore of the channel might be a common site of action. These two reports raise the question of a channel-block mechanism in addition to competitive antagonism for SR95103.

- Does SR95103 act as a channel-blocker?

A simple channel-block mechanism (see Colquhoun and Hqwkes, 1983) predicts, at the current-clamp level, a reduction in the maximum response and a reduction in the slope of dose-response curves at higher antagonist concentrations; we did not observe such an effect with up to 1mM SR95103 (previous paper). At the single-channel level, the effect of channel-block on the burst duration depends on the duration of the blocked state. If the blocked state is brief (less than T_{crit}), then the burst duration and the number of gaps per burst would increase; we did not observe these effects. However, if the blocked state weer longer than T_{crit}, then the observed mean long burst duration would get shorter and the mean open-time in the long bursts would decrease. Although the observed reduction in the mean duration of long bursts did not reach statistical significance, Table 1, there was a significant reduction in the corrected mean open-times in the long bursts.

We assumed that all brief closings occur in the long bursts and then calculated the mean duration of openings in the long bursts (see

methods). Figure 10 shows the observed linear relationship between the reciprocal of this duration and the concentration of SR95103. The reciprocal of this duration will be equal to the sum of the rate constants leading from the long open state Figure 9. The slope of the plot in Figure 10, is then an estimate of the channel blocking rate constant; it was $1.44M^{-6}s^{-1}$.

Although SR95103 may act in part as a channel-blocker, this cannot play a big role in the antagonism of GABA conductance responses because high concentrations of SR95103 did not significantly depress the maximum response seen in current-clamp experiments. Thus, for example, if the mean blocked state lasted 10ms and the blocking rate, k_{+BL} was $1.44M^{-6}s^{-1}$ (see above) then with 3μM GABA and 100μM SR95103, we have estimated that the proportion of time the channel spends in the blocked state is only near 1%. The inclusion of a blocked state would produce a reduction of less than 2% in Popen when compared to a similar model without blocking. This estimate was based on an anlysis of kinetic schemes with plausible rate constants. The illustration shows that such a channel-block would not be detectable at the current-clamp level.

It is concluded that SR95103 acts like a competitive antagonist at low concentrations, but at higher concentrations, there is an additional non-competitive element; this has some properties of channel-block.

ACKNOWLEDGEMENTS

This work was supported by the Wellcome trust, SERC and the Harriet Thompson trust.

REFERENCES

Arunlakshana O. and Schild O. (1959) Some quantitative uses of drug antagonists. Br.J.Pharmacol.Chem. **14**, 48-558.

Barnard E.A., Darlison M.G. and Seeburg P. (1987) Molecular biology of the GABA-A receptor: the receptor channel superfamily. TINS; **10**, 502-509.

Bormann J. and Clapham D.E. (1985) γ-aminobutyric acid receptor channels in adrenal chromaffin cells: a patch-clamp study. Proc.Nat.Acad.Sci.USA **82**,2168-2172.

Chambon J.P., Feltz P., Heaulme M., Restle S., Schliechter R., Biziere K. and Wermuth C.G. (1985) Synthesis of SR95103, an arylaminopyridazine derivative of GABA. Proc.Nat.Acad.Sci. USA **82**, 1832-1836.

Colquhoun D. and Hawkes A.G. (1977) Relaxation and fluctuations of membrane currents that flow through drug operated channels. Proc.R.Soc. B **199**, 231-262.

Colquhoun D. and Hawkes A.G. (1982) On the stochastic properties of bursts of single ion channel openings. Proc.R.Soc. B **211**, 205-235.

Colquhoun D. and Sakmann B. (1985) Fast events in single channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. J.Physiol. (London) **369**, 501-577.

Desarmenien M., Desaulles E., Feltz P. and Hamann M. (1987) Electrophysiological study of SR42641 a novel aminopyridazine derivative of GABA, antagonist properties and receptor selectivity of GABA-A versus GABA-B responses. Br.J.Pharmacol. **90**, 287-298.

Glasbey C. and Martin R.J. (1986) Exploratory and confirmatory plots of single channel records. J.Neurosci.Meth. **16**, 239-249.

Hamann M., Desarmenien M., Desaulles E., Bader M.F. and Feltz P. (1987) Quantitative evaluation of the properties of a pyridazinyl GABA derivative (SR95531), as a GABA-A competitive antagonist. An electrophysiological approach. Brain Res. **442**, 287-296.

