CHARACTERIZATION OF $GABA_B$ RECEPTORS IN THE RAT PERIPHERAL AND CENTRAL NERVOUS SYSTEM.

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Abstract.

Recent electrophysiological studies have led to the proposal of subtypes of $GABA_B$ receptors. In the rat hippocampus the postsynaptic $GABA_B$ receptor is sensitive to the weak $GABA_B$ antagonist phaclofen and to pertussis toxin (PTX) whereas the presynaptic receptor is insensitive to both agents. To investigate the presynaptic receptor further the effect of $GABA_B$ agonists was studied on the K^+-evoked release of endogenous amino acids from rat hippocampal synaptosomes. (-)Baclofen (30-100μM) produced a dose-dependent inhibition of aspartate, glutamate and GABA release evoked by 50mM K^+. 3-Aminopropyl-phosphinic acid (3-APA) (30-300μM) failed to inhibit amino acid release. 3-APA is reported to inhibit [^{3}H]-GABA binding to $GABA_B$ sites in rat whole brain membranes more potently than the prototypic $GABA_B$ ligand (-)baclofen, although in biochemical assays 3-APA is equipotent with (-)baclofen and appears to behave as a partial agonist. Thus 3-APA may distinguish between subtypes of $GABA_B$ receptors.

For comparison, peripheral $GABA_B$ receptors on adrenergic nerve terminals were studied using the electrically stimulated rat anococcygeus muscle preparation. (-)Baclofen, 3-APA and its methyl derivative SKF 97541 produced a dose-dependent inhibition of the electrically-evoked release of preloaded [^{3}H]-noradrenaline (EC_{50} values of 1.4μM, 0.56μM and 1.25μM respectively). CGP 35348, a selective though relatively weak $GABA_B$ antagonist, was compared with two new compounds for their ability to reverse the effect of 30μM (-)baclofen. These compounds were found to antagonize the baclofen response more potently than CGP 35348. Schild analysis of data obtained using the same preparation monitoring antagonism of baclofen-induced inhibition of transmurally-evoked contraction of the muscle indicated the presence of a single receptor type. No evidence was obtained for receptor heterogeneity on adrenergic nerve terminals. Thus, although 3-APA failed to

mimic the inhibitory action of (-)baclofen on transmitter release in hippocampal synaptosomes it did produce the same response in the peripheral tissue.

Experiments were performed to determine whether 3-APA exhibited a differential selectivity for CNS $GABA_B$ receptors in different regions of the rat brain. Since any lack of affinity for hippocampal receptors might go undetected in membrane binding experiments performed in whole brain preparations, studies were performed using receptor autoradiography. 3-APA inhibited [^{3}H]-GABA binding to $GABA_B$ sites to the same extent as (-)baclofen in seventeen brain regions including the hippocampus. The lack of difference between 3-APA and (-)baclofen in the hippocampus contrasted with the findings in the release experiments and the possible reasons for this discrepancy are discussed.

Following incubation of rat brain slices for 24 hours in PTX specific $GABA_B$ binding is reduced throughout the brain with the exception of the corpus striatum. This observation may reflect different post receptor coupling or an inability of the toxin to access striatal neurones. To clarify these observations the effect of PTX on $GABA_B$ binding was determined in membranes prepared from the cortices, striata, hippocampi and cerebella of adult and immature rats. Incubation for 30 min in pre-activated PTX reduced specific $GABA_B$ binding in all areas except the striatum in the adult animals (10-12 weeks), however in animals of 5-6 weeks or 7-9 weeks old the profile of the PTX effect was quite different. $GABA_B$ binding in all brain regions could be reduced by 85% by the inclusion of GTPγS in the incubating medium. Regional and age-related variations in the sensitivity of $GABA_B$ binding to PTX may therefore be due to the presence of $GABA_B$ receptors linked to different inhibitory G-proteins.

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Abbreviations

4-ABPA	4-Aminobutylphosphonic Acid
ACHC	3-Aminocyclohexane carboxylic Acid
AOAA	Amino-oxyacetic Acid
4-AP	4-Aminopyridine
3-APA	3-Aminopropylphosphinic Acid
3-APPA	3-Aminopropylphosphonic Acid
3-APS	3-Aminopropane Sulphonic Acid
ATP	Adenosine Triphosphate
δ-AVA	δ-Aminovaleric Acid
BSA	Bovine Serum Albumin
cyclic AMP : cAMP	Adenosine 3′,5′-Monophosphate
CGP 35348	(p-3-Aminopropyl)-p-diethyloxymethyl Phosphinic Acid
CNS	Central Nervous System
DABA	2,4-Diaminobutyric Acid
DRC	Dose-Response Curve
DRG	Dorsal Root Ganglion
EGTA	Ethylene Glycol-bis(β-Amino Ethyl Ether) N,N,N′,N′,-Tetra Acetic Acid
e.p.s.p.	Excitatory Postsynaptic Potential
FTR	Fractional Tritium Release
GABA	γ-Aminobutyric Acid
GABA-T	GABA-Transaminase
GAD	Glutamic Acid Decarboxylase
GDP	Guanosine Diphosphate
GDP-β-S	5′-O-(2-Thiodiphosphate)
G_i	Inhibitory G-Protein
GMP	Guanosine Monophosphate
G-Protein	Guanyl Nucleotide Binding Protein
G_s	Stimulatory G-Protein
GTP	Guanosine Triphosphate

GTPγS	Guanosine 5′-0-(Thiotriphosphate)
HPLC	High Pressure/Performance Liquid Chromatography
IP_1	Inositol Monophosphate
IP_3	Inositol Triphosphate
i.p.s.p.	Inhibitory Postsynaptic Potential
NMDA	N-Methyl-D-Aspartic Acid
NANC	Non-Adrenergic Non-Cholinergic
OAG	1,2-Oleoyl Acetylglycerol
2-OH-S	2-Hydroxysaclofen
OPA	O-Phthaldialdehyde
PKC	Protein Kinase C
PLA2	Phospholipase A_2
PLC	Phospholipase C
PTX	Pertussis Toxin
SKF 97541	3-Aminopropyl(methyl)phosphinic Acid
SS-LI	Somatostatin-Like Immunoreactivity
SWD	Spike and Wave Discharges
THIP	Tetrahydroisoxasolo-pyridinol
TRIS	Tris(hydroxymethyl)aminomethane
TTX	Tetrodotoxin
VIP	Vasoactive Intestinal Peptide
VSCC	Voltage-Sensitive Calcium Channel
CGP 36742	(p-(3-aminopropyl)p-n-butyl-phosphinic acid
CGP 46381	(p-(3-aminopropyl)-p-cyclohexylmethyl-phosphinic acid

Publications Arising from this Thesis

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A little learning is a dangerous thing;
Drink deep, or taste not the Pierian spring;
There shallow draughts intoxicate the brain,
And drinking largely sobers us again.
Fired at first sight with what the Muse imparts,
In fearless youth we tempt the heights of Arts,
While from the bounded level of our mind
Short views we take, nor see the lengths behind;
But more advanced, behold with strange surprise
New distant scenes of endless science rise!

Alexander Pope

(from an Essay on Criticism)

FOR MUM AND DAD

CHAPTER 1

INTRODUCTION

I. GABA - AN INHIBITORY NEUROTRANSMITTER IN THE MAMMALIAN NERVOUS SYSTEM.

'Simple amino acids do not show startling drug actions'

(Bergen, 1948)

At a time when this statement was widely believed by the scientific community, Eugene Roberts and Sam Frankel (1950) identified an unknown ninhydrin-reactive material, present in extracts of mouse brain, as γ-aminobutyric acid (GABA). Using two-dimensional paper chromatography they were able to demonstrate the presence of relatively large amounts of this amino acid in brain homogenate compared with blood, urine and extracts of normal or neoplastic tissue. Addition of radiolabelled glutamic acid, isolated from algae grown in $^{14}C0_2$, to fresh brain homogenates or acetone powder suspensions accelerated the rate of accumulation of GABA and led to the incorporation of ^{14}C into the GABA molecule. Roberts and Frankel proposed, therefore, that GABA was a normal constituent of the brain and that it was formed from glutamic acid, probably by α-decarboxylation. Indeed it was known already that GABA could be synthesized by the action of glutamic acid decarboxylase (GAD) in plants and bacteria (Gale, 1946; Schales et al., 1946). In further studies Roberts and Frankel (1951) demonstrated that this was also the case in mammalian systems.

Despite these initial observations it was not until the mid 1960's that a role as a central nervous system (CNS) transmitter was seriously considered possible for any such 'simple amino acid'. The ubiquitous distribution of GABA in the CNS seemed, to many, to preclude an important neuromodulatory function (Curtis, 1965; Ryall, 1964). So much so that when in 1966 Krnjevic and Schwartz demonstrated that iontophoretically applied GABA mimicked the action of the cortical inhibitory transmitter, by raising the membrane potential and conductance, their tentative conclusion was that in the cortex, at least, there was no evidence that GABA was *not* the main inhibitory neurotransmitter.

Today the evidence supporting a role for GABA as the most important inhibitory neurotransmitter substance in the mammalian CNS is overwhelming. All of the criteria required for such a role have been fulfilled and are outlined below.

1) Distribution of GABA in the mammalian nervous system.

GABA is present in all regions of the mammalian CNS though its distribution is by no means homogenous. In rat brain GABA is most highly concentrated in the substantia nigra, globus pallidus, hypothalamus and tectum (Okada et al., 1971,; Heyden et al., 1979). Some GABA containing projections have been described, mainly associated with the basal ganglia (Fonnum et al., 1974; Jessell et al., 1978; Walaas and Fonnum, 1980) and cerebellum (Fonnum et al.,

1970), but the majority of GABAergic neurones appear to be interneurones as in the cortex, hippocampus, olfactory bulb, retina and spinal cord (Graham, 1972; Storm-Mathisen, 1972; Miyata and Otsuka, 1975; Ribak et al., 1977; Tappaz, 1978; Hendry and Jones, 1981).

The presence of GABA in peripheral tissues has been more difficult to establish as the concentration of GABA present is typically less than 1% of that found in the CNS (Erdo et al., 1982). However GABA has been detected in over 30 peripheral tissues including the myenteric plexus (Jessen et al., 1979), the superior cervical ganglion (Dobo et al., 1989), the female genital system (Erdo et al., 1982), the bladder (Kusunoki et al., 1984), the kidney (Dobo et al., 1990), and the liver (Minuk, 1986). Evidence for a neurotransmitter role has only been fully established for GABA in the enteric nervous system and is not discussed in the following paragraphs.

2) GABA synthesis.

Glutamic acid decarboxylase (GAD; EC,4.1.1.15) catalyses the conversion of glutamic acid to GABA but unlike other enzymes involved in GABA synthesis it is found primarily in the nervous system (Baxter, 1976). Indeed GAD is apparently restricted to GABAergic neurones (Matsuda et al., 1973a and 1973b; Yu et al., 1984) not being detected in either glutamatergic neurones or in astrocytes (Schousboe et al., 1977). Two genes have been identified encoding separate GAD isoforms that differ in their requirement for the co-factor

pyridoxal-5'-phosphate (Bayon et al., 1977a; Bayon et al., 1977b; Erlander and Tobin, 1991). The functional relevance of these two forms is unclear but it is obviously important to the understanding of how a neurone regulates GABA production. Although GAD is the key enzyme responsible for GABA synthesis it is not the only one, as GABA may also be produced by the action of ornithine δ-aminotransferase (EC2.6.1.13). This enzyme is present in many cell types, but the form detected in GABAergic neurones exhibits a lower affinity (K_m) for GABA than is usual (Drejer and Schousboe, 1984) intimating a possible contribution to overall neuronal GABA production.

3) Neuronal release of GABA.

According to the 'vesicular hypothesis' of transmitter release stimulus secretion coupling is thought to be dependent on the presence of external calcium ions (Katz, 1969; Ceccarelli and Hurlbut, 1980). Accordingly a variety of experimental techniques have been used to demonstrate the calcium-dependent, evoked release of GABA from brain areas *in vivo* (Lehman et al., 1983; Benveniste et al., 1984; Sandberg et al., 1986), from perfused brain slice preparations *in vitro* (Valdes and Orrego, 1978; Szerb, 1983), from synaptosomes (Bradford et al., 1973; DeBelleroche and Bradford, 1977) and from neuronal cell cultures (Pin et al., 1986). In many instances exclusion of Ca^{2+} from the external medium (with or without concomitant addition of EGTA and/or magnesium ions) results in a profound (Burke and Nadler, 1988) or, more typically, a partial (Szerb, 1983; Bonanno et al., 1989) reduction in

GABA release. Examples of calcium-independent evoked transmitter release have been documented - but perhaps only now do we have some insight into the physiological relevance of this phenomenon. In cultured hippocampal neurones preloaded [^{3}H]-GABA can be released by application of the excitatory amino acids glutamate, N-methyl-D-aspartate (NMDA), kainate and by elevated external K^+ or veratridine. In each case this is associated with a rise in intracellular Ca^{2+}-concentration which is completely dependent on the presence of external Ca^{2+} (Harris and Miller, 1989). This suggests that in the hippocampus the release of excitatory amino acids may be physiologically relevant in the regulation of GABAergic transmission. Surprisingly, removal of external Ca^{2+} has little effect on the ability of these agents, with the exception of NMDA, to stimulate [^{3}H]-GABA release. However, in all cases [^{3}H]-GABA release is inhibited in Na^+-free medium or in the presence of the GABA uptake inhibitor nipecotic acid. Thus, it appears that Ca^{2+}-independent release may be mediated by the reversal of the GABA transport system.

Similarly, exocytotic release of vesicular glutamate is Ca^{2+}-dependent (Nicholls and Shira, 1986) and energy-dependent (Sanchez-Prieto et al., 1987). However, under conditions in which the Na^+-electrochemical gradient across the plasma membrane is lowered, cytoplasmic glutamate is released by reversal of the glutamate uptake system in a Ca^{2+}-independent manner (Nicholls et al., 1987). It has been suggested that separate neuronal pools of glutamate are the origin of Ca^{2+}-dependent and -independent release and it is probable that different releasable pools of GABA also exist in nerve terminals. Using isolated growth

cones from neonate rat forebrain it has been demonstrated that K^+-evoked GABA release is Ca^{2+}-independent (Taylor and Gordon-Weeks, 1991) until postnatal day 5 (P5). At this stage of development growth cones contain neither synaptic vesicles nor do they stain for synaptic vesicle antigens. The Ca^{2+}-independent K^+-evoked GABA release is blocked by the uptake inhibitor (RS)-N-(4,4-diphenyl-3-butenyl)nipecotic acid (SKF 89978), once again implying that the reversal of the GABA transport mechanism is responsible for this phenomenon. After P5 a Ca^{2+}-dependent component of K^+-evoked GABA release appears, and is maximal (>50% of total) by P11, coinciding with the appearance of synaptic vesicles in the growth cones (Taylor, et al., 1990).

Where Ca^{2+} is implicated in release mechanisms, studies to determine the type of Ca^{2+}-channel involved have been inconclusive. There is evidence for the involvement of both receptor linked ion channels (Pastuszko et al., 1984; Lazarewicz, 1986; McDermott et al., 1986) and voltage-sensitive calcium channels (VSCC) (Dingledine, 1983). Indirect activation of VSCC's as a result of Ca^{2+}-flux through receptor gated channels has also been postulated (Rivers and Orrego, 1986). The nature of the VSCC's has not yet been determined.

4) Released GABA acts at specific postsynaptic receptors to mediate neuronal inhibition.

Intracellular recording combined with extracellular microiontophoretic drug application revealed that GABA hyperpolarized cortical neurones and this was associated with a rapid rise in membrane conductance. Both the evoked inhibitory postsynaptic potentials (i.p.s.p.'s) and responses to exogenously applied GABA could be reversed by injection of Cl^- into the neurone (Li and Chou, 1962; Krnjevic et al., 1966a; Krnjevic et al., 1966b). Several inorganic anions and even large organic ions were able to substitute for Cl^- which suggested that Cl^--flux occurred via unselective anion channels K^+ was thought unlikely to contribute to the inhibitory effect of GABA as blockers of K^+ movement did not affect the i.p.s.p. (Krnjevic et al., 1971). Since GABA was ineffective when injected intracellularly it was concluded that it must be acting on some cell surface receptor.

The convulsant strychnine was without effect against GABA responses although it had been shown to antagonize the inhibitory action of glycine on spinal cord neurones (Curtis et al., 1968a; Curtis et al., 1968b). A range of other convulsant isoquinoline alkaloids were studied and it was discovered that the effect of GABA on feline central neurones and the strychnine-resistant inhibition of cortical pyramidal cells and cerebellar Purkinje cells could be selectively blocked by bicuculline (Curtis et al., 1970) an alkaloid derived from the *Cordalis* species (Manske, 1933; Welch and Henderson, 1934). Another

antagonist, picrotoxin, already known to be an antagonist of GABA responses in crustacea (Robbins, 1959), also selectively inhibited mammalian GABA receptor activation (Galindo, 1969; Curtis et al., 1971). It is now apparent that bicuculline competes directly with GABA for its binding site on the receptor protein whereas picrotoxin binds to the Cl^- ionophore and antagonizes GABA in a non-competitive manner (Ticku et al., 1978; Simmonds, 1980).

Shortly after the identification of specific GABA receptor antagonists a receptor binding assay was devised (Zukin et al., 1974) which demonstrated specific and reversible binding of [^{3}H]-GABA to crude synaptic membranes prepared from whole rat brain. This specific binding could be displaced to the same extent by both unlabelled GABA (IC_{50} 0.1μM) and bicuculline (IC_{50} 5μM). The density of [^{3}H]-GABA binding was measured in various brain regions and found to be highest in the cerebellum, thalamus, hippocampus, cerebral cortex; moderate in the corpus striatum; and lowest in the medulla-oblongata pons and spinal cord. The density of GABA binding did not correspond to the endogenous levels of GABA.

Specific agonists for GABA receptors were also discovered. Muscimol, a psychotomimetic isoxazole isolated from the mushroom *Amanita muscaria*, was found to be more potent than GABA at activating inhibitory receptor-gated Cl^--flux in all tissues studied (Johnson et al., 1968; Naik et al., 1976; Wheal and Kerkut, 1976). In binding assays muscimol inhibited GABA binding completely with a K_i of 40nM (Greenlee et al., 1978) whilst [^{3}H]-muscimol

itself bound to GABA receptors with a K_D 0f 3nM (Beaumont et al., 1978; Snodgrass, 1978). Muscimol, and another compound, isoguvacine, were found to inhibit feline spinal interneurones (Curtis et al., 1971; Krogsgaard-Larsen et al, 1977). Both of these compounds are semi-rigid GABA analogues which mimic GABA selectively and are antagonized by bicuculline and picrotoxin. Binding of [^{3}H]-GABA can be inhibited by isoguvacine (Enna et al., 1977) and specific binding of [^{3}H]-isoguvacine can be inhibited by GABA, muscimol, bicuculline, but not picrotoxin.

5) GABA uptake and degradation.

Following its release into the synaptic cleft GABA is removed by a sodium-dependent, high affinity uptake system into neurones and glia (Iversen and Neal, 1968; Iversen and Kelly, 1975), and by diffusion into postsynaptic cells (Hyden et al., 1986). The active processes may be distinguished by compounds such as 3-aminocyclohexane carboxylic acid (ACHC) and 2,4-diaminobutyric acid (DABA) which are selective inhibitors of neuronal GABA uptake and by gaboxadol and cis-4-hydroxynipecotic acid which are glial selective. Nipecotic acid is a non-selective GABA uptake inhibitor and, in common with many of these compounds, is itself a substrate for the GABA carrier proteins (Bowery et al., 1976; Krogsgaard-Larsen et al., 1987). β-Alanine, once widely believed to be an inhibitor of glial uptake of GABA (Schon and Kelly, 1975), has now been shown to utilize the taurine carrier in both neurones and glia. Thus, β-alanine can no longer be used as a marker for

GABA uptake into glia, as has been the custom in the past (Larsson et al., 1986). Recently developed nipecotic acid derivatives appear to inhibit GABA uptake without being substrates for the GABA carrier protein (Younger et al., 1984) and may therefore be of importance in conditions which would benefit from enhanced GABA function. It is interesting that selective neuronal GABA uptake inhibitors are apparently proconvulsant (Meldrum et al., 1982) whereas, presumably by increasing the synaptic GABA pool, inhibitors of glial uptake are anticonvulsant (Krogsgaard-Larsen et al., 1981; Schousboe et al., 1986).

It is not surprising that a GABA transporter protein has now been cloned from rat brain (Guastella et al., 1991). This protein, expressed in *Xenopus* oocytes, has been shown to exhibit comparable kinetics to those of the native neuronal and glial proteins. ACHC and DABA were effective inhibitors of [^{3}H]-GABA uptake by the carrier but as they were compared only to β-alanine as a glial inhibitor in this system it is perhaps premature to describe the expressed protein as a *neuronal-like* GABA uptake protein.

Coexistence on nerve terminals of the uptake carriers for GABA and acetylcholine (Bonanno and Raiteri, 1987a; Bonanno et al., 1991) or dopamine (Bonanno and Raiteri, 1987b) provides evidence that major neurotransmitters are co-localized. Supporting evidence is provided, for example, by the visualization of particular neurones in the rat diagonal band which stained positive for both choline-acetyltransferase (ChAT) and GAD in double immunofluorescence studies (Brashear et al., 1986).

Following the active uptake of GABA into nerve terminal further compartmentation into synaptic vesicles occurs. Fyske and Fonnum (1988) have shown that GABA is accumulated by isolated synaptic vesicles in a magnesium-dependent, sodium-independent manner that is inhibited by proton pump inhibitors but not by neuronal or glial GABA uptake inhibitors. GABA is present in nerve terminals at about 50-150mM (Fonnum and Walberg, 1973) but much of this is probably intravesicular and so although the affinity of the vesicular uptake carrier for GABA is low (K_m = 5.6mM) it is appropriate for the local GABA concentration.

The final stage of GABA metabolism is its transamination to succinic semi-aldehyde by GABA-transaminase (GABA-T: E.C.2.6.1.19) an enzyme of both neuronal and glial origin (Schousboe, 1981). A consequence of GABA-T inhibition is the elevation of brain GABA concentrations and this is believed to be the mechanism by which the clinically used anti-epileptic agent γ-vinyl-GABA mediates its effects (Schechter, 1984; Halonen et al., 1990).

By the late 1970's the pharmacology of GABA receptors appeared to be well defined. GABA mediated neuronal inhibition, by either hyperpolarization or depolarization but always by the movement of Cl^-, and its actions were mimicked by muscimol and isoguvacine and antagonized by bicuculline or picrotoxin. However the GABA story was about to evolve further.

II GABA RECEPTOR MULTIPLICITY - The Pharmacology of Baclofen.

A series of GABA analogues were designed which, unlike GABA, would penetrate the CNS following oral administration. One of these compounds was β-p-chlorophenyl γ-aminobutyric acid or baclofen (Keberle and Faigle, 1972). In cats intravenous administration of baclofen was found to reduce mono- and polysynaptic reflexes and to lessen the spasticity induced by mesencephalic transection or ischaemic decerebration (Bein, 1972). Baclofen quickly became the drug of choice clinically for the alleviation of limb spasticity resulting from a variety of spinal injuries.