Hamill O.P., Marty A., Neher E., Sakmann B. and Sigworth,F.J. (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membranes patches. Pfl.Arch.Ges.Physiol. **391**, 85-100.

Heaulme M., Chambon J.P., Leyris R., Molimard J.C., Wermuth C.G. and Biziere K. (1986a) Characterization of three arylaminopyridazine GABA derivatives with the GABA-A receptor site. Brain Res. **384**, 224-231.

Heaulme M., Chambon J.P., Leyris R., Wermuth C.G. and Biziere K. (1986b) Specific binding of a phenylpyridazinium derivative endowed with GABA-A receptor antagonist activity to rat brain. Neuropharmacol. **25**, 1272-1283.

Hille B. (1984) Ionic channels of excitable membranes. Sinauer Sunderland Massachusetts pp226-248.

Jackson M.B., Lecar H., Mathers D.A. and Barker J.L. (1982) Single-channel currents activated by γ-aminobutyric acid, muscimol and (-)pentobarbital in cultured mouse spinal neurons. J.Neurosci. **2**, 889-894.

MacDonald R.L, Rogers C.J. and Twyman R.E. (1989) Kinetic properties of the GABA-A receptor main conductance state of mouse spinal cord neurones in culture. J.Physiol.(London) **410**, 479-499.

Martin R.J. (1985) γ-aminobutyric acid and piperazine-activated single channel currents from *Ascaris* body muscle. Br.J.Pharmacol. **84**, 445-461.

Mathers D.A. (1985) Spontaneous and γ-aminobutyric acid induced single-channel currents in cultured murine spinal cord neurons. Can.J.Physiol.Pharmacol. **63**, 1228-1233.

Michaud J.C., Mienville J.M. and Chambon J.P. (1986) Interaction of three arylaminopyridazine GABA derivatives with rat cortical GABA and glycine receptors: an in vivo microiontophoretic study. Neuropharmacol. **25**, 1197-1203.

Sakmann B., Hamill O.P. and Bormann J. (1983) Patch-clamp measurements of elementary chloride currents activated by the putative inhibitory transmitters GABA and glycine in mammalian spinal neurons. J.Neural Transmission Suppl. **18**, 88-95.

Schofield P.A., Darlison M.G., Fujita N., Burt D.R., Stephenson F.A., Rodriguez H., Rhee L.M., Ramachandron J., Reale V., Glencorse T.A., Seeburg P.H. and Barnard E.A. (1987) Sequence and functional expression of the GABA-A receptor shows a ligand gated receptor superfamily. Nature **328**, 221-227.

Weiss D.S. and Magleby K.L. (1989) Gating schemes for single γ-aminobutyric acid activated chloride channels determined from stability plots, dwell time distribution and adjacent interval deviations. J.Neurosci. **9**, 1314-1324.

Williams T.L., Smith D.A.S., Burton N.R. and Stone T.W. (1988) Amino-acid pharmacology in neocortical slices: evidence for bimolecular actions from an extension of the Hill and Gaddum-Schild equations.; Br.J.Pharmacol. **95**, 805-810.

FIGURES LEGENDS

Figure 1: CUSUM plots of closed and open times.

The CUSUM (for closed times), $Sj = \sum_{i=1}^{j} X_i - X_{mean}$, for j=1,...n; where X_i is the rank order of the ith closed time, *Xmean* is the mean rank. A random fluctuation about zero is expected for channels which show stationary behaviour. The significance of the deviation may be tested (Glasbey and Martin, 1986) by determining D_α, the maximum values of $\frac{Sj}{(1+(a-1).\frac{j}{n})}$; where α=9, 1, and 1/9 and then estimating the three p values for the maximun deviations:

$$p=2\exp.\frac{-24\alpha D_\alpha^2}{n^2.(n+1)}$$

This is also repeated for the open-times. A: closed times; ordinate, CUSUM; abscissa, observation number. The p values were 0.17; 0.95; and 0.13; the closed times were considered to be in random order. B: open times; ordinate, CUSUM; abscissa, observation number. The p values were 0.12; 0.89; and 0.39; the open times were considered to be in random order.

Figure 2: Main state GABA channel I/V relationship. A: Channel openings to the main state at four transpatch potentials from +50 to -50mV. B: Plot of main state channel currents at different potentials. The reversal potential was near +27mV the Nerst Cl potential. The solid line shows a plot of the I/V relationship predicted by the Goldman-Hodgkin-Katz constant field equation with a single permeability of $6.3x10^{-14}cm^3s^{-1}$. Note the inward rectification.