Electrophoretically administered baclofen depressed the firing of spinal interneurones, pyramidal tract neurones and Purkinje cells in the anaesthetized cat with no effect on Renshaw cells (but see Benecke and Meyer-Lohmann, 1974) yet its actions were unaffected by either bicuculline or strychnine (Curtis et al., 1974). The precise mechanism by which baclofen relieved spasticity was therefore unclear but it was proposed that baclofen may inhibit the release of excitatory neurotransmitters within the spinal cord (Davidoff and Sears, 1974; Fox et al., 1978). Though both of these groups concluded that this action of baclofen must be independent of any GABA receptor interaction later studies indicated that baclofen was indeed 'GABA-like'. For example baclofen inhibited the spontaneous firing of rat nigral and ventral tegmental neurones as did GABA and mimicked the rotational behaviour induced by GABA after

injection into the substantia nigra (Olpe et al., 1977b). Yet in all cases baclofen was resistant to antagonism by bicuculline. This paradox was addressed by a series of investigations with the following results. Baclofen was found to inhibit [^{3}H]-GABA binding in membrane preparations weakly; to be ineffective or only a modest inhibitor of GABA uptake into synaptosomes; to elicit a small release of GABA from synaptosomes and to be without effect on GAD activity (Roberts et al., 1978; Olsen et al., 1978). The conclusion drawn from these studies was that, at best, baclofen was an extremely weak GABA agonist but that its effects were more likely to be due to some non-specific action.

The discovery that activation of peripheral bicuculline-sensitive GABA receptors on rat superior cervical ganglia led to the expected increase in chloride conductance but resulted in neuronal depolarization (Bowery and Brown, 1974) prompted Bowery and co-workers to propose that the activation of such receptors on sympathetic nerve terminals should result in a depression of neurotransmitter outflow. These predictions were confirmed by the findings that GABA produced a dose-dependent inhibition of evoked [^{3}H]-noradrenaline release from isolated rat atria, achieving a maximum inhibition of 50-60%. What was unexpected was that this GABA response was resistant to bicuculline and all other recognised GABA antagonists, and was not mimicked by the majority of recognised GABA agonists - with the exception of baclofen (Bowery and Hudson, 1979). Further studies were carried out using a range of peripheral, isolated tissue preparations. In all cases baclofen and GABA dose-dependently inhibited the electrically evoked twitch

responses. Baclofen exhibited stereoselectivity in its effect, with the (-) isomer being approximately 100 times more potent than the (+) isomer. A lack of effect of baclofen on exogenously applied agonist responses indicated a presynaptic location for this atypical GABA receptor. That both baclofen and GABA were acting at the same site to reduce transmitter release was supported by their parallel log dose-response curves, similar maximum responses (40-60% inhibition of twitch response), their cross-desensitization in rat atria and the inability of each to inhibit the twitch response further in the presence of a maximal concentration of the other (Bowery et al., 1981).

It was soon apparent that this novel GABA receptor was also present on central neurones. (-)Baclofen (1-100μM) inhibited the K^+-evoked release of [^{3}H]-noradrenaline from rat cerebellar slices, [^{3}H]-dopamine from striatal slices and [^{3}H]-5-hydroxytryptamine from cortical slices (Bowery et al., 1980). The attenuation of neurotransmitter release by baclofen was stereoselective and mimicked by GABA but not by the GABA agonist 3-aminopropane sulphonic acid (3-APS). At lower concentrations of K^+ (<25mM) GABA enhanced [^{3}H]-noradrenaline release from cerebellar slices unless bicuculline was present, in which case GABA reduced release. 3-APS consistently enhanced transmitter release never producing an inhibition, even in the presence of bicuculline. The conclusion drawn from these results was that two types of GABA receptor were present but only at the lower concentrations of K^+ were both types functionally apparent. Baclofen was also shown to inhibit the release of excitatory amino acids from central neurones: endogenous aspartate and

glutamate from slices of guinea-pig cerebral cortex (Potashner, 1979), and [^{3}H]-D-aspartate from slices of rat cerebral cortex and spinal cord (Johnston et al., 1980).

Evidence for two distinct binding sites for [^{3}H]-GABA in brain synaptic membranes provided the final proof of GABA receptor heterogeneity. It was found that addition of either Ca^{2+} or Mg^{2+} to the incubating medium increased the amount of specific [^{3}H]-GABA binding to rat whole brain membranes. This additional binding component was suppressed by unlabelled GABA but untouched by isoguvacine (Bowery et al., 1983). In the presence of 2.5mM $CaCl_2$, and 40μM isoguvacine to completely inhibit bicuculline sensitive binding sites, [^{3}H]-GABA and [^{3}H]-baclofen were found to exhibit high affinity saturable binding that could be fully displaced by unlabelled GABA and (-) baclofen with equal affinities. Muscimol and 3-APS were much weaker displacers of [^{3}H]-baclofen binding and isoguvacine and bicuculline were devoid of activity (Hill and Bowery, 1981).

It was at this point that Bowery and colleagues designated the classical bicuculline-sensitive GABA site as the $GABA_A$ receptor and the novel baclofen-sensitive, bicuculline-insensitive site as the $GABA_B$ receptor. A summary of the characteristics of each of these receptor is given in Table 1.

Table 1 Characteristics of $GABA_A$ and $GABA_B$ Receptors.

Selective agonists	Isoguvacine	(-) Baclofen
Selective antagonists	Bicuculline	Phaclofen 2-OH-Saclofen CGP 35348
Modulators	Benzodiazepines Barbiturates Bicyclophosphates Steroids	None?
Ion Channels	Receptor activation increases Cl^- conductance	Receptor activation increases K^+ or decreases Ca^{2+} conductance
2nd Messengers	None	Adenylate Cyclase PI Turnover
Dependence of binding on ions	None	Absolute dependence on Ca^{2+} or Mg^{2+}
Effect of guanyl nucleotides	None	Reduce binding affinity

III THE PHARMACOLOGY OF $GABA_B$ RECEPTORS.

1) $GABA_B$ receptor distribution in the mammalian CNS.

Membrane binding assays had demonstrated the existence of two distinct subtypes of GABA receptor in the mammalian CNS however it required autoradiographical studies to make clear that each of these receptors had a unique pattern of distribution (Wilkin et al., 1981; Bowery et al., 1984; Bowery et al., 1987). Several areas have similar densities of both $GABA_A$ and $GABA_B$ binding sites whereas in other regions one receptor type predominates. High levels of both $GABA_A$ and $GABA_B$ binding occur in cerebral cortex, particularly in the outer laminae (I-IV), and in some thalamic nuclei. Moderate levels of both sites are found in the basal ganglia, the Raphe nucleus, amygdala and substantia nigra and throughout hippocampal areas CA1-CA4, with the exception of the pyramidal cell layers which have very few $GABA_B$ receptors. There is a paucity of GABA binding, of either subtype, in the hypothalamus or medulla.

Striking differences in binding distribution occur in the cerebellum. In this brain region $GABA_A$ receptors predominate in the granule cell layer whereas $GABA_B$ receptors are confined to the molecular cell layer (Wilkin et al., 1981). Similarly in the spinal cord, $GABA_A$ receptors show a uniform distribution through both dorsal and ventral laminae (I-X), while $GABA_B$ receptors are concentrated in the dorsal horn (laminae I-IV). $GABA_B$ receptors are

particularly dense in the substantia gelatinosa (laminae II-III) and as $GABA_B$ binding is halved following neonatal capsaicin administration this would imply their location on primary afferent terminals (Price et al., 1984b). The globus pallidus, lateral amygdaloid nucleus, habenulae and superior colliculus contain many more $GABA_B$ than $GABA_A$ receptors. Another region rich in $GABA_B$ binding sites is the interpeduncular nucleus. If either the intrinsic neurones of this structure or the afferent inputs from the habenulae are lesioned then $GABA_B$ binding is reduced, by 85%, by the latter procedure only. This provides evidence for a predominat ly presynaptic location of $GABA_B$ receptors in the interpeduncular nucleus on the terminals of the habenulae input. Similarly, unilateral decortication results in a substantial decrease of $GABA_B$ binding in the caudate putamen on the lesioned side compared to the unlesioned side, indicating the presence of a population of $GABA_B$ receptors on corticostriatal terminals (Moratalla and Bowery, 1991).

2) The development of $GABA_B$ receptor agonists and antagonists.

$GABA_B$ receptors were initially described as baclofen-sensitive, bicuculline-insensitive GABA receptors for the good reason that, with the exception of GABA itself, no other ligands, agonist or antagonist, were known to act at this novel site. In the early 1980's compounds such as muscimol and progabide were shown to mimic baclofen weakly but they were much more potent agonists at the classical $GABA_A$ receptor (Bowery et al., 1982). The first reports of antagonism of $GABA_B$ responses came in 1982. The higher

homologue of GABA, δ-aminovaleric acid (δ-AVA), a known agonist at $GABA_A$ receptors was found to antagonize the $GABA_B$-mediated inhibition of [^{3}H]-noradrenaline released from the isolated rat anococcygeus muscle (Muhyaddin et al., 1982a; Muhyaddin et al., 1982b; Muhyaddin et al., 1983), to inhibit specific [^{3}H]-baclofen binding in rat brain membranes and to reverse the baclofen-induced reduction of population spikes evoked in the rat hippocampal slice (Nakahiro et al., 1985). However, this compound was extremely weak, concentrations in the low mM range being required for any appreciable activity at $GABA_B$ receptors. Another $GABA_A$ agonist, 3-APS, was also reported to competitively inhibit the baclofen depression of the evoked twitch response in the guinea-pig ileum, without any direct effect of its own (Giotti et al., 1983). The authors claimed 3-APS to be more 'potent' in this preparation than δ-AVA (apparent pA_2 values of approximately 4 for both antagonists) but the extreme weakness and lack of specificity of both compounds severely limited their usefulness in determining the physiological relevance of $GABA_B$ receptors in the mammalian nervous system.

As far back as 1965 it had been shown that the phosphonic analogue of GABA, 3-aminopropylphosphonic acid (3-APPA; Fig. 1), depressed the firing of feline spinal neurones (Curtis and Watkins, 1965). A similar effect on rat cerebral and cerebellar cortical neurones was bicuculline-insensitive and therefore presumably not due to $GABA_A$ receptor activation (Bioulac et al., 1979). The corresponding derivative of baclofen, β-(p-chlorophenyl)-3-aminopropyl phosphonic acid (phaclofen; Fig. 1), was synthesized and compared to 3-APPA

in both peripheral and central $GABA_B$ preparations (Kerr et al., 1987). It was observed that whilst neither compound relaxed the guinea-pig ileum nor depressed the transmurally evoked cholinergic twitch response, both antagonized the baclofen-mediated reduction in twitch. This effect was reversible and an apparent pA_2 of approximately 4 was calculated for each compound. In contrast, 3-APPA weakly mimicked baclofen's ability to depress monosynaptic excitation of spinal cord neurones; the action of baclofen and 3-APPA could be reduced by phaclofen (Kerr et al., 1987).

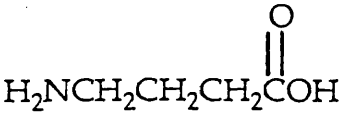
Phaclofen showed an appreciable improvement in selectivity for $GABA_B$ receptors compared to the earlier compounds, enough to make it possible to antagonize both the postsynaptic action of baclofen and the bicuculline-resistant action of GABA, and to abolish the slow i.p.s.p. in hippocampal pyramidal cells, thus establishing an important physiological role for $GABA_B$ receptors in the CNS (Dutar and Nicoll, 1988a). However, phaclofen lacked sufficient potency to make it a universally useful $GABA_B$ antagonist. The synthesis of sulphonic baclofen derivatives addressed this problem. 3-Amino-2(4-chlorophenyl)-2-hydroxy-propylsulphonic acid (2-hydroxysaclofen; 2-OH-S; Fig. 1) showed a ten-fold increase in potency compared to phaclofen at peripheral and central $GABA_B$ receptors (Curtis et al., 1988; Kerr et al., 1988; Lambert et al., 1989; Al-Dahan et al., 1990). The direct sulphonic acid derivative, 3-amino-2-(4-chlorophenyl)-propylsulphonic acid (saclofen) was slightly more potent with an estimated pA_2 of 5.3 against baclofen responses in guinea-pig ileum and rat cortical neurones compared to a pA_2 of 5 for 2-

hydroxysaclofen (Kerr et al., 1989).

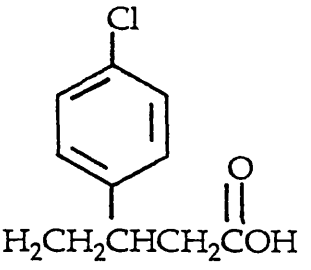
A new compound 3-aminopropylphosphinic acid (3-APA; Fig. 1), synthesized in 1987 (Dingwall et al., 1987) was reported to have higher affinity for $GABA_B$ binding sites in rat brain membranes than baclofen. This was confirmed, and an IC_{50} of 1-3nM obtained in rat brain synaptic membranes and slices for 3-APA compared with 65nM and 30nM for baclofen respectively (Pratt et al., 1989). 3-APA was also selective for $GABA_B$ receptors in this study, the IC_{50} for the displacement of [^{3}H]-GABA from $GABA_A$ receptors in membranes and slices was 420nM and 20μM respectively. 3-APA was a potent $GABA_B$ agonist in both peripheral preparations (Hills et al., 1989) and at presynaptic $GABA_B$ receptors on embryonic rat hippocampal neurones in culture (Ong et al., 1990a). The methyl derivative, 3-aminopropyl(methyl)phosphinic acid (SKF 97541; Fig. 1) was of comparable or greater potency and more selective for $GABA_B$ receptors over $GABA_A$ receptors than 3-APA itself (Hills and Howson, 1990; Seabrook et al., 1990) but its overall profile of activity was similar.

CGP 35348 (p-3-aminopropyl)-p-diethyloxymethyl-phosphinic; Fig. 1) acid represented a breakthrough in $GABA_B$ receptor pharmacology as it was the first $GABA_B$ antagonist, though still a relatively weak compound, to demonstrate appreciable brain penetration following peripheral administration. CGP 35348 was found to selectively inhibit specific [^{3}H]-3-APA binding from rat cortical membranes with an IC_{50} of 34 μM (CGP 35348 was devoid of activity in eleven other receptor binding assays), to inhibit the baclofen

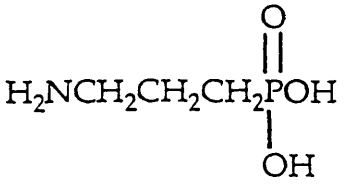
Fig. 1 GABA, Baclofen and Related $GABA_B$ Ligands



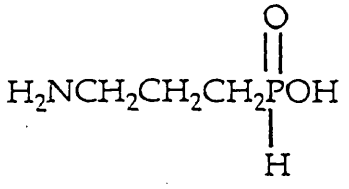
GABA



Baclofen



3-APPA



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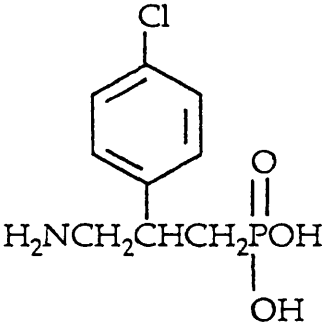
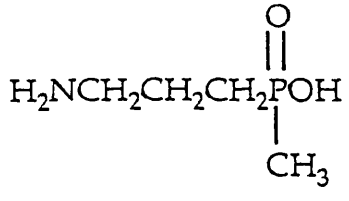
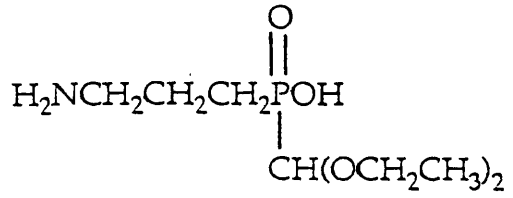


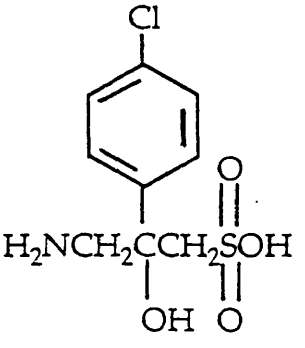
Phaclofen



SKF 97541



CGP 35348



2-OH-Saclofen

potentiation of noradrenaline-stimulated cAMP accumulation in brain slices (at 100-1000μM), to block the late i.p.s.p. and baclofen-induced hyperpolarization of hippocampal neurones (at 30μM), to reverse depression of spinal neurones by baclofen (at 100μM), to prevent impairment of rotorod performance by baclofen (at 30-300mg/kg) and most importantly to inhibit the effect of iontophoretically applied baclofen, but not of the $GABA_A$ agonist THIP, on cortical neurones following peripheral intravenous (10-30mg/kg), oral (600-1000mg/kg) or intraperitoneal (30-100mg/kg) administration (Olpe et al., 1990). Whilst CGP 35348 does not show an increase in potency over saclofen *in vitro* it has the advantage of brain penetration and is without activity at $GABA_A$ receptors.

3) $GABA_B$ receptor-linked intracellular effector systems.

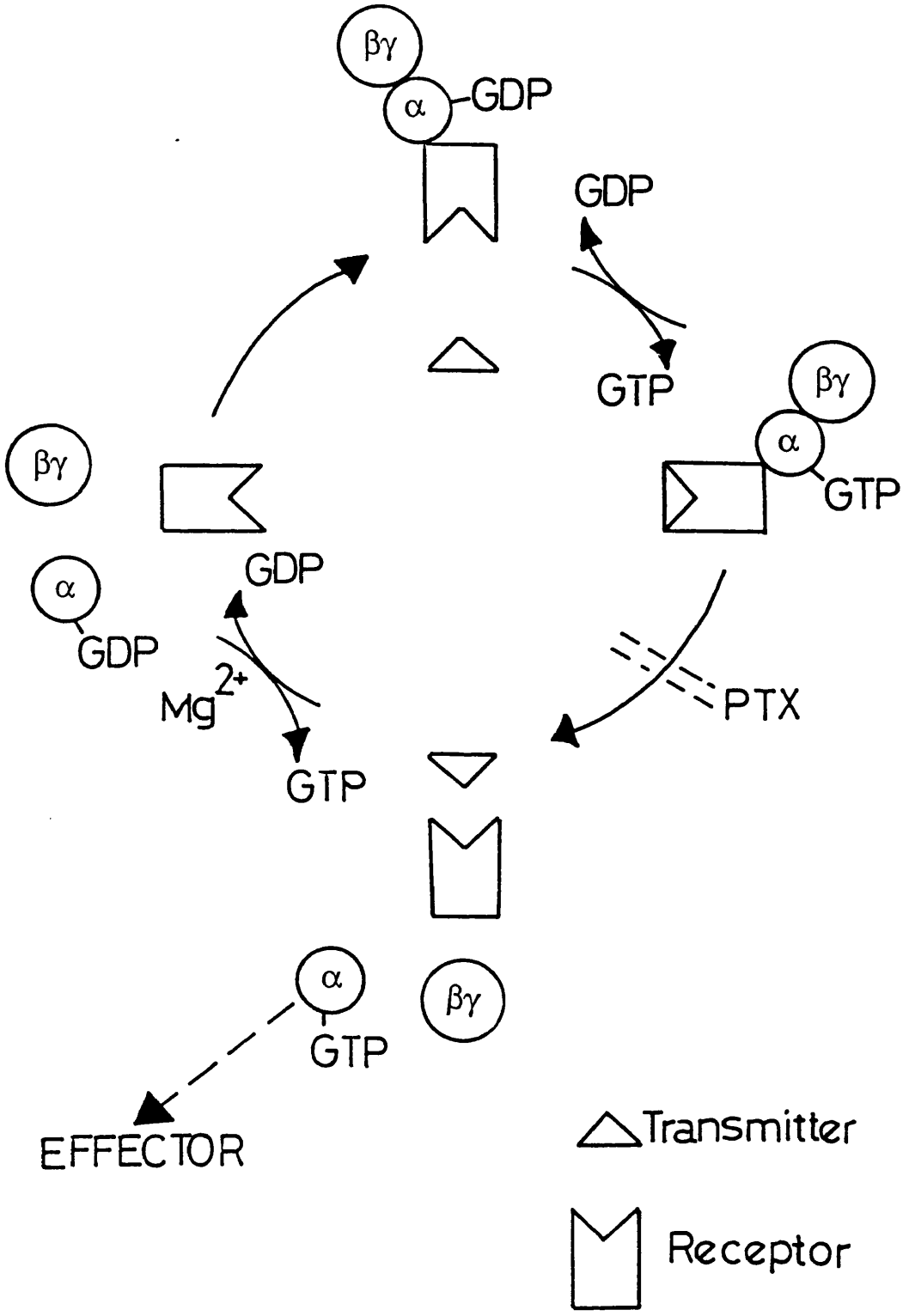
A characteristic of $GABA_B$ binding uncovered by Hill and co-workers (1984) was that it is sensitive to guanyl nucleotides. The inclusion of either guanosine triphosphate (GTP) or guanosine diphosphate (GDP) reduced the saturable binding of [^{3}H]-GABA or [^{3}H]-baclofen to rat brain membranes by 85%. Guanosine monophosphate (GMP) and adenosine triphosphate (ATP) were without effect. In contrast $GABA_A$ binding in the same experiment was unaffected by any of these compounds. The potency of GTP to reduce $GABA_B$ binding was enhanced if the Tris buffer plus Ca^{2+} or Mg^{2+}, which was used for the study, was replaced by a complete physiological saline solution. Rodbell (1980) suggested that an influence of guanyl nucleotides on ligand

binding is indicative of a link between the receptor and the enzyme adenylate cyclase which regulates the formation of the intracellular second messenger adenosine 3',5'-monophosphate (cyclic AMP; cAMP). We now know that this is not strictly correct and that an influence of guanyl nucleotides reflects an association of the receptor with one of a family of membrane bound guanyl nucleotide-binding proteins (G proteins) through which activation of the cell surface receptor effects an intracellular response. The increased effectiveness of GTP in Krebs medium was therefore probably due to the presence of Na^+ which appears to be essential for receptor-G protein interaction (Rodbell, 1980).

G proteins are trimers composed of an α-subunit (39-52kDa) which contains the guanyl nucleotide binding site and intrinsic GTP'ase activity, a β-subunit (35-36kDa) and a γ-subunit (8kDa) (see Stryer and Bourne, 1986 for review). Different α-subunits have been isolated and sequenced and it is this moiety which defines the G protein as stimulatory (G_s) or inhibitory (G_i) with respect to cellular response (Itoh et al., 1986). A series of inhibitory $G_{i\alpha}$ Subunits have been identified: G_{i1}, G_{i2}, G_{i3}, G_o and G_{o^*} (Goldsmith et al., 1988; Neer and Clapham, 1988). Whereas $G_{s\alpha}$ is a substrate for ADP-ribosylation catalysed by cholera toxin (Cassel and Pfeuffer, 1978) the $G_{i\alpha}$'s and $G_{o\alpha}$'s are ADP-ribosylated by pertussis toxin (PTX), an exotoxin of *Bordetella pertussis* (Katada and Ui, 1982; Codina et al., 1983). Specifically, $G_{i\alpha}$-GDP or $G_{i\alpha}$-GTP are the substrates and PTX covalently modifies a cysteine residue (residue 347) close to the carboxy terminus of the α subunit which results in the loss of its ability to bind the activated receptor protein. The inhibition of a receptor mediated

effect by PTX is therefore indicative of an involvement of one or more of the inhibitory G proteins. Two β-chains have been described but appear to show 90% homology, and at least three different γ-chains may exist (Gilman, 1987; Lochrie and Simon, 1988; Jones et al., 1990). G Proteins cycle between an inactive GDP-bound state and an active GTP-bound state. Conversion between the two is usually slow but is accelerated by the proximity of a transmitter-bound receptor protein. GTP is exchanged for the bound GDP and this catalyses the dissociation of the GTP-α-subunit complex from the βγ complex and from the excited receptor. The latter reverts to a low affinity state, the transmitter is then more likely to dissociate and so activation of G proteins is linked to the recycling of receptors. The freed α-subunit is then able to alter the activity of intracellular enzymes or ion channels before it is inactivated by its own ability to convert bound GTP to GDP (Fig. 2). Thus the G protein is recycled for activation by another 'excited' receptor. Receptor proteins can associate with many G proteins just as G proteins can be activated by different receptor types. Therefore this interaction represents a point of convergence and amplification in the physiological regulation of cell activity. Initially the role of the βγ complex was thought to be limited to presenting the G_α-GDP species to the activated receptor. However a more substantial involvement of βγ is likely. As βγ units are functionally interchangeable it may be that free βγ's can 'mop up' active G_α's and thus influence cell activity, this is in addition to any direct effect they may possibly possess (see Taylor, 1990 for review). Asano and colleagues (1985) first demonstrated that PTX ADP-ribosylated 39kDa and 49kDa proteins in bovine cerebral cortex

Fig. 2 Schematic Representation of $GABA_B$ Receptor and Inhibitory G-Protein Interaction.



membranes with the concomitant reduction in high affinity $GABA_B$ binding. Addition of either unpurified G_i or G_o to the incubation medium restored the high affinity binding. A later study, using highly purified G proteins showed that this occurred with added G_{i1}, G_o and G_{o*} but not G_{i2} (Morishita et al., 1990).

Functional evidence for the association of $GABA_B$ receptors to five different intracellular effector systems, all of which may involve G_i activation is discussed below:

i) Inhibition of adenylate cyclase.

Adenylate cyclase activity is regulated by both stimulatory and inhibitory G-proteins, activation of which leads to an increase or decrease respectively in intracellular cAMP formation. In brain slice preparations $GABA_B$ receptor agonists had either no effect on (Hill and Dolphin, 1984; Hill, 1985) or slightly enhanced (Karbon et al., 1984) basal adenylate cyclase activity. However, in crude synaptic membranes prepared from a number of rat brain regions, $GABA_B$ receptor activation resulted in an inhibition of basal adenylate cyclase activity (Wojcik and Neff, 1984) ranging from 5% in hypothalamus to almost 30% in the cerebellum. Furthermore, (-)baclofen (EC_{50} 4μM) was more potent than GABA (EC_{50} 17μM), whilst (+)baclofen, muscimol and other $GABA_A$ agonists were effective only in the low mM range or were inactive. From similar experiments using membranes prepared from the cerebella of mutant

mice lacking either Purkinje cells or granule cells, or from rats in which the cerebellar climbing fibres had previously been lesioned, it was apparent that inhibition of adenylate cyclase in cerebellum requires an intact granule cell layer. Since autoradiographic studies revealed that $GABA_B$ receptors predominate in the cerebellar molecular cell layer rather than the granule cell layer these results imply that $GABA_B$ receptors are located on the terminals of cerebellar granule cells (parallel fibres) which are present in the molecular cell layer (Wojcik and Neff, 1984).

This initial study was verified by the observation that $GABA_B$ agonists inhibited adenylate cyclase in primary cultures of cerebellar granule cells, using either intact cells or cell membrane preparations (Xu and Wojcik, 1986). This response was attenuated by pretreatment of the cells for 14 hours with PTX implying that $GABA_B$ receptor mediated inhibition of adenylate cyclase was due to the activation of an inhibitory G protein.

ii) Modulation of stimulated-adenylate cyclase activity.

The sensitivity of $GABA_B$ binding to guanyl nucleotides intimated a connection between $GABA_B$ receptors and adenylate cyclase. In spite of the lack of effect of either GABA (100μM) or baclofen (100μM) on basal adenylate cyclase activity in slices of rat cerebral and cerebellar cortex (but see Wojcik and Neff,

1984) Hill (1985) found that the accumulation of cAMP triggered by noradrenaline (100μM) was more than doubled in the presence of either compound. This enhancement by baclofen was stereoselective, bicuculline-insensitive and therefore presumably $GABA_B$ receptor mediated. A lack of Ca^{2+}-dependence ruled out the likelihood that this response was due to the synaptic release of some other mediator.

Since these early observations it has been established that activation of $GABA_B$ receptors will enhance cAMP accumulation stimulated by a number of agonists including isoprenaline, histamine, adenosine and vasoactive intestinal peptide (VIP) (Enna and Karbon, 1984; Karbon and Enna, 1985; Watling and Bristow, 1986). The main effect of baclofen seems to be to increase the magnitude of the response to the stimulatory agonist with only a modest enhancement of the agonist affinity for its receptor (Karbon et al., 1984). This is not clearly explained by the finding that $GABA_B$ agonists enhance the affinity of β-adrenoceptor agonists for both high and low affinity β-adrenergic receptors and, whilst the total number of receptors remains unchanged, the proportion of low affinity binding sites is increased (Scherer et al., 1989). Whatever the mechanism this effect of baclofen appears to be limited to intact cell preparations as no effect of baclofen was observed on cAMP production stimulated by either noradrenaline or isoprenaline in rat cortical membranes (Hill and Dolphin, 1984).

There are discrepancies in the published findings concerning some aspects of $GABA_B$ receptor modulation of agonist-stimulated cAMP formation:

a) Hill (1985) claimed that the baclofen augmentation of noradrenaline response was Ca^{2+}-independent. In the same year Karbon and Enna wrote 'the synergistic interaction between baclofen and catecholamines is a calcium-dependent process'.

b) Hill (1985) obtained identical results using slices of rat cerebral cortex and rat cerebellum. Karbon and Enna (1985) however, demonstrated the baclofen augmenting response in slices of rat cerebral cortex, hippocampus and striatum but not cerebellum, spinal cord or pons-midbrain.

c) Bowery and co-workers (1989) failed to show any decrease in the effectiveness of baclofen on noradrenaline-stimulated cAMP formation following *in vivo* intrahippocampal administration of PTX. In contrast, intracerebroventricular administration of PTX did result in a reduction in the baclofen enhancement of isoprenaline-stimulated cAMP formation by 64% compared to control tissue (Wojcik et al., 1989) and ADP-ribosylation of G proteins was verified by back-ribosylation showing that 40-50% of the available G_i had been inactivated. These inconsistencies make it even more difficult to determine the process by which this 'synergistic interaction' occurs. One point on which both groups agree is that, as phosphodiesterase inhibitors are without effect; baclofen is not mediating its response by preventing the breakdown of accumulated cAMP.

α-Adrenergic agonists also potentiate agonist-stimulated cAMP accumulation in brain slice preparations (Duman et al., 1986) whilst in broken cell preparations, they like baclofen, only mediate a direct inhibition of basal adenylate cyclase activity (Kitamura et al., 1985). As some groups found the potentiating effect of $GABA_B$ and α-adrenergic agonists to be abolished by the Ca^{2+} chelator EGTA (Schwabe and Daly, 1977; Karbon and Enna, 1985; Duman et al., 1986) it was proposed that stimulation of both receptor types may increase intracellular Ca^{2+} and thereby activate Ca^{2+}-dependent enzyme systems. This theory is supported by several pieces of indirect evidence. Mepacrine, a non-selective inhibitor of phospholipase A_2 (PLA_2) and protracted administration of corticosteroids which stimulate endogenous PLA_2 inhibitors, both reduced the augmenting response of baclofen and α_1 agonists (Duman et al., 1986). PLA_2 catalyses the release of arachidonic acid from membrane phospholipids and as inhibitors of cyclo-oxygenase or lipoxygenase were ineffective against the augmentation, it is possible that arachidonic acid itself, or some other direct product of PLA_2 activity, is involved (Duman et al., 1986; Schaad et al., 1989). Phorbol esters, which directly activate a second Ca^{2+}-dependent enzyme protein kinase C (PKC), mimic the baclofen enhancement of agonist stimulated cAMP formation (Hollingsworth et al., 1985; Karbon et al., 1985). Although a possible involvement of PKC has been proposed (Enna and Karbon, 1987) on the basis that fatty acids, including arachidonic acid, are able to directly stimulate PKC (Katada et al., 1985; Murukami and Routtenberg, 1985), this is unlikely as the PKC inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) does not block the baclofen

potentiation of VIP-stimulated cAMP accumulation (Scherer et al., 1988; Schaad et al., 1989).

Adenylate cyclase can be directly stimulated by the diterpine forskolin (Saemon and Daly, 1983). In membrane and slice preparations this response is attenuated by baclofen (Hill, 1985; Karbon and Enna, 1985; Xu and Wojcik, 1986) in a PTX-sensitive manner (Bowery et al., 1989) indicating once more an involvement of inhibitory G-proteins. Why baclofen should have two opposing effects, depending on the nature of the adenylate cyclase stimulus, has not been resolved, but is discussed later in terms of $GABA_B$ receptor heterogeneity.

iii) $GABA_B$ receptor-mediated inhibition of calcium channels.

The effect of GABA on calcium currents (I_{Ca}) was first observed in 1978 by Dunlap and Fischbach. GABA decreased the duration of the calcium action potential evoked in cultured embryonic chick sensory neurones by inhibiting calcium conductance through voltage-sensitive channels (Dunlap and Fischbach, 1981). In 1981 Dunlap reported that GABA elicited two distinct responses in these cells: a reduction in resting membrane resistance and/or a reduction in action potential duration, but only 10% of cells showed both responses. The responses could be distinguished by their pharmacological profiles; muscimol evoked only the resistance change whereas the GABA-induced reduction in action potential amplitude was mimicked by baclofen.

Only the change in resistance was sensitive to the $GABA_A$ antagonist bicuculline. Similar results have since been obtained from intracellular recordings from Aδ and C primary afferents in the adult rat dorsal root ganglion (DRG) (Desarmenian et al., 1984), voltage clamp experiments on isolated cat DRG (Robertson and Taylor, 1986) and from whole cell patch clamp studies using cultured mouse DRG (Green and Cotrell, 1987). Given that Bowery and colleagues had previously described the inhibition of neurotransmitter release from sympathetic terminals by activation of $GABA_B$ receptors the mechanism involved could attractively be explained if $GABA_B$ receptor activation reduced calcium entry into nerve terminals as it appears to in the cell soma.

In primary cultures of embryonic chick DRG cells preincubation in PTX (140ng/ml) led to a reduction in the number of cells showing an attenuation of action potential duration by GABA, and the effectiveness of GABA was reduced in those cells still responding (Holz et al., 1986). In the same cells whole cell patch clamp recordings showed that PTX blocked the GABA-induced decrease in calcium current and this effect was specific for a receptor-mediated event as PTX did not reduce the response of the cells to the 1,2-oleoyl acetylglycerol (OAG), an activator of protein kinase C which mimics the effects of GABA on DRG cells. The evidence supporting an involvement of G-proteins in GABA-mediated inhibition of calcium channels was substantiated by the observation that the effect of PTX was mimicked by intracellular administration of 5'-O-(2-thiodiphosphate) (GDP-β-S), a non-hydrolysable GDP

analogue which competes with GTP and thereby inactivates G-proteins. This study provided the first direct evidence of G-protein-mediated inhibition of voltage-dependent calcium channels by a neurotransmitter (Holz et al., 1986). It is conceivable that $GABA_B$ receptor-mediated inhibition of calcium channels is a consequence of $GABA_B$ mediated activation of inhibitory G-proteins and thus a lowering of intracellular cAMP concentration. This is unlikely, however, as neither intracellular cAMP nor forskolin had any detrimental effect on the baclofen-mediated inhibition of the calcium current in cultured rat DRG neurones (Dolphin and Scott, 1987).

The existence of a range of pharmacological tools that distinguish between the known calcium conductances has made the task of determining the type of calcium channel which is blocked by $GABA_B$ receptor activation possible. Two high threshold channels have been identified: the 'L'-type is sensitive to dihydropyridines and is able to open at depolarized potentials (Hess et al., 1984); the N-channel, which has a smaller single channel conductance, gives rise to a transient current and is insensitive to dihydropyridines (Tsien et al., 1988). w-Conotoxin was thought to be an inhibitor of N-channels, however it is more probable that this peptide blocks neuronal but not muscle calcium channels and inhibits both L- and N-currents (McCleskey et al., 1987). In cultures of rat hippocampal pyramidal neurones the calcium current evoked at 0mV from a holding potential of -80mV is reduced by baclofen (10μM) by 33%, an effect blocked by the weak $GABA_B$ antagonist 2-OH-saclofen (Scholz and Miller, 1991). w-Conotoxin also reduced some portion of the calcium

current (24%) but not to the same degree as baclofen, though subsequent application of baclofen was less effective than when previously applied alone. The dihydropyridine calcium channel antagonist nimodipine also reduced the evoked calcium conductance, however it is more effective if the voltage step was made from the more depolarized holding potential of -40mV. Under these conditions nimodipine inhibited 44% of I_{Ca} and partially reduced the response to baclofen, whereas the combination of w-conotoxin and nimodipine produces an almost complete abolition of the baclofen inhibition of I_{Ca}. GTP-γ-S enhanced the effect of baclofen whilst it was attenuated by pretreatment with PTX (250ng/ml) (Scholz and Miller, 1991).

The inhibition of calcium conductance in hippocampal neurones paralleled the ability of baclofen to inhibit both excitatory and inhibitory post synaptic currents evoked by extracellular stimulation of neurones in rat hippocampal slices (Dutar and Nicoll, 1988a). This inhibition was also antagonized by 2-OH-saclofen and prevented by PTX pretreatment. Therefore the receptors mediating presynaptic inhibition in this preparation were indistinguishable from those responsible for mediating inhibition of I_{Ca} (Scholtz and Miller, 1991). To some extent these findings are supported by results obtained in rat cultured cerebellar granule cells and DRG cells. Cerebellar neurones were maintained in a calcium-free depolarizing medium (50mM K^+) and [^{3}H]-glutamate release evoked by a 2-minute incubation in the same medium but containing 5mM Ca^{2+}. Glutamate release was almost completely inhibited (>90%) by the dihydropyridine antagonist (-) 202-791 whereas release was

enhanced by the agonist (+) 202-791. (-) Baclofen (100μM) reduced evoked release by 30-50% (Huston et al., 1990). For comparison in DRG neurones, again under depolarized conditions, both baclofen and (-) 202-791 inhibited the amplitude of the calcium channel current. In both experimental systems although phaclofen was able to reduce the effect of baclofen, 2-OH-saclofen was not, which contrasts to the previous findings. It was concluded that since N-channels should be inactivated at these levels of depolarization the effect of baclofen is probably exerted to a large extent through inactivation of L-type calcium channels. An indirect effect of dihydropyridines on [^{3}H]-baclofen binding has been reported in which stimulation of $GABA_B$ binding is induced by calcium channel agonists whereas binding is inhibited by 75% by a calcium channel antagonist. This inhibition of binding reflects a reduction in $GABA_B$ binding sites rather than a change in the affinity of the receptor for baclofen (Al-Dahan and Thalmann, 1989).

These studies indicate that $GABA_B$ receptors may mediate the inactivation of calcium channels with both L- and N-type profiles. In addition it has recently been observed that a low threshold, transient calcium current (T-current) is also sensitive to baclofen (Dolphin et al., 1990) showing both an inhibition or an enhancement depending on the baclofen concentration. It has been suggested that T-currents are involved in the regulation of Ca^{2+} supply for transmitter release (Seabrook and Adams, 1989) and therefore inhibition by baclofen would result in a reduction in transmitter overflow. A physiological role for $GABA_B$ mediated enhancement of T currents has been proposed in the

priming of thalamocortical cells for burst firing excitation, as observed for example during slow wave sleep (Crunelli and Leresche, 1991).

A second experimental approach has involved measuring the effect of $GABA_B$ receptor activation on Ca^{2+} influx into rat brain synaptosomes using the fluorescent probe Quin-2. Cerebellar synaptosomes loaded with Quin-2 ester when depolarised produce a K^+-concentration dependent increase in fluorescence indicating a concomitant rise in intracellular Ca^{2+} concentration (Bowery et al., 1987). This signal is reduced by simultaneous application of either (-)baclofen, GABA, or nitrendipine but not (+)baclofen or isoguvacine. Identical results were obtained in rat cerebral cortical synaptosomes though it was noted that the weak $GABA_B$ antagonist phaclofen behaved as an agonist (Stirling et al., 1989).

iv) $GABA_B$ receptor-mediated increase in potassium conductance.

Baclofen induced hyperpolarizations of central neurones mediated by an increase in potassium conductance have been observed in hippocampal CA1 (Newberry and Nicoll, 1984; Nicoll and Newberry, 1984) and CA3 cells (Inoue et al., 1985; Brown and Gahwiler, 1987) and in neurones of the cerebral cortex (Howe, 1987; Howe et al., 1987), locus coeruleus (Osmanovic and Shefner, 1988), lateral parabrachial (Christie and North, 1988) and dorsolateral septal (Gallagher et al., 1984) nuclei. The effect of baclofen is stereoselective, bicuculline-insensitive and mimicked by GABA and is therefore presumably

due to activation of $GABA_B$ receptors. Electrical stimulation of excitatory afferent pathways in, for example, hippocampus, thalamus and cerebral cortex evokes a characteristic series of post synaptic potentials consisting of an excitatory post synaptic potential (e.p.s.p.), a fast inhibitory post synaptic potential (i.p.s.p.) and a late, slow i.p.s.p. (Satou et al., 1982; Newberry and Nicoll, 1984; Crunelli et al., 1987). The fast i.p.s.p. is Cl^-- dependent, bicuculline-sensitive mimicked by applied GABA and muscimol and therefore due to activation of $GABA_A$ receptors. The late i.p.s.p. is K^+-dependent, resistant to biculline, antagonized by phaclofen (Dutar and Nicoll, 1988; Soltesz et al., 1988) and has identical properties to the baclofen-induced hyperpolarization described above and thus it is thought to be mediated by $GABA_B$ receptor activation (Newberry and Nicoll, 1985). In the hippocampus, at least, the $GABA_B$ agonist-induced hyperpolarization and the electrically-evoked late i.p.s.p.(but not the fast i.p.s.p.) are blocked by pretreatment with PTX or by intracellular injection of GTP-γ-S into post synaptic neurones (Andrade et al., 1986; Thalmann, 1988). This provides good evidence that $GABA_B$-mediated increases in K^+-conductance are activated via an inhibitory G-protein in the same way as previously described for $GABA_B$-inactivation of Ca^{2+}-conductance.

In addition to the direct hyperpolarization of neurones baclofen will inhibit postsynaptic potentials by reducing the amount of excitatory and inhibitory neurotransmitters available for release (Ault and Nadler, 1982). In rat neocortical neurones this was not associated with a shortening of the duration

of the calcium spike (Howe et al., 1987) and therefore not due to inactivation of calcium channels. It is not inconceivable that hyperpolarization of presynaptic terminals by a $GABA_B$-induced opening of K^+-channels, reducing neuronal excitability might result in a reduction in transmitter release. As yet, however, there is no strong evidence to support this possibility.

The precise nature of the K^+-channels opened by $GABA_B$ receptor activation is not known. The reversal potential for the baclofen-induced increase in membrane conductance has been typically found to be about -85mV, close to that of the slow after-potential, recorded following a series of action potentials, which is thought to be due to a calcium-activated potassium conductance (Alger and Nicoll, 1980). There is no evidence, however, that baclofen activates such a conductance. In hippocampus the baclofen-induced K^+-current was unaffected by the removal of calcium from the external medium (Inoue et al., 1985) or by the injection of Ca^{2+}-chelators directly into the cell (Andrade et al., 1986). Additionally, in purified synaptosomes prepared from rat cerebral cortex baclofen inhibited rather than enhanced K^+-stimulated [^{86}Rb]-efflux (a marker for Ca^{2+}-activated potassium channels). This was probably due to an inhibition of voltage-sensitive calcium-channels rather than any direct effect on K^+-channels (Ticku and Delgado, 1989).

Studies indicate that the K^+ current activated by $GABA_B$ agonists is sensitive to low concentrations of 4-aminopyridine (4-AP), is voltage-dependent and therefore distinguishable from 'M' type K^+ currents, and shows inward

rectification (Inoue et al., 1985; Newberry and Nicoll, 1985). These are properties displayed by a novel transient K^+ current, the A current, first described in invertebrate neurones (Connor and Stevens, 1971) but also present in the mammalian CNS (Gustafsson et al., 1982). In cultured rat hippocampal neurones A currents are almost totally inactive at resting membrane potentials. GABA or baclofen removes this inactivation (Saint et al., 1990). If A currents are present presynaptically and affected in this way by $GABA_B$ agonists, the resulting transient rise in K^+ conductance may reduce action potential duration by increasing the rate of repolarization and may thus depress transmitter release.

This effect on A current is reportedly not sensitive to PTX and may therefore represent a direct effect of $GABA_B$ receptor activation on K^+-channel opening. As discussed above a proportion of the $GABA_B$ agonist-induced increase in K^+ conductance is PTX sensitive and these K^+ channels may be linked directly to inhibitory G-proteins or to a second messenger system. Activation of phospholipase A_2 (PLA_2) to release arachidonic acid from membrane phospholipids has been implicated, because application of arachidonic acid to the inner surface of an inside out hippocampal neurone patch increased K^+ conductance in a similar manner to that induced by $GABA_B$ agonists (Premkumar et al., 1990). However this argument is not supported by the observation that PLA2 inhibitors do not block the baclofen-mediated reduction of e.p.s.p.'s in the hippocampal slice. (Dunwiddie et al., 1990).

v) $GABA_B$ receptor-mediated modulation of phospholipid metabolism.

The evidence that $GABA_B$ receptors are linked to the metabolism of membrane bound phospholipids is not as conclusive as it is for the effector systems discussed above but it is implied by a few recent studies. Baclofen has no effect on basal accumulation of inositol monophosphate (IP_1) in lithium-treated cerebral cortex slices of either rat (Crawford and Young, 1988) or mouse (Godfrey et al., 1988). However, baclofen potently (IC_{50}<1μM) inhibits IP_1 accumulation stimulated by histamine H_1-receptor or 5-hydroxytryptamine 5-HT_2-receptor activation respectively. This effect is stereoselective , not mimicked by $GABA_A$ agonists and resistant to bicuculline. The mechanism by which baclofen modulates neurotransmitter-stimulated IP_1 formation is a matter for speculation at present and its determination is made more difficult by the fact that the mode of action of histamine itself is not well understood. An interesting observation was made by Michler and Erdo (1989) in cultured chick tectum neurones, neither GABA, baclofen nor the $GABA_A$ agonist tetrahydroisoxasolo-pyridinol (THIP) had any effect on IP_3 accumulation but surprisingly phaclofen potently elevated IP_3 levels. That phaclofen was effective at concentrations much lower than usually required to antagonize $GABA_B$ responses, low micromolar rather than low millimolar, suggests a pharmacological activity of this compound that is additional to its ability to block $GABA_B$ receptors.

IV $GABA_B$ RECEPTOR HETEROGENEITY.

The availability of new $GABA_B$ ligands and the use of pharmacological tools such as PTX has led to findings which are not easily explained in terms of our current knowledge of $GABA_B$ receptor pharmacology. Increasingly such apparently anomalous results are being put forward as evidence for $GABA_B$ receptor heterogeneity. In this the $GABA_B$ field trails far behind advances in $GABA_A$ receptor pharmacology; not only has the $GABA_A$ receptor been purified and partially sequenced, but the isolation of a family of clones and the identification of gene products makes the likelihood of multiple receptor subtypes almost certain. Evidence for $GABA_B$ receptor subtypes is more circumstantial.

1) $GABA_B$ modulation of agonist- and forskolin-stimulated cAMP formation can be distinguished by $GABA_B$ agonists and PTX.