Figure 3: Observed and binomial distribution of the proportion of time channels were, or predicted to be, open in a patch.
The horizontal bars are the observed values of the % time spent with 0,1,2, and 3 channels open. The arrows indicate the values predicted by the binomial distribution with n=3, the total number of channels present in the patch, and p=0.082, the probability of channel opening.

Figure 4: Inhibitory effect of SR95103 on opening frequency of GABA channels.
A: Control GABA 3μM; P_{open}=0.036. B: GABA 3μM + SR95103 30μM; P_{open}=0.007. C: GABA 3μM + SR95103 100μM; P_{open}=0.001. D: wash; GABA 3μM alone. SR95103 reduces the opening frequency of GABA channels.

Figure 5: GABA channel current-amplitude histograms and the lack of effect of SR95103.
A: Control GABA 3μM; I=-1.74±0.02pA (mean±SE). B: GABA 3μM +SR95103 30μM; I=-1.64±0.04pA (mean±SE). C: GABA 3μM + SR95103 100μM; I=-1.72±0.03pA (mean±SE).

Figure 6: Percent inhibition dose-response curve.
Ordinate: percent inhibition (see methods) in the presence of 3μM GABA. Abscissa: SR95103 concentration. The line fitted was a least-squares estimate of parameters for the modified Hill equation: maximum percent inhibition, 97%; IC50, 24μM; N, 0.98.

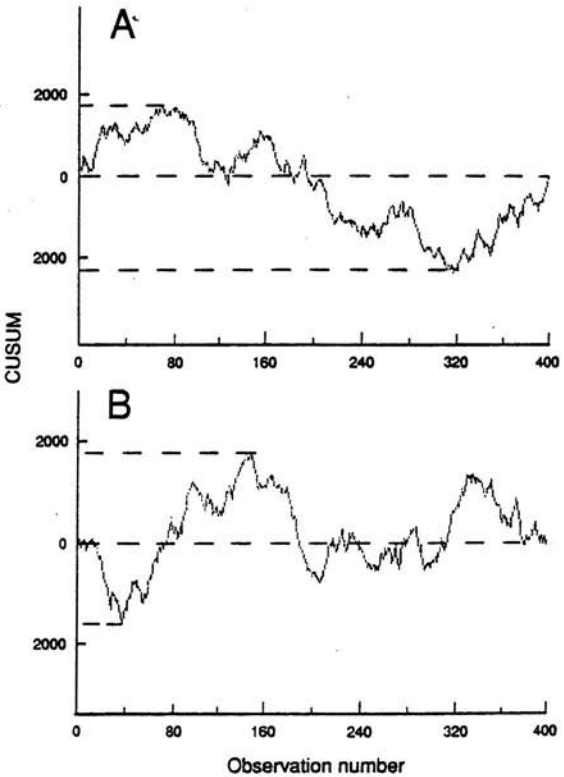
Figure 7: Effects of SR95103 on channel currents seen at high time resolution.
A: Control; GABA 3μM. B: GABA 3μM + SR95103 30μM. C: GABA 3μM + SR95103 100μM. D: wash; GABA 3μM alone. Note the short bursts in C.

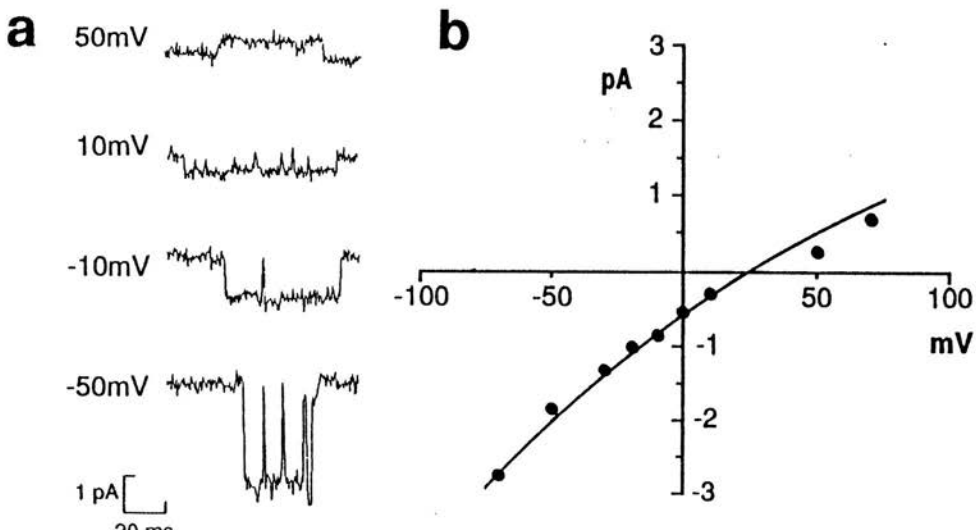
Figure 8: Frequency histograms showing the effect of SR95103 on channel burst distribution.

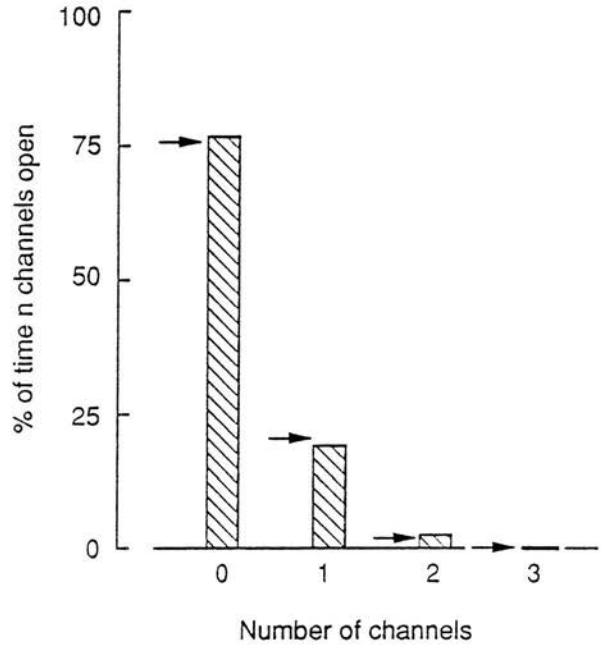
A: Control GABA 3μM; p.d.f. a_1=0.56, T_1=3.1ms,a_2=0.44, T_2=33.2ms; mean burst duration=16.1ms. B: GABA 3μM + SR95103 30μM; p.d.f. a_1=0.30, T_1=1.6ms, a_2=0.70, T_2=26.7ms; mean burst duration=19.4ms. C: GABA 3μM +SR95103 100μM; p.d.f. a_1=0.82, T_1=1.6ms, a_2=0.18, T_2=41.9ms; mean burst duration=10.2ms.

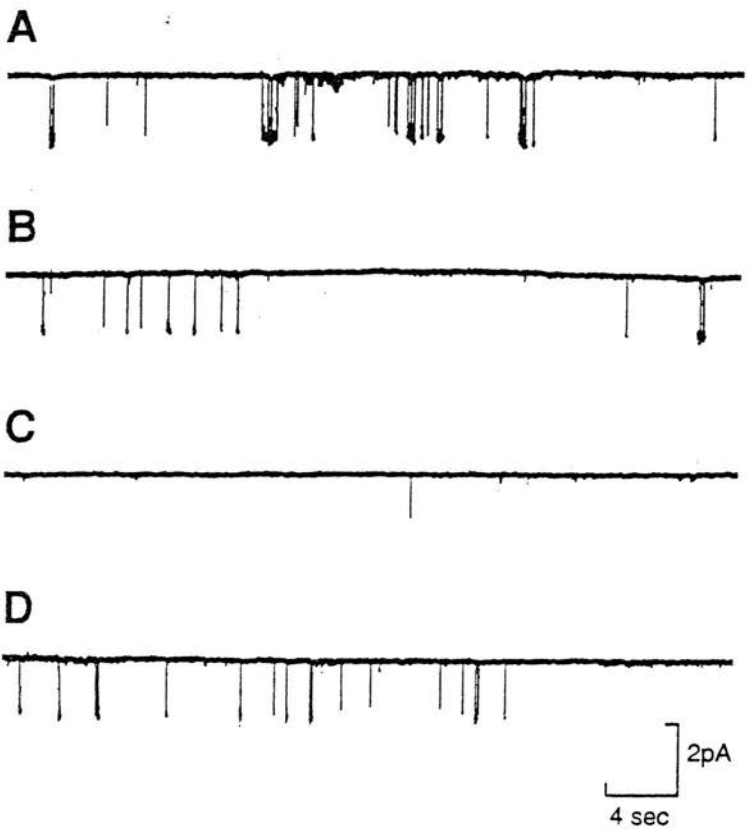
Figure 9: Minimal kinetic scheme for agonist and antagonist action.
The receptor-channel complex (R) has two sites to which the agonist (A), but one site to which the antagonist (B) combines. Open states (*) may occur only after isomerization of the single-bound or double-bound agonist receptor-channel complex. The dissociation constant for the first agonist molecule to combine (K_{D1}) is much larger than for the second agonist molecule (K_{D2}); the dose-response relationship would then be approximated by the modified Hill equation with N=2. The channel closing rates are $\alpha 1$ and $\alpha 2$. $\alpha 1$ is larger than $\alpha 2$ so that RA_2^* is the long open state. the channel opening rates are $\beta 1$ and $\beta 2$. The antagonist dissociation constant is K_{B1}. The putative blocked channel state (RA_2^{BL}) is also shown in brackets.