The observation that baclofen is able to potentiate agonist-stimulated cAMP formation in brain slice preparations yet in the same system it attenuates the stimulation of adenylate cyclase by forskolin has never been easy to explain. In broken cell preparations baclofen inhibits basal cAMP accumulation, probably through the activation of G_i or G_o, and this may account for the reduction in forskolin response but is contradictory to the augmenting response. Support for the involvement of two distinct $GABA_B$ receptor subtypes comes from a number of sources. (-)Baclofen and a number of amino

acid analogues were compared for their ability to inhibit $GABA_B$ binding in rat brain membranes and to modulate stimulated adenylate cyclase activity (Scherer et al., 1988a). Of the seven compounds tested only two, in addition to baclofen, had appreciable affinity for $GABA_B$ binding sites. These were 3-APPA and its higher homologue 4-aminobutylphosphonic acid (4-ABPA). The remaining four compounds, one of which was phaclofen, had no effect on isoprenaline- or forskolin-stimulated cAMP accumulation in rat brain cortical slices, in the absence or presence of (-)baclofen. However, whereas 3-APPA mimicked (-)baclofen's ability to inhibit the forskolin response it neither enhanced the isoprenaline response, nor did it inhibit the ability of baclofen to do so. 4-ABPA was inactive in both assays. The more potent agonist 3-APA also distinguished between these $GABA_B$ mediated effects. It potently (EC_{50} 3μM) inhibited forskolin-stimulated cAMP accumulation to the same extent as (-)baclofen yet was much less potent as an enhancer of noradrenaline-stimulated cAMP accumulation (EC_{50} 25μM) and produced only 60% of the maximal response to (-)baclofen. Antagonism of the baclofen response by high concentrations of 3-APA support the claim that the compound may be a partial agonist in this system (Pratt et al., 1989).

The effect of PTX pretreatment on $GABA_B$ modulation of adenylate cyclase activity has also been investigated, with conflicting results. In hippocampal slices, taken from the brains of rats sacrificed 3-8 days after intrahippocampal administration of PTX (4μg), (-)baclofen failed to inhibit the forskolin-stimulated accumulation of cAMP but was still able to enhance noradrenaline-

stimulated cAMP accumulation as in control tissue (Bowery et al., 1989). This would indicate that only $GABA_B$-mediated inhibition of forskolin-stimulated adenylate cyclase activity is mediated via G_i/G_o. However, in rat cortical slices *in vivo* PTX pretreatment reduced the baclofen enhancement of isoprenaline-stimulated cAMP formation. No effect on the $GABA_B$ inhibition of forskolin stimulated cyclase was detectable as PTX attenuated the forskolin response directly (Wojcik et al., 1989). To propose the involvement of both PTX-sensitive and PTX-insensitive $GABA_B$ receptors in the modulation of stimulated adenylate cyclase activity is therefore difficult, but not inconceivable.

2) Central $GABA_B$ receptors located pre-and postsynaptically exhibit different pharmacological characteristics.

It would be convenient if the presynaptic $GABA_B$-mediated reduction in transmitter release was due to an inhibition of calcium conductance, and the postsynaptic hyperpolarization due solely to the opening of potassium channels. However there is no clear indication that pre- and postsynaptic $GABA_B$ receptors can be subdivided on the basis of their associated ion channels alone. The first evidence of receptor heterogeneity based on synaptic location came from the elegant study of Dutar and Nicoll (1988b). They reported that in the CA1 region of the rat hippocampal slice both the baclofen-induced hyperpolarization and the late i.p.s.p. were reduced by high concentrations of phaclofen or pretreatment of the animals with PTX. The

presynaptically mediated baclofen attenuation of evoked e.p.s.p. was unaffected by either agent. Subsequent investigations have confirmed this observation; in the hippocampus presynaptic inhibition by neuropeptide Y, or baclofen, was insensitive to PTX whereas the postsynaptic effects of baclofen and 5-hydroxytryptamine were sensitive to the toxin (Colmers and Pittman, 1989); in the dorsal raphe nucleus PTX selectively inhibited the postsynaptic action of baclofen with no effect on its presynaptic action (Colmers and Williams, 1988); in cortical neurones the presynaptic $GABA_B$ response, measured as a paired pulse depression of the early i.p.s.p., was unresponsive to phaclofen, 2-OH-saclofen or CGP 35348 but the late i.p.s.p. was antagonized by all three (Deisz et al., 1992); in the corticostriatal slice preparation the presynaptic effect of endogenous GABA was bicuculline-resistant, mimicked by baclofen but phaclofen-insensitive (Calabresi et al., 1990). Taken together these results provide strong evidence that presynaptic $GABA_B$ receptors in the CNS may not be linked to G_i/G_o, or they are linked to an, as yet, unidentified G protein that is not a substrate for ADP-ribosylation by PTX. The pharmacological specificity of this receptor appears to differ from the postsynaptic $GABA_B$ site.

3) Other evidence.

The pharmacological profile of $GABA_B$ receptors in the cerebellum appears to differ from that of $GABA_B$ receptors in other brain regions. Not only do $GABA_B$ agonists inhibit basal adenylate cyclase activity in the cerebellum (Wojcik and Neff, 1984) but there is also some disagreement as to whether or not cerebellar $GABA_B$ receptors mediate the facilitation of agonist-stimulated cAMP formation (Hill, 1985; Karbon and Enna, 1985). A recent study investigated the ability of $GABA_B$ ligands to inhibit specific [^{3}H]-baclofen binding from rat cerebellar membranes (Drew et al., 1990). The results indicated that the order of potency of phaclofen, 2-OH-saclofen and saclofen mirrored their known potencies as $GABA_B$ antagonists. However 3-APPA and 4-ABPA were very much more potent than expected, with IC_{50} values of 1.5μM and 3.9μM respectively compared to 0.14μM for (±)baclofen. Contamination by 3-APA may have explained these results but 3-APA itself was found to be inactive at $GABA_B$ receptors on cultured cerebellar neurones. Ca^{2+}-evoked glutamate release from these cells, under depolarizing conditions, was inhibited by (-)baclofen (100μM) but not 3APA (50μM) and the baclofen response was inhibited by phaclofen and PTX, but not 2-OH-saclofen (Huston et al., 1990). Presynaptic $GABA_B$ receptors on these neurones are therefore distinct from the presynaptic PTX-insensitive, phaclofen-insensitive hippocampal $GABA_B$ receptors described above.

There are other individual observations which also support the hypothesis of

$GABA_B$ receptor heterogeneity. In the rat neocortical slice, maintained in Mg^{2+}-free Krebs solution, baclofen will suppress spontaneous paroxysmal discharges and this effect can be antagonized by 2-OH-saclofen. 3-APA appears to have an additional effect in that though it produces some attenuation of discharge amplitude, with little effect on frequency, its main effect is a rapid hyperpolarization, a response only occasionally elicited by baclofen. Though the 3-APA diminution of amplitude was reversed by 2-OH-saclofen the hyperpolarization was not (Ong et al., 1990). A reason for this difference has yet to be determined, $GABA_B$ receptor heterogeneity is one possibility.

The results of a recent study persuaded Raiteri and colleagues that they had identified no less than three distinct presynaptic $GABA_B$ receptors on rat cortical synaptosomes with differing sensitivities to phaclofen and CGP 35348 (Bonanno and Raiteri, 1992). They investigated the effect of these antagonists on the inhibitory effect of (-)baclofen (10μM) on the K^+-evoked release of endogenous GABA, accumulated [^{3}H]-GABA, endogenous glutamate and somatostatin-like immunoreactivity (SS-LI). Phaclofen inhibited baclofen effects on all the systems except on glutamate release, even at 1mM. CGP 35348 antagonized the baclofen inhibition of glutamate and SS-LI release, was less potent against the inhibition of [^{3}H]-GABA and unable to block the reduction in endogenous GABA release produced by baclofen. Alone phaclofen increased the release of endogenous GABA but not glutamate, the reverse was true for CGP 35348. From this the authors suggested the existence

of a phaclofen-sensitive, CGP 35348-insensitive receptor present on GABAergic terminals; a phaclofen-insensitive, CGP 35348-sensitive receptor on glutamatergic terminals and a receptor sensitive to both antagonists on somatostatin containing neurones. They proposed to classify these receptors as $GABA_{B1}$, $GABA_{B2}$ and $GABA_{B3}$ respectively. Whether such receptors have physiological relevance remains to be seen.

V AIMS OF THESIS.

Given the premise that $GABA_B$ receptors located presynaptically in the CNS exhibit a pharmacology distinct from those located postsynaptically, the first part of this work was devoted to an investigation of central presynaptic $GABA_B$ receptors using the inhibition of neurotransmitter release as an index of presynaptic function. A subsequent comparison was made with peripheral presynaptic $GABA_B$ receptors in the classical $GABA_B$ preparation, the rat anococcygeus muscle. In the same preparation, monitoring both the antagonism of baclofen-induced inhibition of transmurally-stimulated [^{3}H]-noradrenaline overflow and twitch contraction, the potency of two novel $GABA_B$ ligands was compared to that of CGP 35348. Experiments were also performed, using receptor autoradiography, to determine whether any of the compounds under investigation exhibited a differential selectivity for CNS $GABA_B$ binding sites. It was hoped that such a study may highlight regional variations in the sensitivity of $GABA_B$ sites to particular compounds that would otherwise remain undetected in membrane binding studies.

The final part of the thesis is devoted to membrane binding experiments carried out following pretreatment of the tissue with PTX. Studies in this laboratory had previously demonstrated that in brain slices, cut from tissue blocks incubated *in vitro* with PTX, specific $GABA_B$ binding was reduced in all regions with the exception of the corpus striatum (Bowery et al., 1990). To clarify whether or not this was merely due to a particular inability of the toxin to access striatal neurones this study looked at the effect of PTX on $GABA_B$ binding in membranes prepared from rat corpus striatum and three brain regions previously shown to be PTX sensitive, the cerebral cortex, the hippocampus and the cerebellum.

CHAPTER TWO: METHODS

I CHARACTERIZATION OF PRESYNAPTIC $GABA_B$ RECEPTORS IN THE RAT HIPPOCAMPUS.

1) Tissue Preparation.

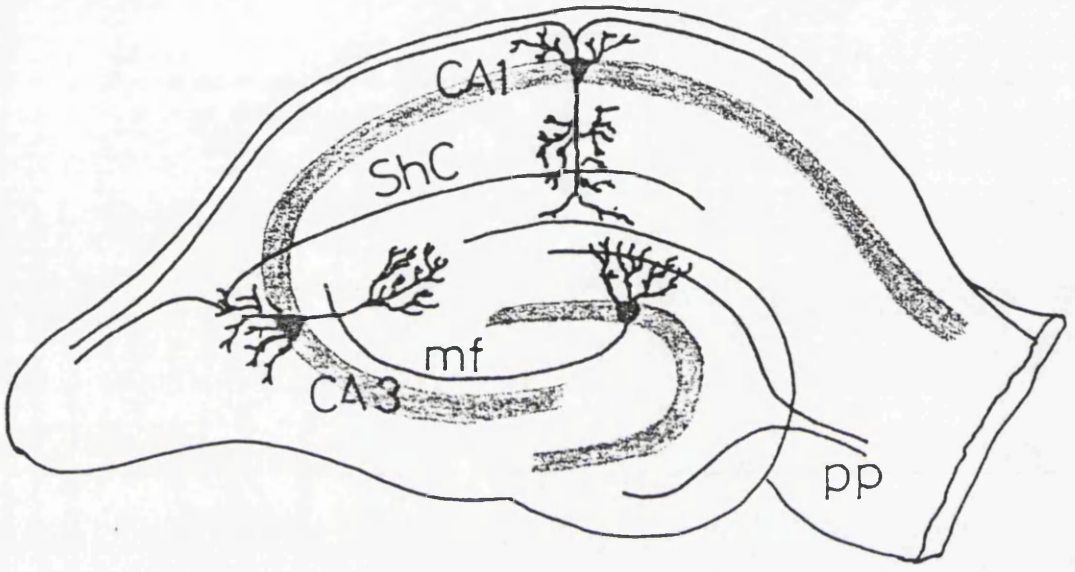
i) Preparation of rat hippocampal slices.

One male, Wistar rat (220-240g), was sacrificed for each experiment by stunning and decapitation. The brain was rapidly removed onto ice, halved and the hippocampi carefully dissected out. 350µm slices were cut using a M^{c}Ilwain tissue chopper. The tissue was transferred to a petri-dish containing warm, oxygenated Krebs-Henseleit solution (NaCl, 120mM; KCl, 3mM; $MgSO_4.7H_2O$, 1.2mM; $CaCl_2$, 1.8mM; NaH_2PO_4, 1.2mM; $NaHCO_3$, 25mM; glucose, 11mM: pH 7.4) and dispersed into individual slices with a small paint brush and fine forceps. A schematic representation of the preparation is shown in Fig. 3. This procedure was used for the preparation of hippocampal slices in all subsequent studies.

ii) Preparation of rat hippocampal synaptosomes.

Crude synaptosomes were prepared essentially as described by Gray and Whittaker (1962). Male, Wistar rats (220-240g) were sacrificed and hippocampi were removed. Tissue pooled from two animals was homogenized in 20 volumes of ice-cold 0.32M sucrose solution using a teflon/glass homogenizer.

Fig. 3 Schematic diagram of the rat hippocampal slice.



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The homogenate was centrifuged at 1000g, for 10 min, at 4°C. The resulting P_1 pellet, comprising nuclei and cell debris, was discarded and the supernatant (S_1) centrifuged for 20 min at 12,000g. Removal of the supernatant, S_2, yielded the crude synaptosomal pellet, P_2, which was then resuspended in 20 volumes of oxygenated Krebs-Henseleit solution, at 37°C.

2) K^+-Evoked Release of [^{3}H]-D-Aspartate from Rat Hippocampal Slices.

i) Determination of the time-course of accumulation of [^{3}H]-D-aspartate.

Prepared hippocampal slices were incubated, 4 slices per tube, in 0.5ml oxygenated Krebs-Henseleit solution containing 67nM [^{3}H]-D-aspartate, at 37°C, for up to 60 min. At t = 5, 10, 20, 30, 40 and 60 min the incubation medium was aspirated from three of the tubes, the slices were rinsed in 3 x 1ml aliquots of normal Krebs-Henseleit solution and solubilized in 200µl Soluene-350 overnight. All tissue samples were neutralized with 0.5ml of 0.2M HCl and their tritium content was determined by liquid scintillation counting.

ii) Determination of the time required for basal release of [^{3}H]-D-aspartate to stabilize.

Hippocampal slices were incubated in 3ml of oxygenated Krebs-Henseleit solution containing 67nM [^{3}H]-D-aspartate for 40 min at 37°C. The slices were

rinsed briefly in normal Krebs-Henseleit solution and 5 slices were transferred to each of 4 superfusion chambers. The chambers were submerged in a water bath maintained at 37°C for the duration of the experiment and the slices were perfused continuously with oxygenated Krebs-Henseleit (0.4ml/minute). To determine the time required for basal tritium release to stabilize 5 min fractions were collected for 160 min. At the end of the experiment the hippocampal slices from each chamber were solubilized in 200µl Soluene-350. The tissue was neutralized with 0.5ml of 0.2M HCl and counted, together with the 32 fractions, for tritium content.

Released tritium was expressed as 'fractional tritium release' (FTR), i.e. the amount of tritium released in each fraction expressed as a percent of the total tritium content of the tissue remaining at the beginning of that fraction.

$$\text{Fractional Tritium Release (FTR)} = \left(\frac{n_x}{t + (n_x + n_{x+1} + \ldots + n_{32})} \right) \times 100$$

where n_x = tritium content (dpm) of any one of the fractions 1-32.

t = tritium content of the solubilized slices at the end of the experiment.

iii) K^+-Evoked release of [^{3}H]-D-aspartate from rat hippocampal slices.

Hippocampal slices were incubated for 40 min in oxygenated Krebs-Henseleit solution containing 67nM [^{3}H]-D-aspartate. Slices were rinsed briefly and five transferred to each of four superfusion chambers. The preparations were then perfused with oxygenated Krebs-Henseleit solution (at 37°C) for 60 min to allow basal tritium release to stabilize. 5 Min perfusate fractions were collected over a period of 190 min. At times t=40, 90 and 140 min the perfusion medium was changed to a 'depolarizing' Krebs-Henseleit solution containing 10, 20, 35 or 50mM KCl, with correspondingly lowered NaCl concentration, for the duration of that fraction. At the end of the experiment slices were solubilized in 200µl Soluene-350, neutralized with 0.2M HCl and the tritium content of tissue and collected fractions determined.

FTR was calculated as previously described (section ii). Due to the time required (10-15 min) for the depolarizing medium to travel from the reservoir to the superfusion chamber and to then reach the collection vial, elevated FTR elicited by increased K^+ was not detected until two or three fractions after its introduction into the system. FTR returned to basal levels four fractions later. Thus the combined FTR in these four fractions represented the K^+-evoked release of [^{3}H]-D-aspartate. Basal release was determined by combining the FTR measured in the two fractions immediately preceding with that in the two fractions immediately following the K^+-evoked release (Fig. 4).

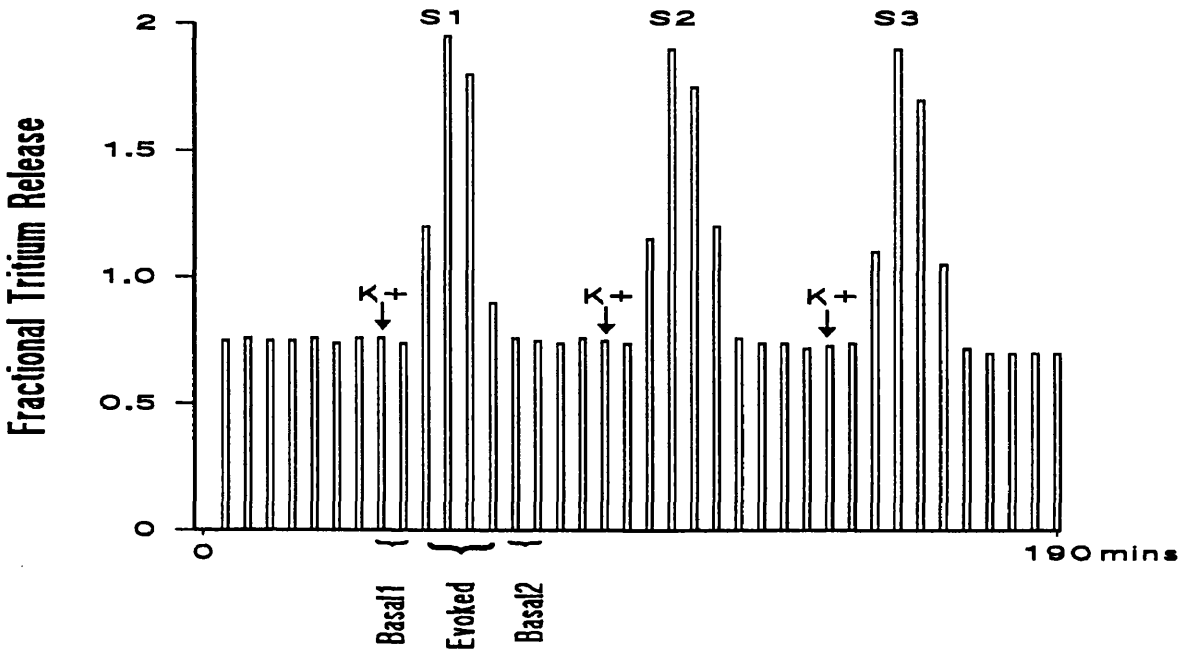


Fig. 4 Schematic representation of evoked tritium overflow.

Evoked tritium overflow = evoked release (4 fractions) - {basal 1+basal 2} (4 fractions).

Evoked tritium overflow produced by the three successive K^+ pulses was designated S_1, S_2 and S_3 respectively.

Additional studies were carried out in nominally Ca^{2+}-free Krebs-Henseleit solution with or without the addition of 1mM EGTA. Values for S_1, S_2 and S_3 were compared in the absence and presence of 1.8mM $CaCl_2$ and the portion of K^+-evoked release dependent on the presence of external Ca^{2+} was determined as follows:

% evoked tritium overflow dependent on Ca^{2+} $\left(\frac{S_x(+Ca^{2+}) - S_x(-Ca^{2+})}{S_x(+Ca^{2+})} \right)$ x100 $S_x = S_1$, S_2 or S_3

To determine the effect of test compounds on K^+-evoked [^{3}H]-D-aspartate release compounds were introduced into the perfusion medium 2 min prior to, and during, the second stimulation period. The S_2/S_1 ratios were calculated and compared to the S_2/S_1 ratio obtained under control conditions (i.e. no additions).

3) K^+-Evoked Release of [^{3}H]-D-Aspartate from Rat Hippocampal Synaptosomes.

i) Determination of the time-course of accumulation of [^{3}H]-D-aspartate.

Rat hippocampal synaptosomes were resuspended in 6ml of Krebs-Henseleit solution maintained at 37°C and oxygenated with 95%O_2/5%CO_2. [^{3}H]-D-

Aspartate (final concentration 67nM) was added to the synaptosomal suspension and vortexed. At t = 0.5, 2, 5, 10, 15 and 30 min 200µl aliquots of the suspension were removed and filtered under moderate vacuum onto 0.65µm nitrocellulose filters in a Millipore filtration unit. The experiment was performed in triplicate. The synaptosomes were washed with 3 x 1ml aliquots of normal Krebs-Henseleit solution and counted for tritium content by liquid scintillation counting.

ii) Determination of the time required for basal release of [^{3}H]-D-aspartate to stabilize.

Rat hippocampal synaptosomes were incubated for 10-15 min in 3ml of oxygenated Krebs-Henseleit solution containing 67nM [^{3}H]-D-aspartate (at 37°C). Equal aliquots were dispensed onto Whatman GF/C filters in each of four superfusion chambers, maintained at 37°C. The tissue was perfused at 0.4ml/min and 2 min fractions were collected for 60 min. At the end of the experiment the tritium content of both the synaptosomes remaining on the filters and the fractions were determined. FRT was determined as described above (I.2.ii).

iii) K^+-Evoked release of [^{3}H]-D-aspartate from rat hippocampal synaptosomes.

Equal aliquots of rat hippocampal synaptosomal suspension were transferred to four superfusion chambers and washed for 30 min, at 0.4ml/min, to allow basal release to stabilize. Two minute perfusate fractions were collected for a period of 70 min and the tissues were pulsed with Krebs-Henseleit solution containing either 10mM KCl or 25mM KCl, for 2 min, at t = 10, 30 and 50 min. Filters holding the synaptosomes and all fractions were counted for their tritium content. FTR and tritium overflow (S_1, S_2 and S_3) were determined for both concentrations of KCl.

Experiments were repeated in nominally Ca^{2+}-free Krebs-Henseleit solution with or without addition of 1mM EGTA. FTR and tritium overflow were calculated, and S_1, S_2 and S_3 values were compared in the absence and presence of 1.8mM $CaCl_2$.

As in the rat hippocampal slice study, test compounds were introduced into the perfusion medium 2 min prior to, and during, the second stimulation period. For each compound the S_2/S_1 ratio was compared to control values.

4) K^+-Evoked Release of [^{3}H]-GABA from Rat Cortical Synaptosomes: A Comparison with the Evoked Release of [^{3}H]-GABA and [^{3}H]-D-Aspartate from Rat Hippocampal Synaptosomes.

To determine why (-)baclofen may fail to modulate K^+-evoked release of [^{3}H]-D-aspartate from rat hippocampal preparations using the above techniques whilst experiments carried out by Bonanno et al. (1989) showed that (-) baclofen inhibited K^+-evoked release of [^{3}H]-GABA from rat cerebrocortical synaptosomes the methods used by this group were first replicated and then repeated using hippocampal synaptosomes preloaded with either [^{3}H]-GABA or [^{3}H]-D-aspartate.

i) K^+-Evoked [^{3}H]-GABA release from rat cerebrocortical synaptosomes.