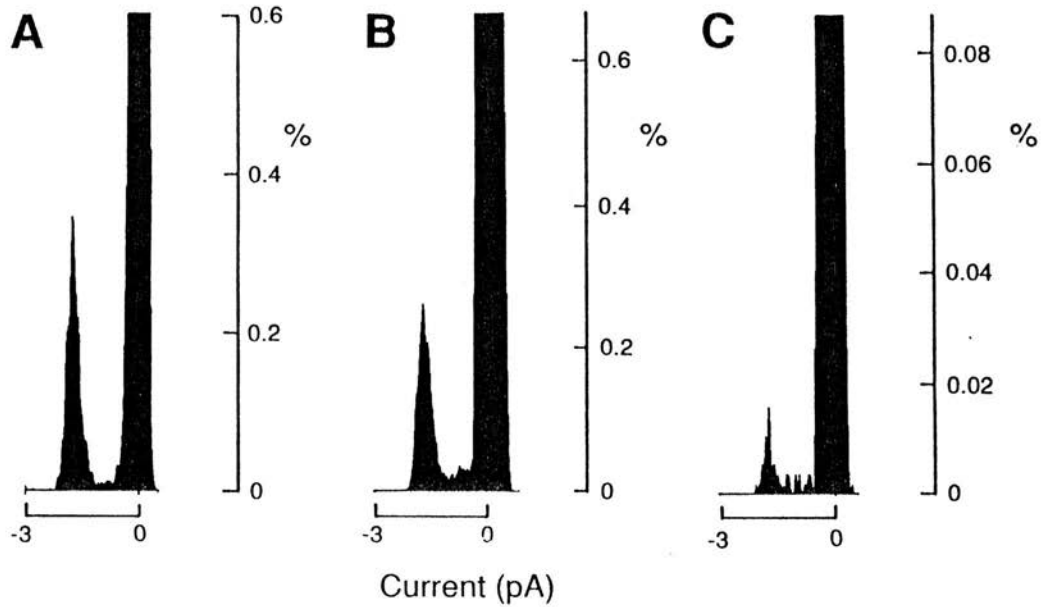
Figure 10: Effects of SR95103 on mean open-times in long bursts.
Ordinate: Reciprocal of mean open times in long bursts. Abscissa: Concentration pf SR95103. The slope (obtained by least squares regression) was $1.44M^{-6}s^{-1}$ and could be interpreted as the channel blocking rate at -50mV.

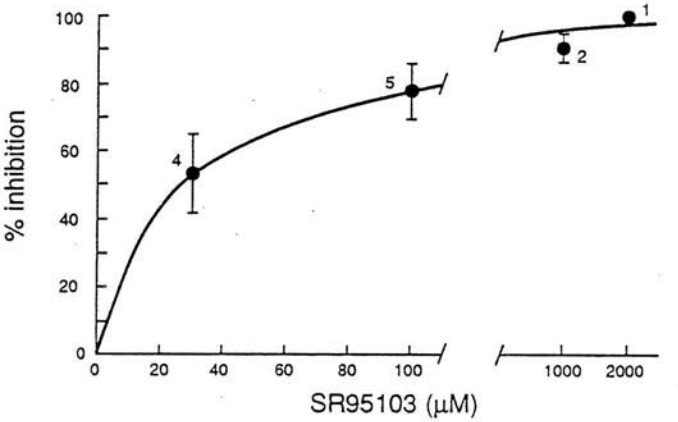

A
2000
0
2000
CUSUM
0
80
160
240
320
400
B
2000
0
2000
0
80
160
240
320
400
Observation number

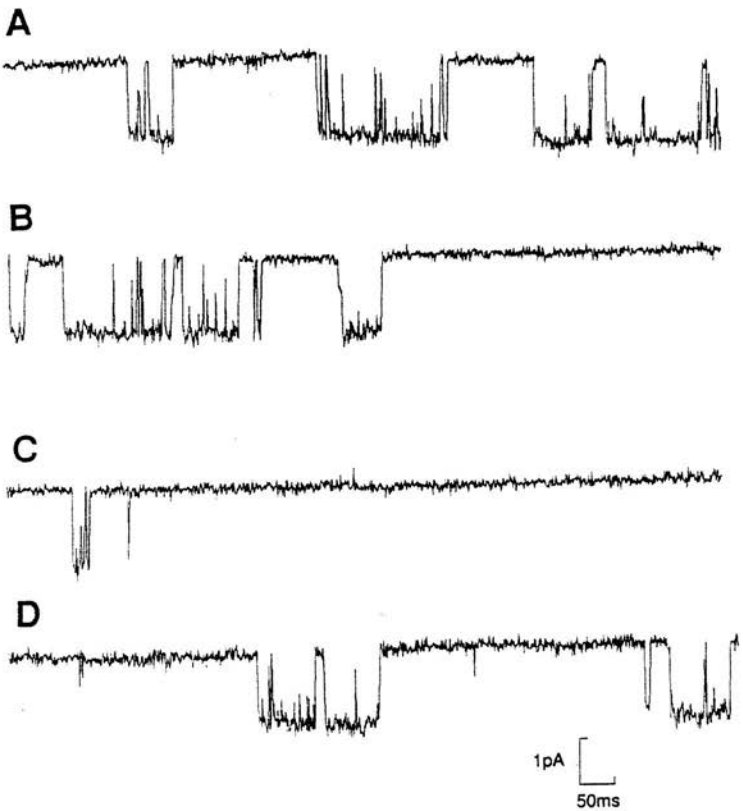

a
50mV
10mV
-10mV
-50mV
1 pA
20 ms
b
pA
3
2
1
-1
-2
-3
-100
-50
50
100
mV


100
75
50
25
0
% of time n channels open
0
1
2
3
Number of channels

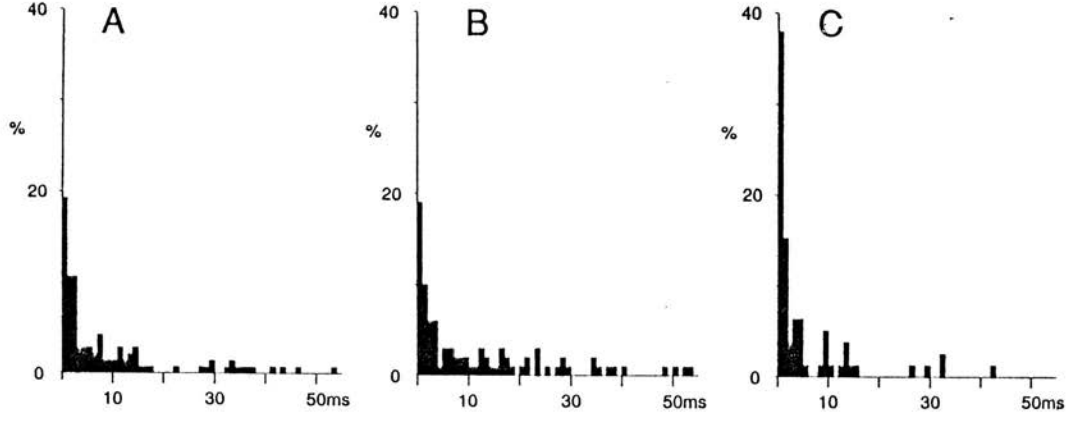

A
B
C
D
2pA
4 sec

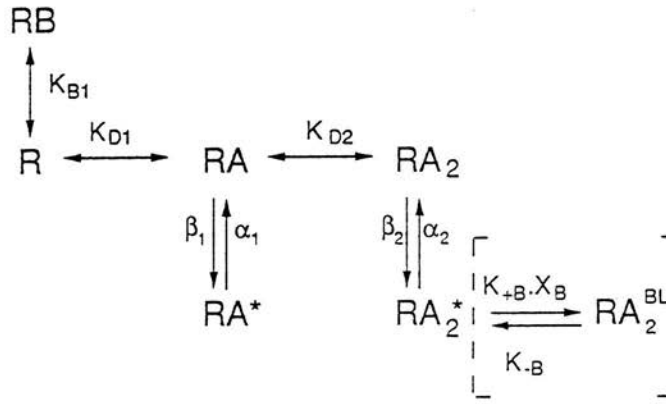

A
B
C
0.6
0.4
0.2
0
%
0.08
0.06
0.04
0.02
-3
0
Current (pA)


% inhibition
100
80
60
40
20
0
0
20
40
60
80
100
1000
2000
SR95103 (μM)
1
2
4
5


A
B
C
D
1pA
50ms

Bursts




RB
K_{B1}
R
K_{D1}
RA
K_{D2}
RA_2
β_1
α_1
β_2
α_2
RA*
RA_2^*
$K_{+B}.X_B$
K_{-B}
RA_2^{BL}

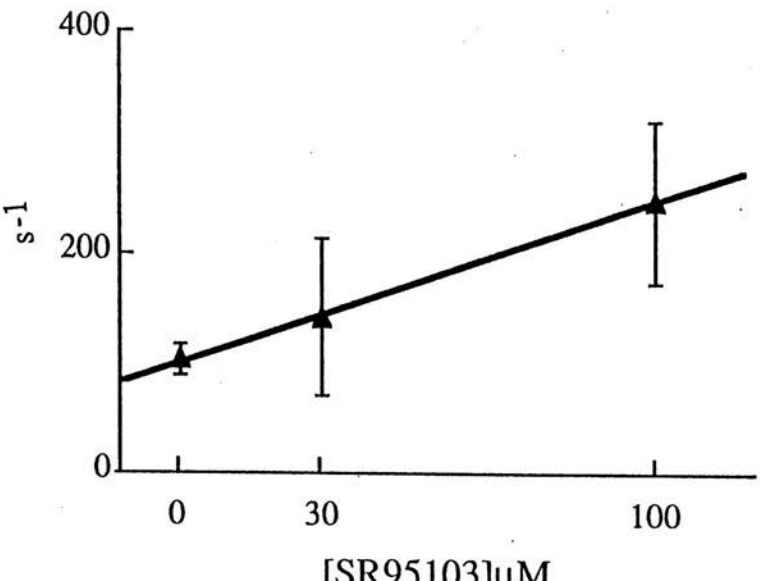

400
200
0
s-1
0
30
100
[SR95103]µM

Table 1: Summary of GABA-activated channel properties in the absence and presence of SR95103. Each observation is the mean from experiments on at least four patches. * $p \leq 0.05$ T-test.

		Control GABA 3μM	GABA 3μM + SR95103 30μM	GABA 3μM+ SR95103 100μM