Rat cerebrocortical synaptosomes were incubated for 15 min, at 37°C, in 20 volumes of oxygenated Krebs medium containing 50nM [^{3}H]-GABA (91.5Ci/mmol, Amersham). 200µl Tissue aliquots were transferred onto 0.65µm nitrocellulose filters in each well of an eight chamber perfusion system and washed for 40 min at 0.6ml/min. 50µM Amino-oxyacetic acid (AOAA) was present in the perfusing medium throughout, to reduce degradation of the released [^{3}H]-GABA by GABA-T. After 40 min equilibration, six 3 min fractions were collected with a 90 sec period of depolarization (15mM K^+) applied at the beginning of the second fraction. In these experiments increased tritium release produced by 15mM K^+ was detectable in fractions three and

four, and returned to basal levels by fraction five. Fractional release was determined as described above (I.2.ii). Subtraction of basal FTR (fractions 2 and 5 combined) from K^+-evoked FTR (fractions 3 and 4 combined) gave a measure of K^+-evoked tritium overflow (S_1).

Each tissue sample was stimulated only once and therefore within the eight chambers two acted as controls (depolarizing medium alone). The remaining six received depolarizing medium to which (-)baclofen was added. Tritium overflow in the presence of (-)baclofen was compared to that obtained under control conditions by Student's 2-tailed t-test.

ii) K^+-Evoked release of [^{3}H]-D-aspartate or [^{3}H]-GABA from rat hippocampal synaptosomes.

The experiment was repeated exactly as for cortical [^{3}H]-GABA release (I.4.i), using rat hippocampal synaptosomes preloaded with either 67nM [^{3}H]-D-aspartate, or batches of hippocampal synaptosomes were divided and half incubated in 67nM [^{3}H]-D-aspartate and half in 50nM [^{3}H]-GABA as before. The effect of (-)baclofen on [^{3}H]-amino acid release stimulated by 15mM K^+ was compared to control values.

5) K^+-Evoked Release of Endogenous Amino Acids from Rat Hippocampal Synaptosomes.

Preliminary experiments were carried out to determine whether it was possible to detect the release of endogenous amino acids from rat hippocampal synaptosomes using the superfusion technique outlined above (I.4.i). Synaptosomes were prepared and aliquots transferred to the superfusion chambers and washed at 0.6ml/min for 30 min. Six 3 min fractions were then collected and analyzed for endogenous amino acid content by high pressure/performance liquid chromatography (HPLC) as outlined below (I.5.iii). No amino acids were detected in these samples. Increasing the concentration of protein in the chambers or changing the flow rate of the perfusing medium failed to increase amino acid concentrations to levels detectable with the available HPLC system. It was decided therefore to use an alternative experimental method based on that described by Neal and Shah (1989).

i) Measurement of basal release of endogenous amino acids.

Rat hippocampal synaptosomes were resuspended in 20 volumes of oxygenated Krebs-Henseleit solution and incubated for 15 min, at 37°C. Synaptosomes (400µl) were transferred onto 0.65µm nitrocellulose filters in a twelve chamber Millipore filtration unit and rinsed under moderate vacuum with 10 x 2ml aliquots of warm, oxygenated Krebs-Henseleit solution. Basal

release was determined at this point by incubation of the tissue with 0.5ml Krebs-Henseleit solution for 5 min. To ensure basal release was stable this solution was removed by filtration and replaced, for a further 5 min, with 0.5ml of fresh Krebs-Henseleit solution. Amino acid content of all samples was determined by HPLC analysis (see I.5.iii), protein content was determined by the Bradford assay (see IV.3) and the amounts of endogenous amino acid present in the samples expressed as pmoles/mg protein/5 min. The amino acid content of the second sample was compared to that of the first (Student's 2-tailed t-test).

ii) K^+-evoked release of endogenous amino acids.

Rat hippocampal synaptosomes were incubated and washed as outlined above (section I.5.i). Basal release was determined by incubation with 0.5ml Krebs-Henseleit solution for 5 min. This solution was removed by filtration and replaced for a further 5 min by 0.5ml Krebs-Henseleit solution containing 15mM, 25mM or 50mM KCl, with appropriately lowered NaCl concentrations, with or without concomitant addition of test compounds. The endogenous amino acid content in basal and stimulated fractions was measured by HPLC analysis and calculated as pmoles/mg protein/5 min.

The experiment was repeated using nominally Ca^{2+}-free Krebs-Henseleit solution throughout. Basal and stimulated levels of aspartate, glutamate and

GABA were determined by HPLC and compared to those obtained in the presence of 1.8mM $CaCl_2$.

iii) Quantification of endogenous amino acid content by HPLC.

Detection of amino acids was achieved by precolumn derivitization of each sample with o-phthaldialdehyde (OPA, Fluka) (Lindroth and Mopper, 1979). OPA reacts rapidly with primary amines in the presence of 2-mercaptoethanol at alkaline pH to form highly fluorescent indole derivatives which may be separated and quantified by HPLC with fluorescence detection at wavelengths in the range 340-455nm.

a) Instrumentation

A Gilson high pressure liquid chromatograph equipped with two piston pumps, mixing unit, sample injection valve and 20µl sample loop was used in these experiments. A Gilson fluorometric detector was connected to the Drew Roseate DS4000 integration and data collection software system. Amino acids were identified by retention time and peak areas were measured and stored on disk.

b) Procedure.

150µl Aliquots of amino acid standards or samples were pipetted into sealed brown glass vials (Anachem) and loaded into the refrigerated (4°C) autosampler unit. The autosampler was programmed to add 100µl OPA (54mg OPA/1ml absolute alcohol + 10ml 0.1M sodium tetraborate dehydrate + 100µl mercaptoethanol; 'age' for 24 hours before use) to 25µl sample aliquots and after 1 min reaction time to inject 100µl of the mixture onto a 20µl injection loop. The amino acids were separated using a 15cm, 5µm C-18 reversed phase Microsorb column protected by a 5µm C-18 Microsorb guard column (both Dynamax, Rainin Instrument Co.). Amino acid separation was achieved by gradient elution by increasing the proportion of buffer B (100% methanol) in buffer A (50mM NaH_2PO_4 pH 5.5:Methanol, 4:1) from 0% at t=0, to 35% at t=35 min (flow rate 1ml/min). The last peak of interest, GABA, was typically eluted at 28 min. With the inclusion of a 5 min wash in 100% methanol analysis of each sample was eluted in 40 min (Fig. 5). All reagents were of HPLC grade. Solutions were filtered through 0.25µm nitrocellulose filters prior to use and degassed under helium throughout the run.

Fig. 5 Profile of HPLC gradient.

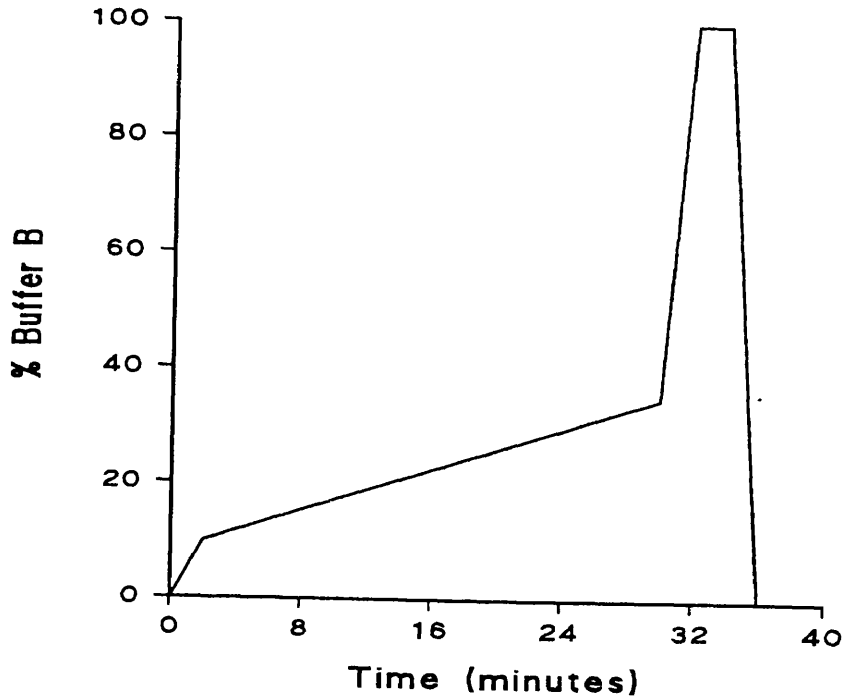
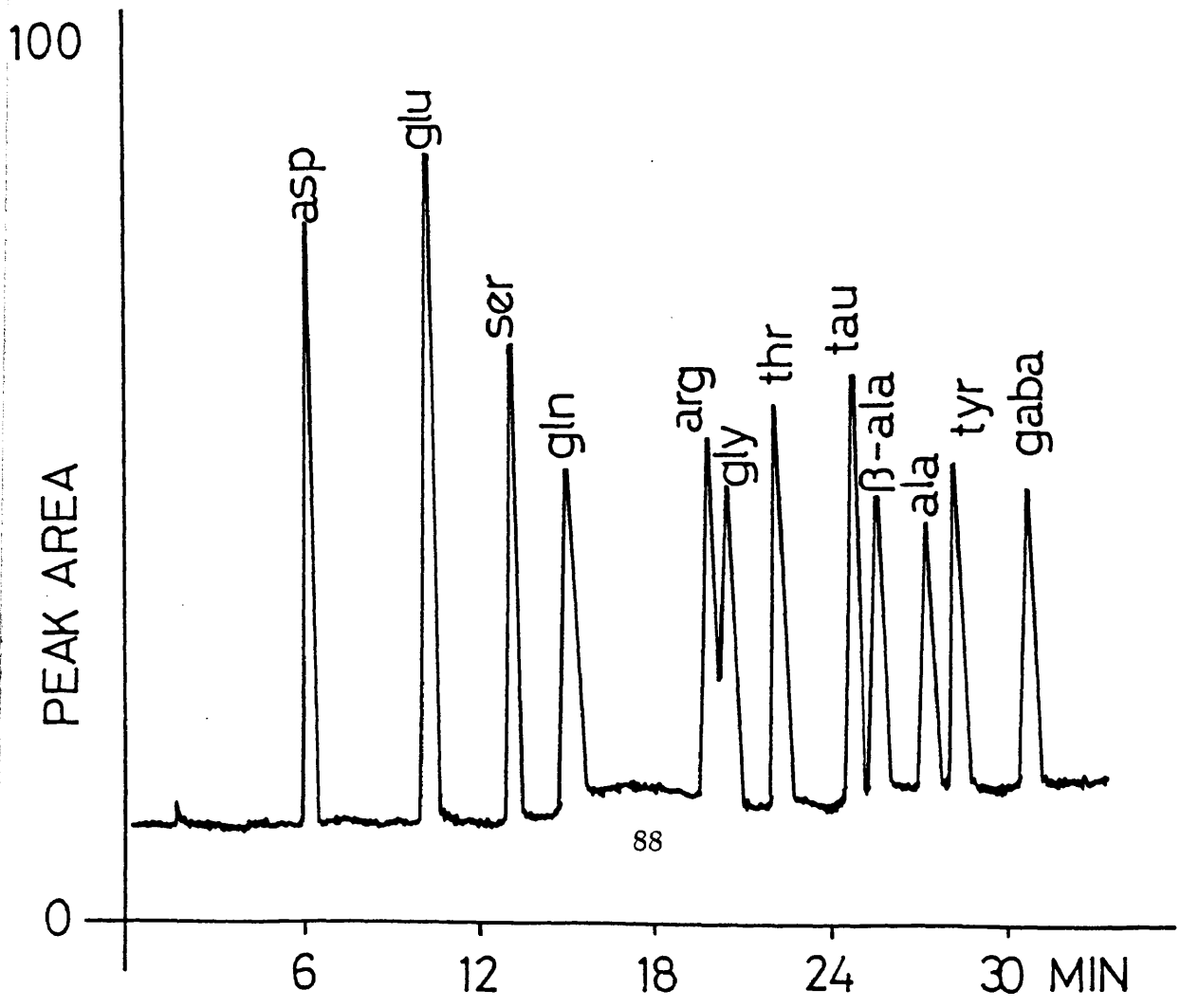


Fig. 6 Chromatogram of 10pmole amino acid standard solution.



c) System Calibration using Amino Acid Standards.

From commercially available L-amino acid standards (Sigma) solutions containing 2.5μM aspartic acid, glutamic acid, serine, glutamine, glycine, threonine, arginine, taurine, β-alanine, tyrosine, alanine and GABA were prepared and stored at -20°C in 1ml aliquots for up to 2 months without decomposition. Working standards were prepared daily by dilution of the stock solutions. At the beginning of each run of experimental samples three or four concentrations (1.25-10pmoles on the column) of amino acid standards were used to calibrate the system. Fig. 6 shows a representative chromatogram for the 10pmole standard solution. For each amino acid there was a linear relationship between peak area and concentration up to 20 pmoles (highest concentration measured) and regression lines intercepted the origin (Fig. 7).

d) Quantification of Unknown Samples.

Amino acid peaks in the experimental samples were identified by retention time on the column. Peak areas were integrated and the concentrations of individual amino acids were calculated from the slopes of the standard curves.

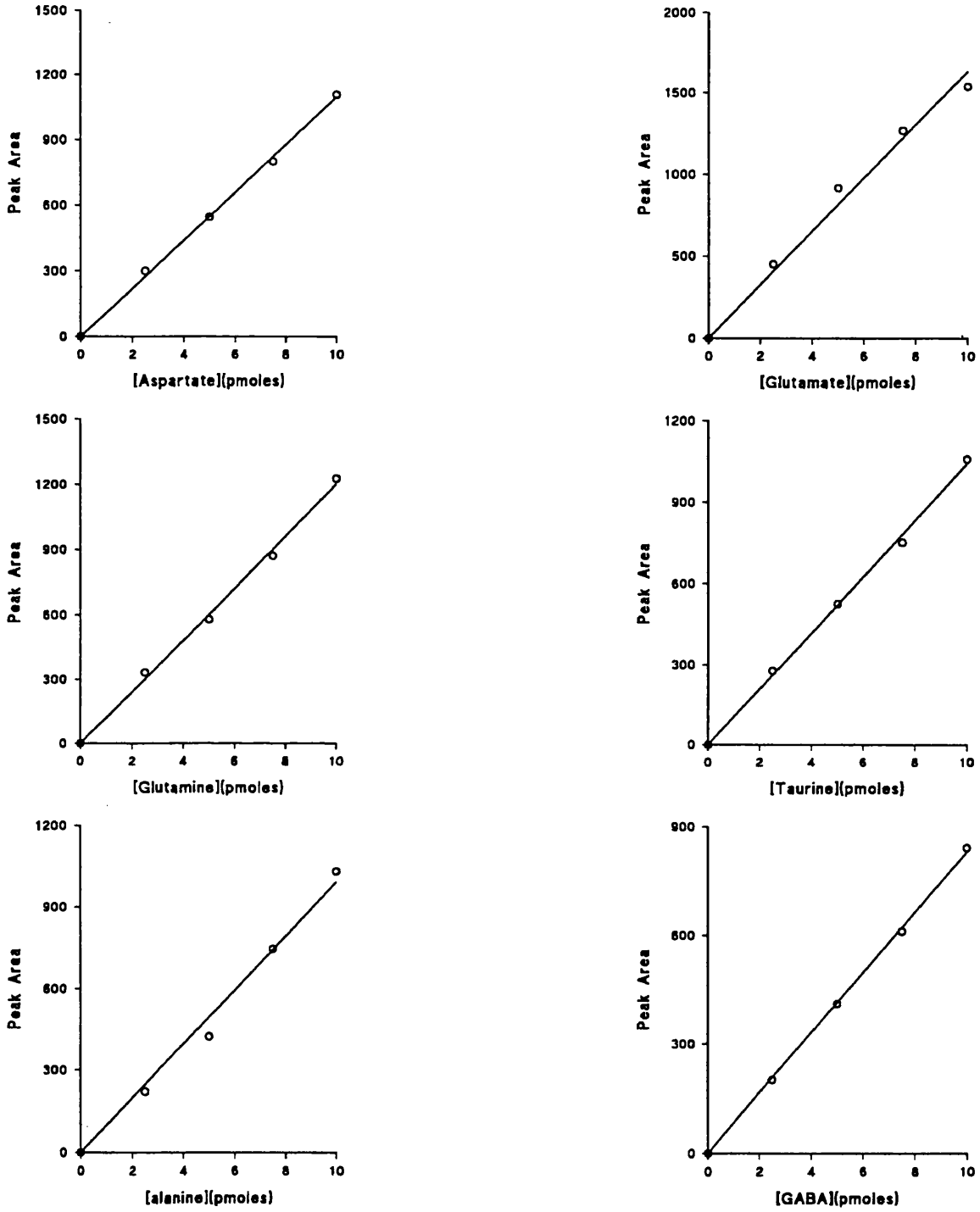


Fig. 7 Representative standard curves for known amino acids.

HPLC analysis of amino acid standards was performed prior to each run. The amino acid composition of unknown samples was determined from peak retention time and concentration from peak area.

II) CHARACTERIZATION OF PRESYNAPTIC $GABA_B$ RECEPTORS IN THE RAT ANOCOCCYGEUS MUSCLE.

1) Tissue Preparation.

i) Dissection of the rat anococcygeus muscle.

The paired anococcygeus muscles arise from either side of the upper coccygeal vertebrae and pass caudally, lying first behind and then each to one side of the colon, finally joining above the colon to form the 'ventral bar' which is found close to the anal opening. This smooth muscle preparation has a dense adrenergic innervation with no apparent cholinergic input, though cholinergic muscarinic receptors are located on the smooth muscle cells (Gillespie, 1972). Inhibitory neurones are also present which mediate powerful relaxations via the release of nitric oxide (Gillespie et al., 1989; Hobbs and Gibson, 1990).

The rat anococcygeus muscles were removed as described by Gillespie (1972). Male, Wistar rats (220-240g) were stunned and exanguinated. The abdomen was opened and the bladder, vasa, testes and seminal vesicles removed. The pelvic bone was split, and the delicate connective tissue cleared from behind the colon until the paired anococcygeus muscles were visible. The muscles were freed from their points of attachment and transferred, as a single unit, to a petri dish. In warm, oxygenated Krebs-Henseleit solution all remaining connective tissue was removed and the muscles divided at the ventral bar to

give two separate preparations which, since they were removed from the same animal, conveniently acted as paired test and control tissues in some experiments.

2) The Electrically-Evoked Release of [^{3}H]-Noradrenaline.

This method is an adaptation of that used to demonstrate the novel pharmacological profile of GABA receptors on the terminals of sympathetic neurones (Bowery et al., 1981). Anococcygeus muscles from one animal were incubated for 40 min, at 37°C, in 1ml of continuously oxygenated (95%O_2/5%CO_2) Krebs-Henseleit solution containing 250nM [^{3}H]-L-noradrenaline (38Ci/mmol, Amersham). The preparations were transferred to two 10ml glass organ baths, maintained at 37°C, and suspended from an isometric force transducer through platinum ring electrodes (Fig. 8). A resting tension of 0.5-1.0g was applied to each muscle which was then washed for 90 min with warm, oxygenated Krebs-Henseleit solution by which time basal release had stabilized. The perfusing medium was allowed to 'drip' over the preparations at a constant flow rate of 1ml/minute. Yohimbine (2.5µM) was added to the Krebs-Henseleit solution to prevent feedback inhibition by released noradrenaline acting on presynaptic α_2-adrenergic receptors. Pargyline and ascorbic acid were also added to the perfusion medium, both at 500µM, to reduce the breakdown of released noradrenaline by monoamine oxidase and oxidation respectively. In some experiments the force transducer was connected, via an amplifier, to a flat-bed chart recorder and the response

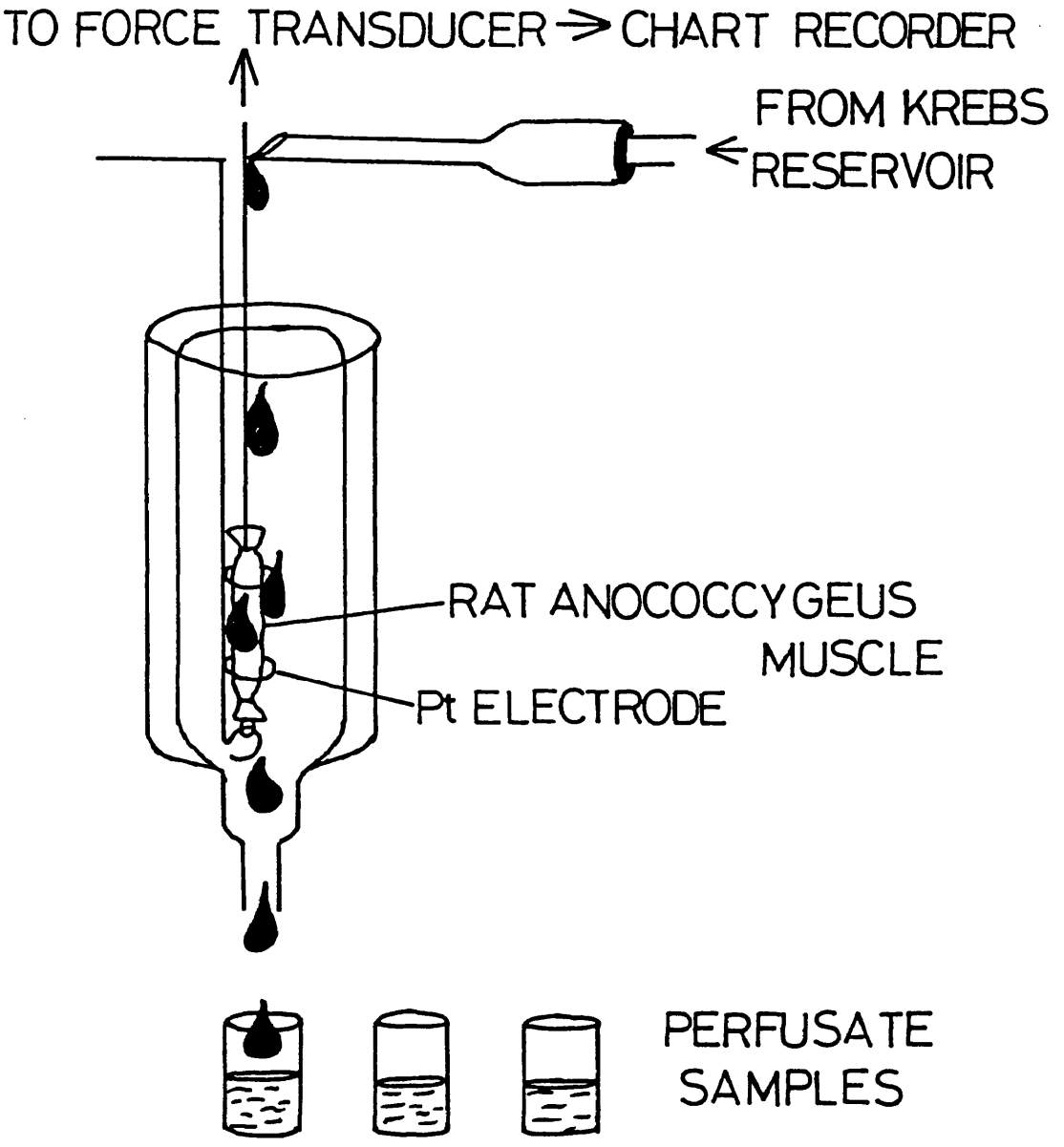


Fig. 8 Experimental set-up for the measurement of the electrically-evoked release of [^{3}H]-noradrenaline from the rat anococcygeus muscle.

of the anococcygeus muscle to electrical stimulation was recorded simultaneously with the collection of released tritium.

At 90 min the flow rate was reduced to 0.5ml/min and 5min perfusate fractions were collected for 3 hours 20 min. The preparations were transmurally stimulated at time t = 30, 55, 80, 105, 130, 155 and 180 min using the following stimulus parameters: one 10 sec train of 0.5 msec pulses delivered at variable frequencies and supramaximal voltage by a Grass S88 stimulator.

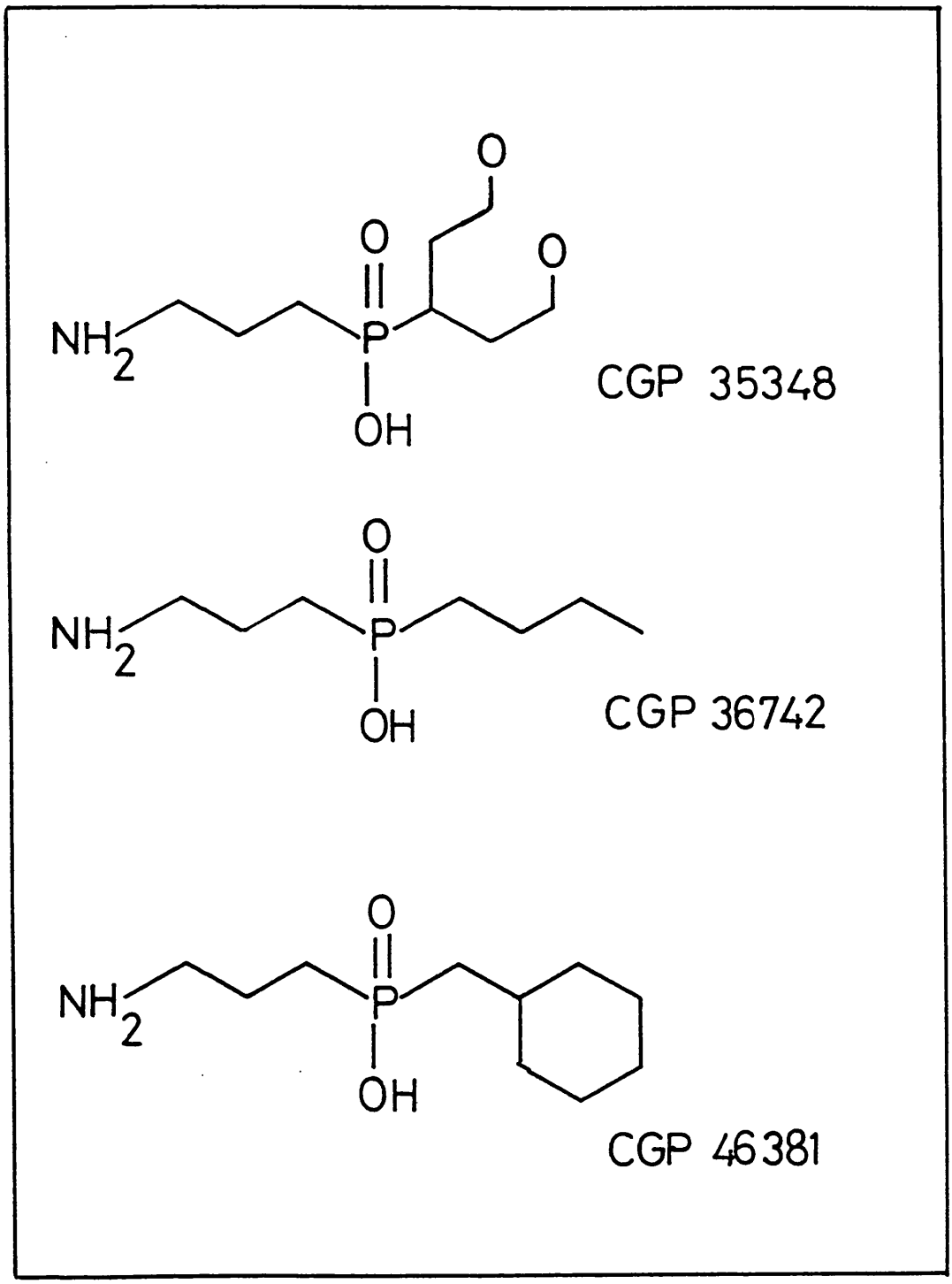
In one study the experiment was carried out as described with the exception that one of the two preparations was maintained in Ca^{2+}-free Krebs-Henseleit until after the third stimulation period. 1.8mM $CaCl_2$ was then returned to the perfusion medium for the remainder of the experiment.

At the end of the experiments, tissues were solubilized in 500µl Soluene-350 and neutralized with 0.2M HCl. Tissue and perfusate samples were added to 10ml of scintillation fluid and counted for tritium content. FTR of [^{3}H]-noradrenaline was determined as previously described for [^{3}H]-D-aspartate release from rat hippocampal slices (I.2.ii). As the stimulus was applied directly to the muscle via the platinum electrodes, the 'dead space' in the system consisted only of the time required for the perfusate to leave the organ bath and reach the collection vial. Evoked [^{3}H]-noradrenaline release was detectable in the fraction collected during the stimulation period with some small spillover into the next fraction. Thus evoked release was calculated by

the addition of the FTR in these two samples. Basal release was determined by addition of the FTR in the samples either side of these two fractions. Subtraction of 'basal' from 'evoked' gave the amount of tritium released above basal levels by electrical stimulation (tritium overflow) as a percent of tissue content and this was designated S_1 - S_7 for the seven stimulation periods. Test agonists were introduced into the perfusion medium 90 sec prior to the third and sixth stimulation periods, for each preparation the effect of the same concentration of one compound was evaluated. S_3/S_2 ratios were calculated and used to assess the viability of the preparation. S_6/S_5 ratios were calculated and used to assess the effect of the test agonist. Mean S_6/S_5 ratios were compared for each agonist concentration to control S_6/S_5 ratios by Student's 2-tailed t-test.

The effect of three putative $GABA_B$ antagonists, CGP 35348, CGP 36742 and CGP 46381 (Fig. 9), was assessed against (-)baclofen and 3-APA. Concentrations of the test agonists producing just submaximal decreases in electrically-evoked [^{3}H]-noradrenaline release were chosen; 30μM (-)baclofen or 3μM 3-APA. The agonists were introduced into the perfusion medium 90 seconds prior to the third and sixth stimulation periods as described previously. Each of the antagonists was introduced into the perfusion medium immediately following the fifth stimulation to allow a 20 min equilibration period. For the last 90 sec of this period both agonist and antagonist were present concomitantly. The S_3/S_2 ratio was calculated to assess tissue viability. Results from preparations in which this ratio was significantly different from

Fig. 9 The Structures of CGP 35348, CGP 36742 and CGP 46381.



that obtained previously for these concentrations of agonist were discarded. In those preparations responding to the agonists, the S_6/S_5 ratios were calculated and mean values compared to ratios obtained for the agonist alone and to ratios from control tissues. Preliminary experiments were carried out to determine the effect of the antagonists alone.

3) The Electrically-Evoked Twitch Response in the Rat Anococcygeus Muscle.

Preparations of anococcygeus muscles from three animals were set up for electrical field stimulation in six 3ml glass organ baths containing continuously oxygenated Krebs-Henseleit solution, at 37°C. Stimulation, using 1 sec trains of 0.5 msec pulses at 10Hz delivered every 20 sec at supramaximal voltage, produced powerful and reproducible contractions, measured isometrically and recorded in grammes of tension developed (g tension) on flat bed chart recorders.

Tissues were allowed to equilibrate for 60 min before exposure to sequentially increasing concentrations of the $GABA_B$ agonists (-)baclofen, 3-APA and SKF 97541. Drugs were added cumulatively until no further decrease in twitch height was obtained and care was taken to ensure that the response to each concentration had stabilized before subsequent additions. The response to each concentration was calculated as the percent decrease in electrically-evoked

twitch contraction compared to the control twitch height. Graphs of the logarithm$_{10}$ (log) of the agonist concentration plotted against % decrease in twitch height were constructed from which the concentration producing 50% of the possible maximum depression in response (EC_{50}) was determined. Dose-response curves (DRC) were repeated a number of times and the geometric mean EC_{50} values were calculated.

To determine the potency of the putative $GABA_B$ antagonists CGP 35348, CGP 36742 and CGP 46381 DRC's were constructed to each of the three agonists in the absence and presence of increasing concentrations of each antagonist. No more than three or four dose DRC's were constructed on any one tissue. Antagonists were added to the bathing medium 15-20 min before repetition of the agonist DRC in the continuing presence of the antagonist. Increasing concentrations of antagonists produced progressive rightward shifts of the control DRC as measured by the dose ratio (EC_{50} in presence of antagonist/control EC_{50}). The affinity of the antagonist (PK_B) was then determined by the line of best fit (least squares) to the Schild regression (Arunlakshana and Schild, 1959) using three or more observations at each of three or four concentrations of antagonist. For a competitive antagonist the plot of log antagonist concentration against the log mean(DR-1) gives a straight line with a slope of unity, and when y=0, x=-logK_B.

III) DISPLACEMENT OF [^{3}H]-GABA FROM $GABA_A$ AND $GABA_B$ BINDING SITES IN WHOLE RAT BRAIN SLICES BY PUTATIVE $GABA_B$ LIGANDS.

1) Preparation of rat brain sections for receptor autoradiography.

Male, Wistar rats (220-240g) were anaesthetized with pentobarbitone sodium (Sagittal 0.1ml/100ml i.p.) and the brains perfused via the right ventricle with 250ml ice-cold 0.01M phosphate buffered saline, pH 7.4. When completely blood free the brains were carefully removed, mounted on corks with a small quantity of Tissue Tek (Miles Diagnostics Ltd.) and frozen in isopentane which was cooled to -35°C in liquid nitrogen. The brains were stored at -70°C until required.

For receptor autoradiography, brains were equilibrated at -20°C, for 30 min, and 10µm sagittal sections were cut at the level of the hippocampus (3.4mm lateral from midline, Paxinos and Watson, 1986) using a Frigocut 2800 cryostat, at -20°C. Sections were thaw mounted onto glass microscope slides and approximately 100 sections were obtained from each brain. Racks of mounted sections were air-dried for 2 hours before storing, at -20°C, for at least 12 hours before use.

2) GABA Binding Assays in Rat Brain Sections.

i) $GABA_A$ binding assay.

Frozen brain sections were thawed at room temperature and washed in 50mM Tris-HCl, pH 7.4, for 2 x 30 min periods (30 slides per 250ml buffer). The sections were thoroughly air-dried prior to the binding assay. For the detection of $GABA_A$ binding, sections were incubated, for 20 min, with 100µl of Tris-HCl containing 30nM [^{3}H]-GABA (91.5Ci/mmol) and 100µM (-) baclofen to saturate $GABA_B$ sites. Non-specific binding was defined by the inclusion of isoguvacine (100µM). For displacement studies triplicate sections were incubated in the total binding medium containing 0.1, 1, 10 or 100µM of each of the test compounds, (-)baclofen, 3-APA, SKF 97541, CGP 35348, CGP 36742 and CGP 46381. After 20 min the incubation medium was aspirated off and each section was washed twice, for 3 sec, in ice-cold Tris-HCl and for 1 sec in distilled water. Sections were added to scintillation fluid and counted for tritium content using a β-counter.

Non-specific binding (dpm) was subtracted from all other values. Specific binding remaining in the presence of the test compounds was compared to that in the absence of drug additions and the % decrease in specific $GABA_A$ calculated for each concentration of displacing ligand:

$$\% \text{ decrease in specific } GABA_A \text{ binding} = \left[1 - \left(\frac{\text{specific dpm in presence of ligand}}{\text{specific dpm in absence of ligand}}\right)\right] \times 100\%$$

ii) $GABA_B$ binding assay.

Detection of $GABA_B$ binding was carried out essentially as for the $GABA_A$ binding assay. Thawed sections were washed for 2 x 30 min periods in 50mM TRIS-HCl, pH 7.4, containing 2.5mM $CaCl_2$, and then thoroughly dried before use. Divalent cations are essential for the detection of $GABA_B$ binding sites. Air-dried brain sections were incubated for 20 min with 100µl TRIS-HCl, pH 7.4, containing 2.5mM $CaCl_2$, 30nM [^{3}H]-GABA (91.5Ci/mmol) and 40µM isoguvacine to saturate $GABA_A$ binding sites. For the displacement studies increasing concentrations of the following compounds were included in the incubation medium; (-)baclofen, 3-APA, SKF 97541, CGP 35348, CGP 36742 and CGP 46381. Non-specific binding was defined by the presence of (-)baclofen (100µM). Each concentration was tested in triplicate and these sections apposed to tritium-sensitive film (III.3) a further duplicate was added to scintillation fluid and counted for tritium content to immediately determine the viability of the experiment. Compounds were tested in three or four separate experiments.

3) Autoradiographic visualization of $GABA_B$ binding in rat brain slices.

Following incubation the sections were dried and, together with commercial tritium standards (^{3}H-Micro-scales Amersham), were apposed to tritium sensitive Hyperfilm (Amersham) in X-ray cassettes for 8 weeks at room temperature. The exposed film was then immersed, emulsion side uppermost, in Kodak D-19 developer until the brain images were clearly visible. After rinsing briefly in distilled water, the negatives were fixed in 25% sodium thiosulphate solution and left for double the clearing time. Films were rinsed in running water for 30 min and dried.

Specific $GABA_B$ binding was quantified in several brain regions by reference to the standards exposed to the same film using a Quantimet 970 Image Analyzer. Optical densities of the sections were automatically converted to nCi/mg and, given the specific activity of the radioligand, this was finally expressed as fmoles of [^{3}H]-GABA bound per mg of protein (fmol/mg protein) for a given brain area. Triplicate values were calculated at each concentration of displacing ligand. The concentration of test compound which inhibited 50% of specific $GABA_B$ binding (IC_{50}) was determined from graphs of $\log_{10}$ of the concentration of displacer against % specific $GABA_B$ binding.

IV REGIONAL EFFECTS OF PERTUSSIS TOXIN ON $GABA_B$ BINDING IN RAT BRAIN.

1) Tissue Preparation.

i) Preparation of rat brain synaptic membranes.

Male, Wistar rats (5-12 weeks old; 100-320g) were sacrificed by stunning and decapitation and the brains rapidly removed onto ice. The cerebral cortices, hippocampi, corpus striata cerebella and were dissected and tissue pooled from 15-20 animals for each experiment. The tissue was homogenised in 40ml ice-cold 0.32M sucrose and centrifuged at 1,000g for 10 min, at 4°C. The pellet was discarded and the supernatant recentrifuged at 20,000g, for 20 min. The resulting pellet was lysed with 40ml ice-cold distilled water and kept on ice for 15 min in the refrigerator. Centrifugation at 8,000g, for 20 min, resulted in a pellet and supernatant with a 'buffy coat' layer at their interface. This 'buffy coat' was collected with the supernatant and centrifuged at 48,000g for 20 min. The final pellet was washed once with water then resuspended in 4ml of distilled water and frozen, at -20°C, until required.

ii) Treatment of rat brain membranes with PTX.

The prepared membrane pellets were thawed at room temperature. PTX (400µl, Porton Products) was preactivated by incubation in 4ml TRIS-HCl (100mM; pH 7.2), containing dithiothreitol (50mM), for 2-3 hours at 29°C. Meanwhile, the rat brain membrane pellets were washed three times by resuspending in 40ml ice-cold distilled water, followed by centrifugation at 20,000g for 10 min. Pellets were incubated at room temperature in TRIS-HCl (50mM, pH 7.4, 2.5mM $CaCl_2$, 1mM $MgCl_2$) for 45 min and centrifuged at 20,000g for 10 min to remove as much endogenous GABA from the preparations as possible . After two further 15 minute washings at room temperature followed by centrifugation, the pellets were resuspended in 2ml of TRIS-HCl (100mM, pH 7.2) and each divided into two 1ml portions. One portion from each brain region was incubated with 1ml of preactivated PTX solution and 2ml of reaction buffer (100mM TRIS-HCl, pH 7.2 containing NAD, 2mM; ATP, 1mM; thymidine, 10mM; EGTA, 1mM; $MgCl_2$) and the other portion in 1ml PTX vehicle (50% glycerol, 50% phosphate buffer , pH 7.2 containing NaCl) and 2ml reaction buffer, for 30 min at 29°C. The ADP-ribosylation reaction was terminated by the addition of 40ml ice-cold TRIS-HCl (50mM, pH 7.4) and centrifugation, at 20,000g, for 10 min. The supernatant was discarded and the pellets were stored on ice in the refrigerator until required for assay.

2) $GABA_B$ binding assay in control and PTX-treated rat brain membranes.

Assay tubes were set up in triplicate. Tubes contained either 100µl distilled water (total $GABA_B$ binding), 100µl (-)baclofen (final concentration 100µM to define non-specific $GABA_B$ binding) or 100µl GTPγS (final concentration 2µM). The reaction was initiated by the addition of 100µl of [^{3}H]-GABA (final concentration 5nM, 91.5Ci/mmol) to all tubes. Each brain region for control and PTX-treated tissue was assayed individually. Pellets were resuspended in an appropriate volume of TRIS-HCl (50mM. pH 7.4), containing 40µM isoguvacine to saturate $GABA_A$ binding sites, and 800µl was added to each assay tube. Samples of tissue homogenate were retained for protein assay and in some cases determination of the tissue GABA content. After a 10 min incubation period the reaction was terminated by centrifugation, at 10,000g, for 3 min. The supernatant was carefully removed by suction, the pellets superficially rinsed in ice-cold distilled water and solubilized overnight in 100µl Soluene-350. 0.2M HCl (400µl) was added to neutralize the Soluene-350 and the contents of each tube was added to 10ml scintillant and the samples counted for tritium content. Triplicate values were averaged and non-specific values subtracted from all others to give specific $GABA_B$ binding in dpm/tube. $GABA_B$ binding was finally expressed as fmol GABA bound per mg protein (fmol/mg).

3) Protein estimation using the Bradford method.

This method is based on the rapid binding of the azo dye Coomassie Brilliant Blue G-250 to protein causing an increase in the maximum absorbance of the dye from 465nm to 595nm. The reaction is complete in 2 min and the colour change stable for 1 hour. The advantage of this method is that cations, carbohydrates and chelating agents produce little interference (Bradford, 1976).

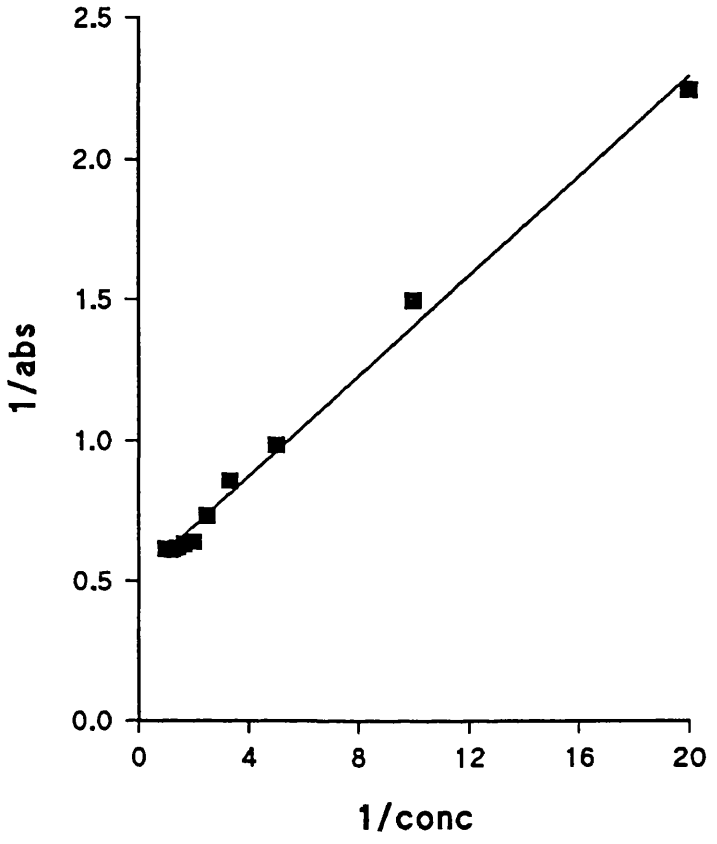
The Bradford reagent was prepared by dissolving 100mg Coomassie Brilliant Blue G-250 in 50ml 95% ethanol, adding 100ml 85%(w/v) phosphoric acid and making this solution up to 1l with distilled water. The reagent was stirred continuously overnight, filtered and stored at 4°C for up to one month. Bovine serum albumin (BSA) standards (0-1mg) were prepared, in triplicate, in 0.2M NaOH solution from a stock solution of 1mg/ml BSA. The reaction was initiated by the addition of 1ml Bradford reagent, samples were vortexed and incubated for 30 min. Absorption was read at 595nm using a spectrophotometer (Cecil Instruments). A plot of 1/concentration vs 1/absorbance was constructed from which the concentrations of unknown samples were calculated. 50µl Aliquots of protein samples were added to 50µl 0.2M NaOH and 1ml Bradford reagent added. The samples were vortexed and the absorbance read after 30 min. Protein content in mg/ml was determined from a calibration curve (Fig. 10) which was determined in parallel with each assay.

V) SOURCE OF NOVEL COMPOUNDS

All reagents unless otherwise stated were obtained from Sigma or BDH and were of Analar grade.

(-) Baclofen, (+) baclofen, 3-APA, SKF 97541, CGP 35348, CGP 36742, and CGP 46381 were kindly donated by Ciba-Geigy, Switzerland.

Fig. 10 Calibration curve for BSA protein standards.



BSA standard solutions (0-1mg/ml) were prepared in 0.2M NaOH. The reaction was initiated by the addition of 1ml Bradford reagent, samples wre vortexed and incubated for 30 min. Absorption was read at 595nm and the reciprocal of protein concentration plotted against the reciprocal of absorbance. The protein concentration of unknown samples was derived from the regression line.

CHAPTER THREE: RESULTS

CHARACTERIZATION OF PRESYNAPTIC $GABA_B$ RECEPTORS IN RAT HIPPOCAMPAL PREPARATIONS

1) $GABA_B$-Modulation of the K^+-Evoked Release of [^{3}H]-D-Aspartate from Rat Hippocampal Slices.

D-Aspartate is a substrate for the Na^+-dependent, high affinity carrier protein which is responsible for the accumulation of L-aspartate and L-glutamate by neurones and glial cells, although, unlike these amino acids D-aspartate is not metabolized for several hours following uptake (Davies and Johnston, 1976). Therefore, D-aspartate has frequently been used as a 'marker' for L-glutamate and L-aspartate containing neurones and its evoked, Ca^{2+}-dependent release has been studied in a wide range of *in vitro* CNS preparations including rat and guinea-pig cortical, striatal, hippocampal and cerebella slices (Mangano et al., 1991; Potashner and Gerard, 1983) and synaptosomes (Levi and Gallo, 1981), cat spinal cord slices (Johnston et al., 1980), cultured cerebellar granule cells (Zhu and Chuang, 1987) and *in vivo* using intrahippocampal microdialysis (Nielsen et al., 1989).

D-Aspartate was therefore chosen to investigate the effect of (-)baclofen on the K^+-evoked release of excitatory amino acids from rat hippocampal preparations. The initial aim was to characterize the presynaptic $GABA_B$ receptor using release techniques and to compare the results to those of Dutar and Nicoll (1988b) who had investigated pre-and postsynaptic $GABA_B$ receptors in the rat hippocampus using electrophysiological methods and found evidence of receptor heterogeneity.

i) Accumulation of [^{3}H]-D-aspartate.

The graph of accumulation of tritium with time (Fig. 11) shows that uptake of [^{3}H]-D-aspartate by rat hippocampal slices was maximal at 40 min. This incubation period was used in all subsequent experiments.