Popen		0.053±0.011	0.028±0.008	0.009±0.004
Channel conductance (pS)		22.4±1.0	22.6±1.0	23.3±0.6
Mean open time (ms)		8.45±1.11	9.90±2.44	4.54±1.41*
Mean burst duration (ms)		16.94±2.54	20.15±3.93	7.96±3.63
pdf_{burst}	abf	0.418±0.083	0.389±0.090	0.745±0.088*
	T_{bf}(ms)	2.3±0.6	1.6±0.5	1.3±0.5
	abs	0.581±0.083	0.610±0.090	0.255±0.880*
	T_{bs}(ms)	26.6±2.0	27.4±2.2	22.0±7.2
Brief closed times				
	acf	0.459±0.090	0.477±0.122	0.440±0.067
	T_{cf}(ms)	0.66±0.20	0.85±0.30	0.66±0.27

Table 4.2: Effects of SR95103 on GABA channel burst distributions and the partial reversal by increasing GABA concentration

	GABA 3μM control	GABA 3μM SR95103 30μM	GABA 3μM SR95103 60μM	GABA10μM SR95103 60μM
Corrected mean burst duration (ms)	23.8	20.2	2.3	8.7
pdfburst				
abf	0.246	0.241	0.829	0.591
T_{bf} (ms)	0.5	0.7	0.4	1.0
a_{bs}	0.754	0.759	0.171	0.409
T_{bs} (ms)	31.4	26.4	11.7	19.8