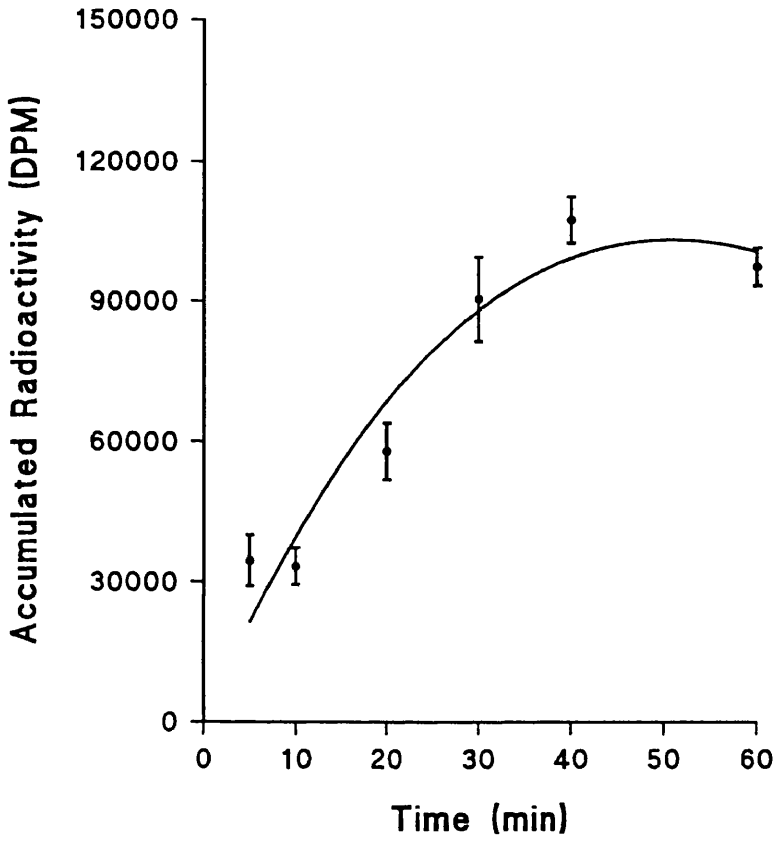
ii) Determination of the time required for basal release of [^{3}H]-D-aspartate to stabilize.

Under these experimental conditions efflux of [^{3}H]-D-aspartate from perfused rat hippocampal slices fell from 2% of total tissue content per fraction to stabilize at approximately 0.8% after 90 min (Fig. 12). This probably represents the removal of tritium from non-releasable tissue compartments in addition to the measurement of neuronal basal tritium release. During the next 70 min basal tritium release declined at a much slower rate to 0.6%. A wash period of 90 min was therefore determined as sufficient to allow basal release of [^{3}H]-D-aspartate from rat hippocampal slices to stabilize and was used in all subsequent experiments.

iii) The effect of increasing K^+-concentrations on basal release.

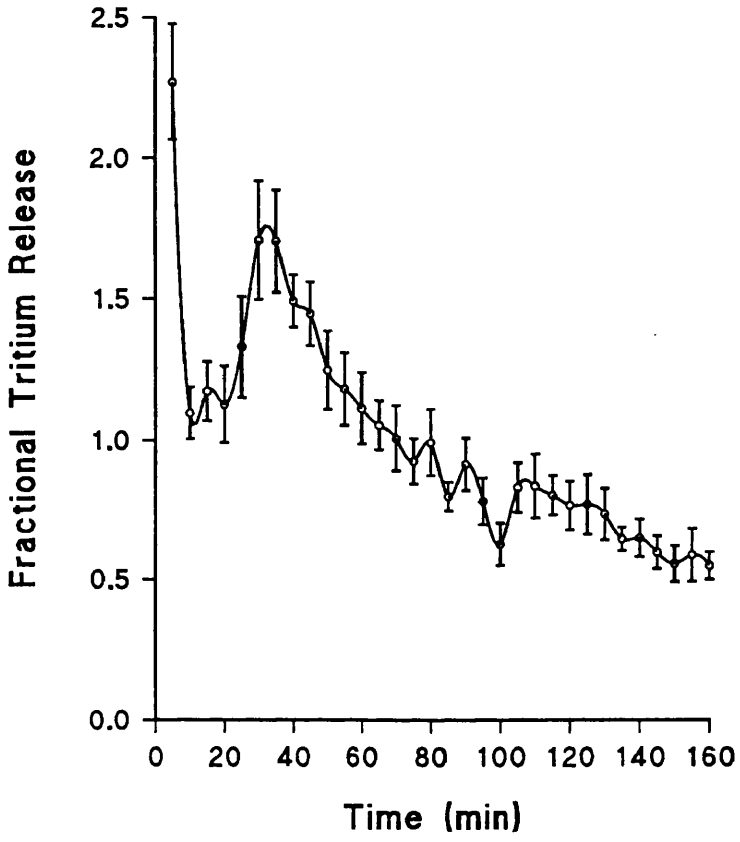
Initial studies investigated the effect of 2 minute pulses of increasing concentrations of K^+, 10mM, 20mM, 35mM and 50mM, on [^{3}H]-D-aspartate release. The 'depolarizing' medium was introduced into the perfusion system,

Fig. 11 Time-course of accumulation of [^{3}H]-D-aspartate by rat hippocampal slices.



Rat hippocampal slices, 4 per time point, were incubated with [^{3}H]-D-aspartate (67nM) for up to 60 min. Accumulated tritium was determined, following tissue solubilization, by liquid scintillation counting. Accumulated tritium (dpm) is plotted against time. Values are the mean ± s.e.m. (n=4).

Fig. 12 Decline of basal release of [^{3}H]-D-aspartate from rat hippocampal slices with time.



Rat hippocampal slices were incubated with [^{3}H]-D-aspartate (67nM) for 40 min, 5 slices transferred to each of four perfusion chambers and washed with warm, oxygenated Krebs-Henseleit solution for 160 min. Fractional tritium release was determined and plotted against time (min). Values are the mean ± s.e.m. (n=4).

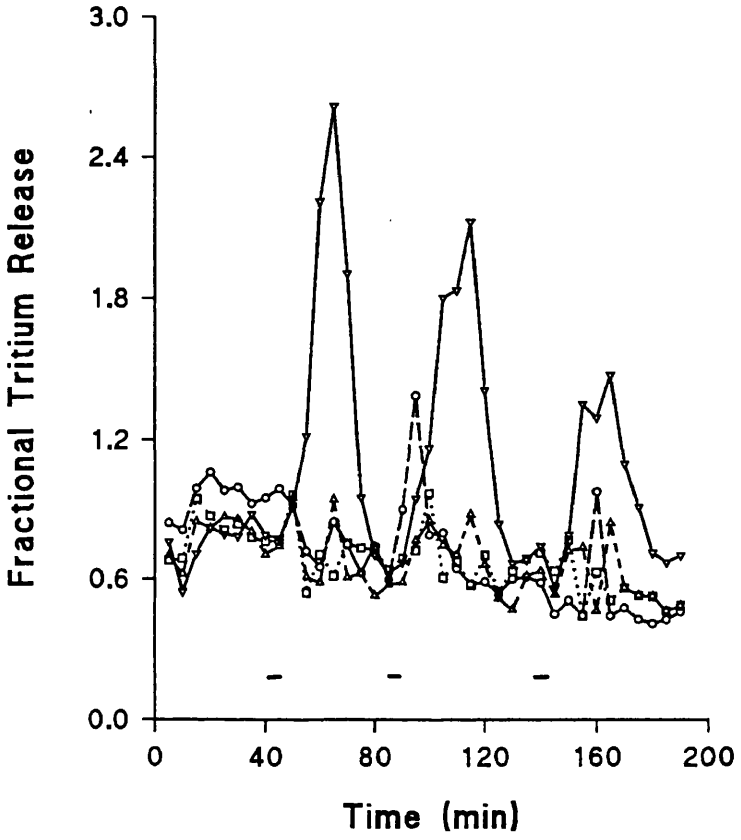
for 2 min, at t=40, 90 and 140 min. Irrespective of whether 2 min or 5 min fractions were collected, no increase in tritium release was observed above basal levels at any of the K^+ concentrations tested (data not shown). It was therefore decided to apply the K^+ pulses for a longer time period. In a single experiment preloaded and washed hippocampal slices were stimulated for 5 min with Krebs-Henseleit solution containing either 10mM, 20mM, 35mM or 50mM KCl at t=40, 90 and 140 min.

The results indicated that only at the highest K^+ concentration was the release of tritium clearly enhanced above basal levels (Fig. 13). At this concentration 5.4% of the tissue content was released during the first stimulation period, 4.81% during the second period and 2,85% during the third period (Table 2). Lower K^+ concentrations evoked the release of between 0.12-0.5% of tissue tritium content. The S_2/S_1 ratios from a further 8 control experiments using 50mM K^+ were obtained to give a mean value of 0.95 ± 0.12 (n=10; Table 5). These data indicate that the amount of tritium released by the second K^+ pulse was similar to that released by the first (Fig. 15). Krebs-Henseleit solution containing 50mM KCl was therefore used to evoke [^{3}H]-D-aspartate release from rat hippocampal slices throughout the remainder of this study.

iv) Calcium-dependency of K^+-evoked release of [^{3}H]-D-aspartate.

Experiments were carried out to determine to what extent K^+-evoked release of [^{3}H]-D-aspartate from rat hippocampal slices was dependent on the presence of calcium ions in the superfusion medium. Evoked tritium overflow

Fig. 13 The concentration-dependent, K^+-evoked release of [^{3}H]-D-aspartate from rat hippocampal slices.



Rat hippocampal slices were pulsed at 50 min intervals (solid bars) with Krebs-Henseleit solution containing 10mM (○), 20mM (□), 35mM (△) or 50mM (▽) KCl in the presence of 1.8mM $CaCl_2$. Values of fractional tritium release are single observations.

Table 2 Tritium overflow from rat hippocampal slices evoked by increasing concentrations of external K^+.

	Evoked Tritium Overflow (% Tissue Content)			
	10mM K^+	20mM K^+	35mM K^+	50mM K^+
S_1	0.24	0.42	0.23	5.38
S_2	0.46	0.40	0.5	4.81
S_3	0.12	0.32	0.36	2.85

Tritium overflow was compared at increasing concentrations of K^+ in the perfusate. The values of S_1, S_2 and S_3 were obtained from the experiment obtained described in Fig. 13 above.

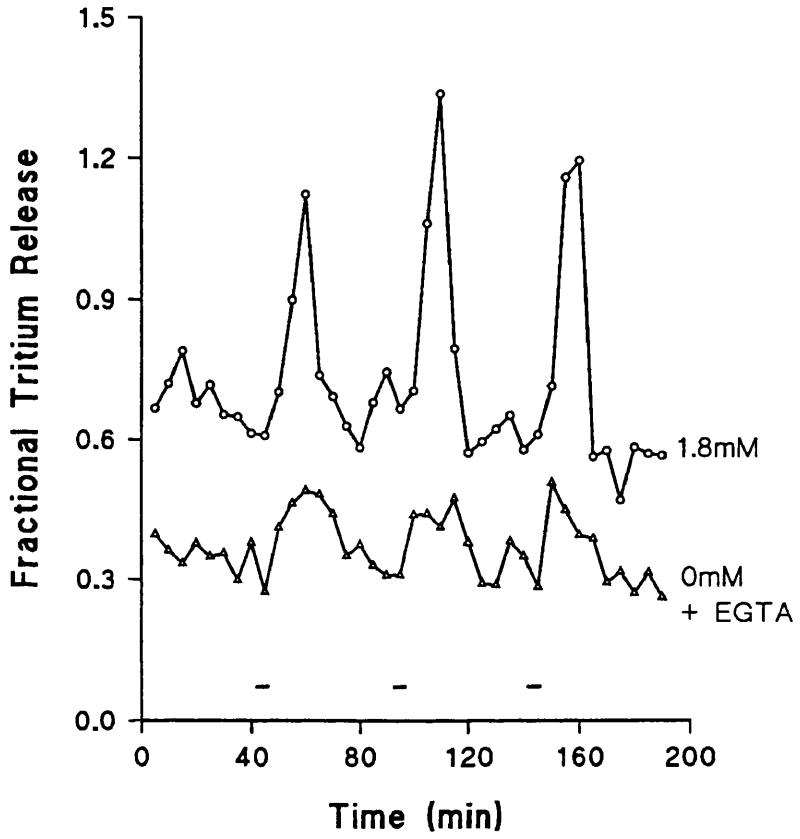
was determined for the three successive K^+ pulses in nominally Ca^{2+}-free and normal (1.8mM Ca^{2+}) Krebs-Henseleit solution. A second experiment was performed with the addition of the calcium chelating agent EGTA (1mM) to the Ca^{2+}-free medium (Fig. 14).

K^+-Evoked tritium release was reduced by 22-50% in nominally Ca^{2+}-free medium (Table 3). This value was increased to 46-70% when EGTA was present (Table 4). Though the basal release of [^{3}H]-D-aspartate was lower in the absence of Ca^{2+} (Fig. 14) this is a single observation that probably reflects the variability in FTR measured in different perfusion chambers. All subsequent studies were carried out in the presence of 1.8mM $CaCl_2$.

v) The effect of (-)baclofen on K^+-evoked release of [^{3}H]-D-aspartate.

(-)Baclofen (10-100μM) was added to the perfusion medium 2 min prior to, and during, the second stimulation period. S_2/S_1 Ratios were calculated and compared to the control S_2/S_1 obtained in (iii) above (Table 5). The results indicated that (-)baclofen, at concentrations up to 100μM, did not significantly inhibit the K^+-evoked release of [^{3}H]-D-aspartate from rat hippocampal slices. A decrease in evoked [^{3}H]-D-aspartate was not obtained by increasing the time of exposure to (-)baclofen prior to S_2 from 2 min to up to 20 min. Prolonged exposure (20 min) to 100μM (-)baclofen appeared to enhance [^{3}H]-D-aspartate release; the S_2/S_1 ratio was 1.53 ± 0.55 (n=4) compared the control value of 0.95 ± 0.12 (n=10), although these two ratios were not significantly different.

Fig. 14 Calcium-dependency of the K^+-evoked [^{3}H]-D-aspartate release from rat hippocampal slices.



Rat hippocampal slices were superfused with normal Krebs-Henseleit solution (1.8mM $CaCl_2$) or nominally Ca^{2+}-free medium containing 1mM EGTA. Preparations were stimulated for 5 min every 50 min with the appropriate Krebs-Henseleit containing 50mM KCl (solid bars). Tritium efflux expressed as a % of tissue content (FTR) was determined over the 190 min collection period. Values are from 1 experiment.

Table 3 Evoked [^{3}H]-D-aspartate release in the absence of calcium ions.

	Evoked Tritium Overflow		% Evoked Release Dependent on Ca^{2+}
	+1.8mM Ca^{2+}	+0mM Ca^{2+}	
S_1	1.07	0.84	22%
S_2	1.25	0.63	50%
S_3	1.32	0.69	48%

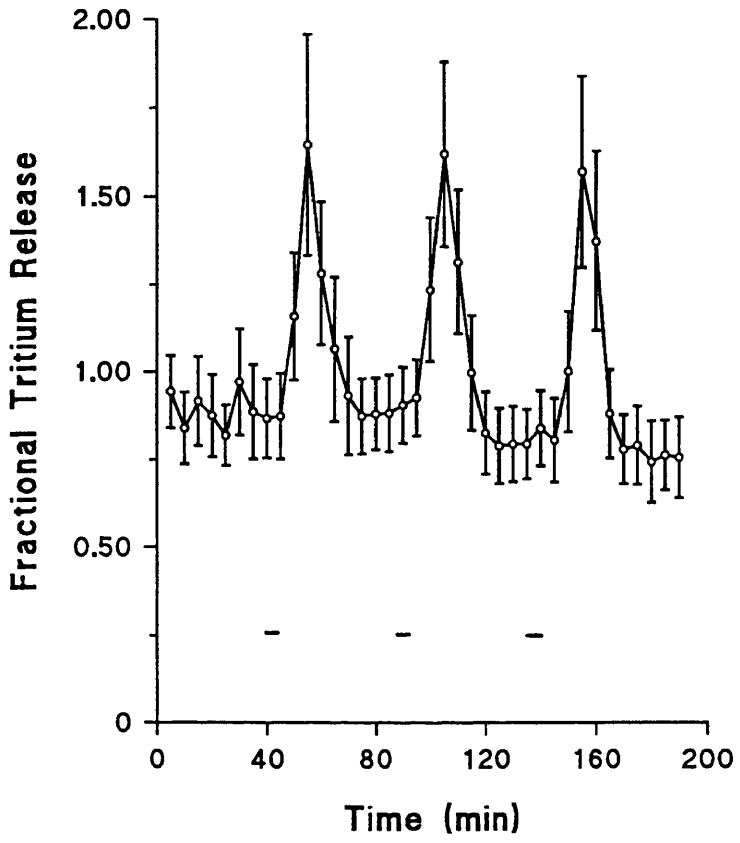
Table 4 Evoked [^{3}H]-D-aspartate release in the absence of calcium ions (+EGTA).

	Evoked Tritium Overflow		% Evoked Release Dependent on Ca^{2+}
	+1.8mM Ca^{2+}	0mM Ca^{2+} +EGTA	
S_1	1.12	0.61	46%
S_2	1.56	0.48	70%
S_3	0.99	0.50	50%

[^{3}H]-D-Aspartate release was evoked by 50mM K^+ in normal (1.8mM $CaCl_2$) and nominally Ca^{2+}-free Krebs-Henseleit solution (n=1;Table 3). The experiment was repeated with the addition of 1mM EGTA to the Ca^{2+}-free medium (n=1;Table 4). The portion of K^+-evoked release dependent on the presence of calcium ions was calculated as follows:

$$\% \text{ Evoked Release Dependent on } Ca^{2+} = \left(\frac{S_x(+Ca^{2+}) - S_x(-Ca^{2+})}{S_x(+Ca^{2+})} \right) \times 100 \qquad S_x = S_1, S_2 \text{ or } S_3$$

Fig. 15 K^+-Evoked release of [^{3}H]-D-aspartate from rat hippocampal slices: control responses to 50mM K^+.



Depolarizing Krebs-Henseleit solution containing 50mM KCl was introduced into the superfusion chambers at 50 min intervals for 5 min (solid bars). Values are the mean ± s.e.m. of the fractional tritium release measured at each time point (n=10)

Table 5 The effect of (-)baclofen on the K^+-evoked release of [^{3}H]-D-aspartate from rat hippocampal slices.

	S_2/S_1	n	
Control	0.95 ± 0.12	10	NSD
10μM (-)Baclofen	0.85 ± 0.07	3	NSD
30μM (-)Baclofen	0.76 ± 0.06	3	NSD
100μM (-)Baclofen	0.96 ± 0.16	5	NSD

(-)Baclofen (10-100μM) was introduced into the superfusion medium 2 min prior to, and during, the second stimulation period. S_2/S_1 Ratios were calculated in the presence of (-)baclofen and compared to the control value (obtained in section iii above) using Student's 2-tailed t-test. Values are the mean ± s.e.m. (n=3-10).

NSD = no significant difference from control at p = 0.05

One possible explanation for the lack of inhibitory effect of (-)baclofen on K^+-evoked [^{3}H]-D-aspartate from rat hippocampal slices is that 50mM K^+ is too strong a depolarizing stimulus against which to detect subtle modulation of transmitter release by (-)baclofen. However lower concentrations of K^+ were ineffective at producing measurable increases in tritium efflux above basal level (Fig. 13). It was therefore decided to repeat these experiments using crude hippocampal synaptosomes, a preparation which may respond to less aggressive chemical stimulation and which may therefore be more useful for the detection of $GABA_B$ modulation of K^+-evoked [^{3}H]-D-aspartate release.

2) $GABA_B$-Modulation of the K^+-Evoked Release of [^{3}H]-D-Aspartate from Rat Hippocampal Synaptosomes.

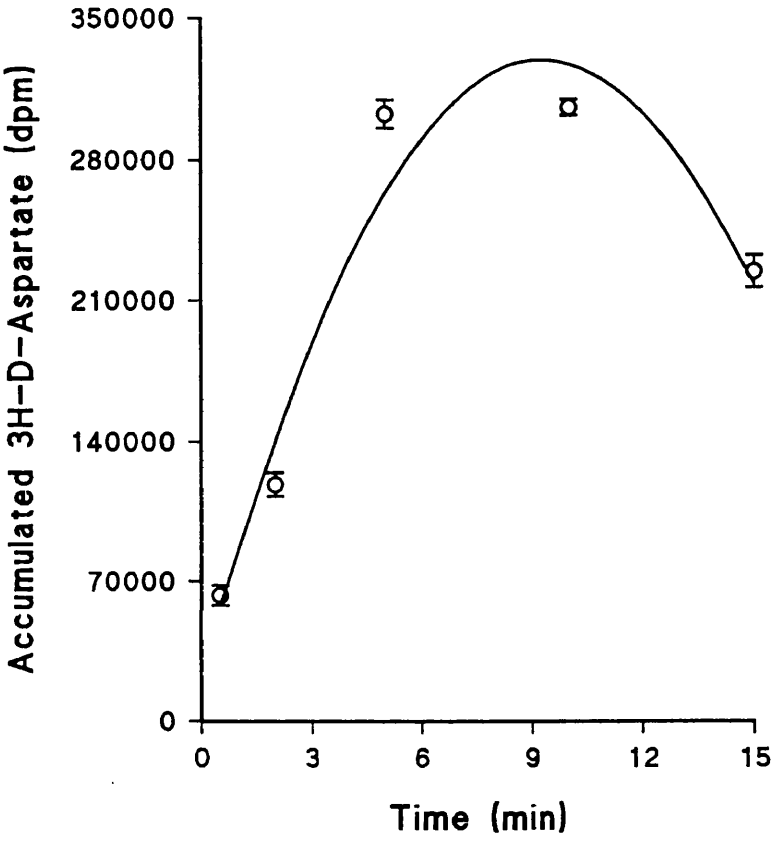
i) Accumulation of [^{3}H]-D-Aspartate.

The accumulation of [^{3}H]-D-aspartate by rat hippocampal synaptosomes was maximal at 10 min (Fig. 16). An incubation period of no more than 15 min was therefore used for all subsequent studies.

ii) Determination of the time required for basal release to stabilize.

Efflux of [^{3}H]-D-aspartate from rat hippocampal synaptosomes fell from 28% of tissue content per 5 minute fraction to approximately 1% after 25 min. As before this is thought to represent both the removal of tritium from non-

Fig. 16 Time-course of accumulation of [^{3}H]-D-aspartate by rat hippocampal synaptosomes.



Rat hippocampal synaptosomes were incubated with 67nM [^{3}H]-D-aspartate, at 37°C, for up to 15 min. 200µl aliquots were removed at t = 0.5, 2, 5, 10 and 15 min and washed under vacuum before the tissue tritium content was measured by scintillation counting. Values are mean ± s.e.m. (n=3).

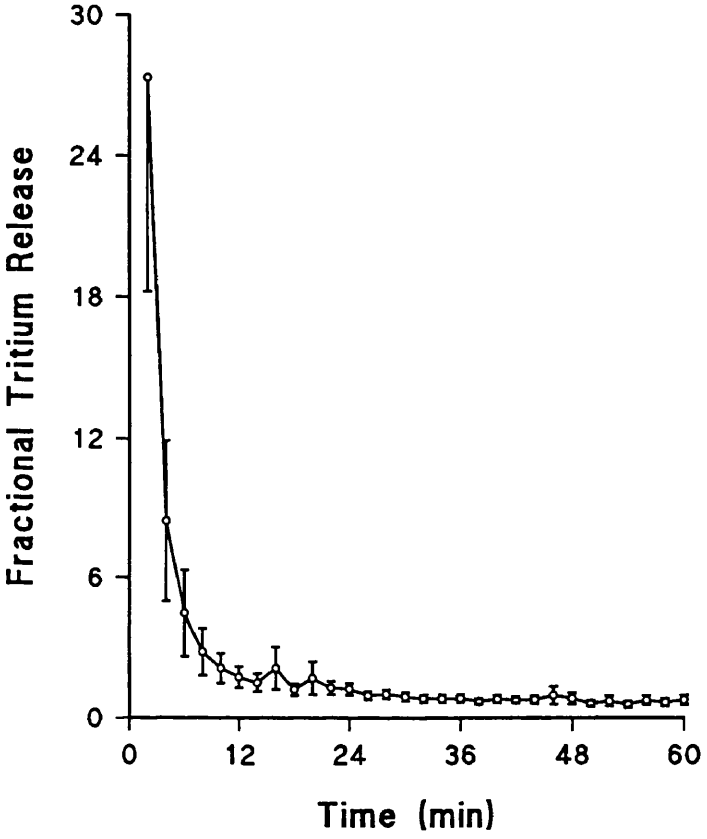
releasable tissue pools as well as basal neuronal tritium efflux. Release was then stable, falling to only 0.6% of tissue content over the next 50 min (Fig. 17). A wash period of 30 min was determined to be sufficient to allow basal tritium efflux to stabilize and was used for all subsequent experiments.

iii) The effect of increasing concentrations of K^+ on basal release of [^{3}H]-D-aspartate.

For these experiments 2 min fractions were collected to enable the detection of small changes in tritium overflow. The depolarizing Krebs-Henseleit medium, containing either 10mM or 25mM KCl, was introduced into the superfusion system for 2 min at t = 10, 30 and 50 min.

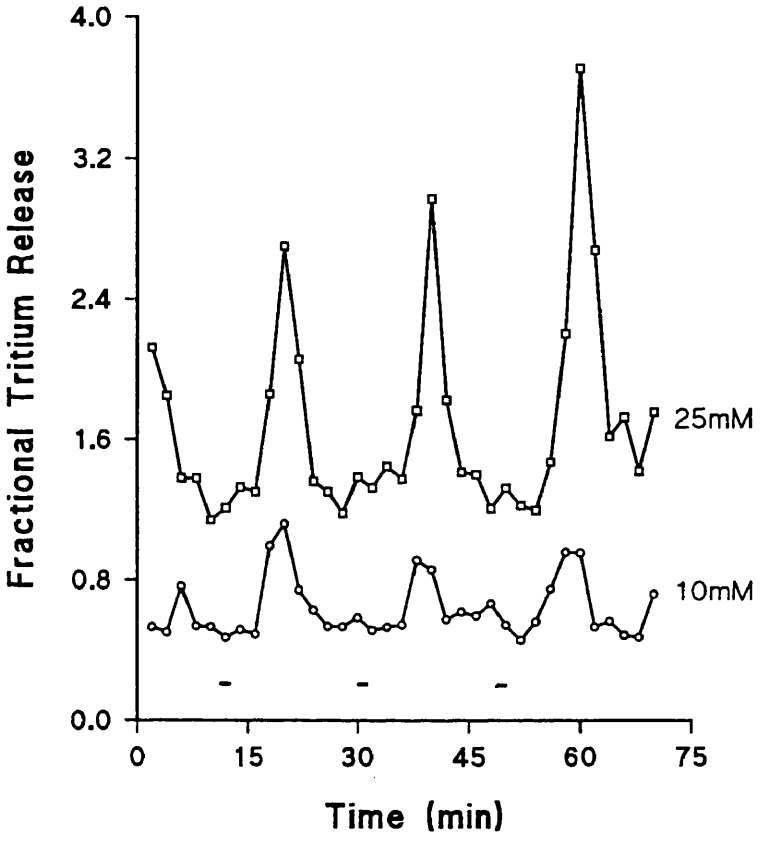
Elevated K^+ concentrations produced a dose-dependent increase in tritium release, detectable in the collected perfusate 8 min after introduction of the depolarizing medium into the system (Fig. 18). Tritium release returned to basal levels 2 or 3 fractions later. At 10mM K^+ tritium overflow comprised 1.23% (S_1), 0.7% (S_2) and 1.13% (S_3) of tissue tritium content, at 25mM K^+ this was increased to 2.80% (S_1), 2.78% (S_2) and 1.29% (S_3) (Table 6). The graph of FTR plotted against time for both K^+ concentrations (Fig. 18) gives an example of the variability in basal level of FTR in these experiments. This is likely to be due to slight differences in the rate at which perfusion medium is pumped through the four chambers and possibly to uneven distribution of the synaptosomal suspension on the nitrocellulose filters.

Fig. 17 Decline of efflux of [^{3}H]-D-aspartate from rat hippocampal synaptosomes with time.



Rat hippocampal synaptosomes preloaded with [^{3}H]-D-aspartate were transferred to perfusion chambers and washed for 60 min with warm, oxygenated Krebs-Henseleit solution. Perfusate samples were collected every 2 min. Fractional tritium release was determined from the tritium content of the fractions and synaptosomal aliquots and plotted against time. Values are the mean ± s.e.m. (n=4).

Fig. 18 The concentration-dependent, K^+-evoked release of [^{3}H]-D-aspartate from rat hippocampal synaptosomes.



Rat hippocampal synaptosomes were pulsed for 2 min, at 20 min intervals (solid bars), with Krebs-Henseleit solution containing either 10mM or 25mM KCl. Values are the mean of duplicate observations from separate experiments.

Table 6 Tritium overflow from rat hippocampal synaptosomes evoked by increasing concentrations of external K^+.

	Evoked Tritium Overflow (% Tissue Content)	
	10mM K^+	25mM K^+
S_1	1.23	2.8
S_2	0.70	2.78
S_3	1.13	1.29

Evoked tritium overflow was compared for increasing concentrations of K^+ in the superfusion medium. Values of S_1, S_2 and S_3 are from the experiment described in Fig. 18 above.

iv) Calcium-dependency of K^+-evoked release of [^{3}H]-D-aspartate.

The effect of 2 min pulses of 25mM K^+ Krebs-Henseleit medium on the release of [^{3}H]-D-aspartate from rat hippocampal synaptosomes was determined in the absence and presence of 1.8mM $CaCl_2$. 1mM EGTA was added to the Ca^{2+}-free solution (Fig. 19). In contrast to the corresponding hippocampal slice experiments, evoked tritium release was not reduced in Ca^{2+}-free medium, even with the addition of EGTA, rather it was enhanced (Table 7). This was unexpected and the explanation not clear.

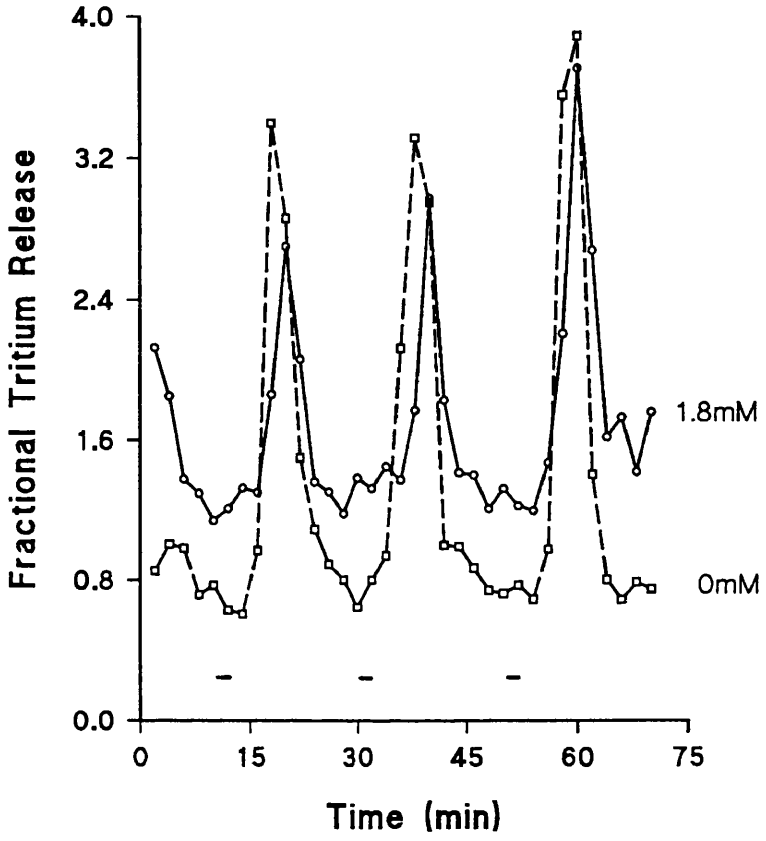
v) The effect of (-)baclofen on K^+-evoked release of [^{3}H]-D-aspartate

The effect of (-)baclofen was determined against [^{3}H]-D-aspartate release evoked by 10mM K^+. (-)Baclofen was introduced into the superfusion medium 2 min prior to, and during, S_2. Calculated S_2/S_1 ratios were compared to control values (Table 8). There was no inhibitory effect of (-) baclofen (30-300μM) on evoked tritium release, rather the S_2/S_1 ratios were greater in the presence of (-)baclofen than in its absence. 100μM (-)Baclofen was also unable to reduce [^{3}H]-D-aspartate release evoked by 25mM K^+ (Table 9).

vi) The effect of $GABA_B$ agonists on the K^+-evoked release of [^{3}H]-GABA.

For comparison the effect of (-)baclofen was determined using rat hippocampal synaptosomes preloaded with [^{3}H]-GABA (50nM; 91.5Ci/mmol). The experimental design was identical to that for [^{3}H]-D-aspartate release. (-)

Fig. 19 Lack of calcium-dependence of the K^+-evoked release of [^{3}H]-D-aspartate from rat hippocampal synaptosomes.



Rat hippocampal synaptosomes were stimulated with 2 min pulses of 25mM K^+, at 20 min intervals (solid bars), in the absence or presence of 1.8mM $CaCl_2$. Ca^{2+}-free medium contained 1mM EGTA. Values are duplicate observations from 1 experiment.

Table 7 Tritium overflow evoked by 25mM K^+ in rat hippocampal synaptosomes: lack of calcium-dependence.

	Evoked Tritium Overflow	
	+ Ca^{2+}	- Ca^{2+} + EGTA
S_1	2.80	5.51
S_2	2.78	5.59
S_3	4.29	6.88

Tritium overflow, evoked by 25mM K^+, was determined in the absence and presence of 1.8mM $CaCl_2$. 1mM EGTA was added to the Ca^{2+}-free medium. Values are from the experiment described in Fig. 19 above.

Table 8 The effect of (-)baclofen on the release of [^{3}H]-D-aspartate from rat hippocampal synaptosomes evoked by 10mM K^+.

	S_2/S_1
Control	0.56
+ 30μM (-)Baclofen	1.07
+ 100μM (-)Baclofen	1.19
+ 300μM (-)Baclofen	1.54

[^{3}H]-D-Aspartate release was evoked by 10mM K^+. (-)Baclofen (30-300μM) was introduced into the superfusion medium 2 min prior to, and during, S_2. S_2/S_1 ratios were calculated for control and (-)baclofen treated synaptosomes. Values are the mean of duplicate observations.

Table 9 The effect of (-)baclofen on the release of [^{3}H]-D-aspartate from rat hippocampal synaptosomes evoked by 25mM K^+.

	S_2/S_1
Control	0.99
+ 100μM (-)Baclofen	1.37

The experiment was repeated using 25mM K^+ as the depolarizing stimulus. (-)Baclofen (100μM) was introduced into the medium 2 min prior to, and during, S_2. Values are the mean of duplicate determinations.

Baclofen (10-100μM) appeared to enhance [^{3}H]-GABA release evoked by 10mM K^+, although the mean S_2/S_1 ratios were not significantly different from control (Table 10). 100μM GABA and 100μM 3-APA also produced an apparent enhancement of release and this was significantly different from control in the presence of GABA (Table 11). Therefore $GABA_B$ receptor mediated inhibition of [^{3}H]-GABA release could not be detected using this experimental approach.

3) $GABA_B$ Modulation of the K^+-Evoked Release of [^{3}H]-GABA from Rat Cerebrocortical and Hippocampal Synaptosomes: a Comparison with the K^+-Evoked Release of [^{3}H]-D-Aspartate from Rat Hippocampal Synaptosomes.

i) The K^+-evoked release of [^{3}H]-GABA from rat cerebrocortical synaptosomes.

Since $GABA_B$ agonists failed to inhibit K^+-evoked [^{3}H]-amino acid release from rat hippocampal preparations using the above methodology a comparison with the method of Bonanno et al (1989) was performed.

(-)Baclofen (1-100μM) produced a dose-dependent decrease in the release of [^{3}H]-GABA evoked by 15mM K^+ from rat cerebrocortical synaptosomes. A maximum inhibition of 55% was produced by 30μM (-)baclofen (Fig. 20) and these results are comparable with those obtained by Bonanno and coworkers (1989).

Table 10 The effect of (-)baclofen on K^+-evoked release of [^{3}H]-GABA from rat hippocampal synaptosomes.

	S_2/S_1	n	
Control	0.49 ± 0.16	4	
+ 10μM (-)Baclofen	1.49 ± 1.48	3	NSD
+ 30μM (-)Baclofen	0.56 ± 0.47	3	NSD
+ 100μM (-)Baclofen	0.81 ± 0.11	4	NSD

[^{3}H]-GABA release was evoked by 2 min pulses of 10mM K^+ at 20 min intervals. (-)Baclofen (10-100μM) was introduced into the superfusion medium 2 min prior to, and during, S_2. S_2/S_1 ratios were calculated in the absence and presence of (-)baclofen and compared (Student's 2-tailed t-test). Values are the mean ± s.e.m. (n=3-4).

NSD = no significant difference from control at p = 0.05.

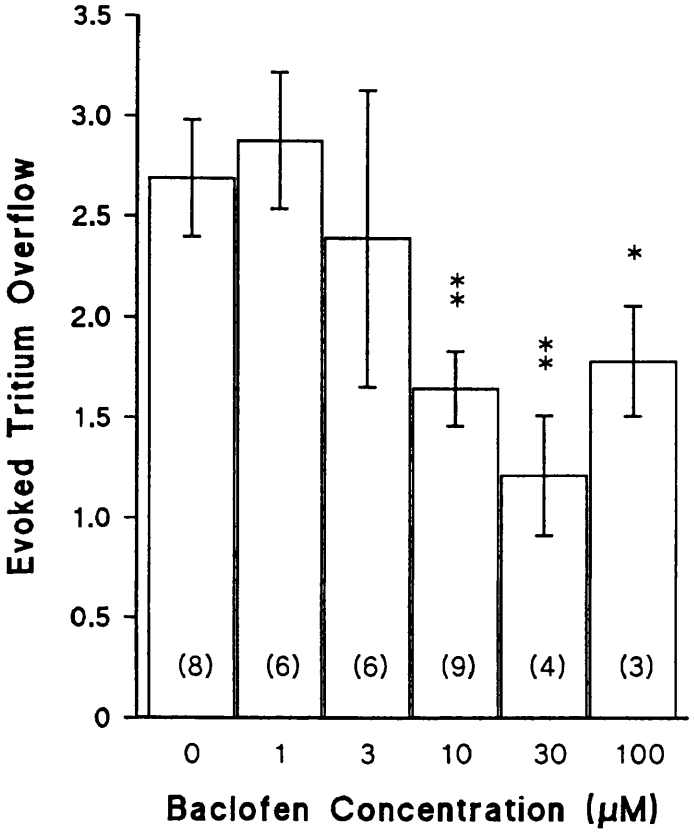
Table 11 The effect of 100μM (-)baclofen, GABA and 3-APA on K^+-evoked release of [^{3}H]-GABA from rat hippocampal synaptosomes.

	S_2/S_1	n	
Control	0.49 ± 0.16	4	
+ 100μM (-)Baclofen	0.81 ± 0.11	4	NSD
+ 100μM GABA	2.18 ± 0.21	3	$p < 0.005$
+ 100μM 3-APA	0.89 ± 0.34	3	NSD

[^{3}H]-GABA release was evoked by 10mM K^+ and test agonists were introduced into the superfusion medium 2 min prior to, and during, S_2. S_2/S_1 ratios were calculated for each test agonist and compared to control (Student's 2-tailed t-test). Values are the mean ± s.e.m. (n=3-4).

NSD = no significant difference from control at p = 0.05.

Fig. 20 Inhibition of the K^+-evoked release of [^{3}H]-GABA by (-)baclofen in rat cerebrocortical synaptosomes.



[^{3}H]-GABA release from rat cerebrocortical synaptosomes was elicited by a 90 sec application of 15mM K^+. (-)Baclofen (1-100μM) was added concomitantly with the depolarizing medium and its effect on evoked tritium overflow compared to that produced in its absence. Values are the mean ± s.e.m. of 3-9 observations from 6 experiments.

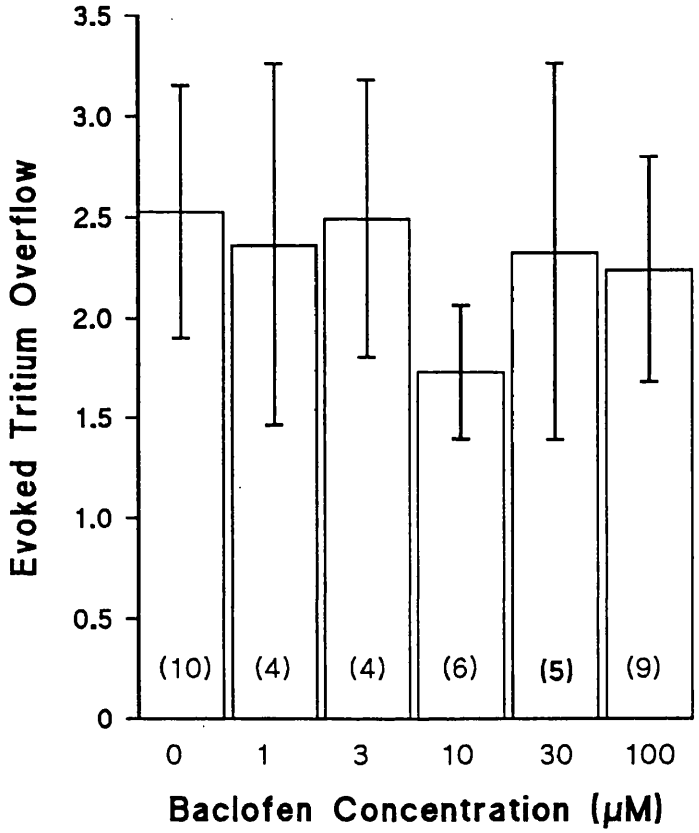
Student's 2-tailed t-test * $p < 0.05$ ** $p < 0.01$.

ii) The effect of (-)baclofen on the K^+-evoked release of [^{3}H]-D-aspartate from rat hippocampal synaptosomes.

Having assessed that the experimental method was sufficiently sensitive to detect $GABA_B$-mediated inhibition of [^{3}H]-GABA release in cortical synaptosomes the experiments were repeated using rat hippocampal synaptosomes preloaded with [^{3}H]-D-aspartate. As in previous experiments using hippocampal preparations, (-)baclofen (1-100μM) failed to significantly modulate the evoked release of [^{3}H]-D-aspartate compared to control (Fig. 21). A final series of experiments were carried out in which batches of hippocampal synaptosomes were divided and incubated with either [^{3}H]-GABA or [^{3}H]-D-aspartate. The effect of (-)baclofen on release of both tritiated amino acids evoked by 15mM K^+ was compared in the same experiments. As before, (-)baclofen (10-100μM) had no significant effect on [^{3}H]-D-aspartate release (Fig. 22a). Surprisingly, compared to the cortex, (-) baclofen did not inhibit [^{3}H]-GABA release in the hippocampus (Fig. 22b)

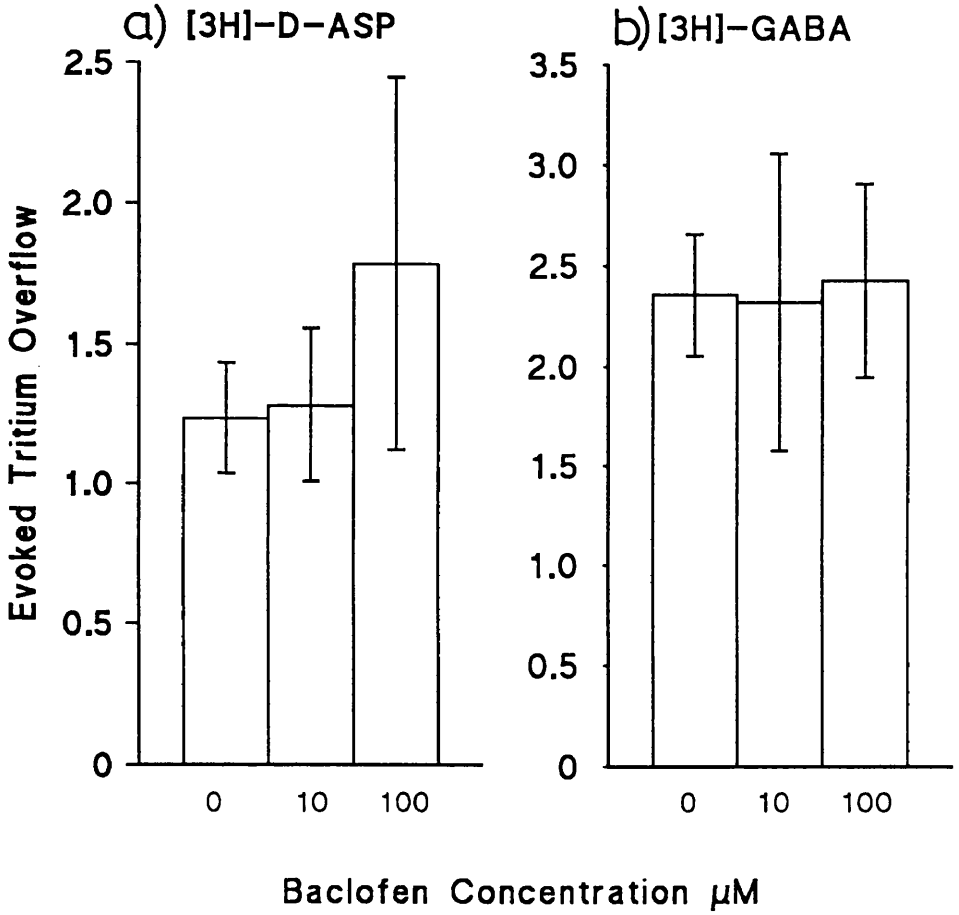
It is apparent that, irrespective of the experimental method used, $GABA_B$ agonists did not inhibit the K^+-evoked release of [^{3}H]-D-aspartate in rat hippocampal preparations. This was not due to any apparent inadequacy of the superfusion system or the depolarizing stimulus employed, as (-) baclofen did inhibit the release of [^{3}H]-GABA from rat cortical synaptosomes. Rather this seems to be due to the choice of tissue preparation or to the use of the neuronal marker D-aspartate.

Fig. 21 Effect of (-)baclofen on K^+-evoked release of [^{3}H]-D-aspartate in rat hippocampal synaptosomes.



[^{3}H]-D-Aspartate release from rat hippocampal synaptosomes was evoked by a 90 sec pulse of 15mM K^+. (-)Baclofen (1-100μM) was added concomitantly with the depolarizing medium and its effect compared to control (Student's 2-tailed t-test). Values are the mean ± s.e.m. of 4-10 observations from 5 experiments.

Fig. 22 Lack of effect of (-)baclofen on the K^+-evoked release of [^{3}H]-D-aspartate or [^{3}H]-GABA from rat hippocampal synaptosomes.



Release of either [^{3}H]-D-aspartate (a) or [^{3}H]-GABA (b) was elicited by a 90 sec pulse of 15mM K^+. (-)Baclofen (10-100μM) was added concomitantly with the depolarizing medium and its effect compared to control tissue (Student's 2-tailed t-test). Values are the mean ± s.e.m. (n=3-4).

4) The Effect of $GABA_B$ Agonists on the K^+-Evoked Release of Endogenous Amino Acids from Rat Hippocampal Synaptosomes.

i) Measurement of basal endogenous amino acid content.

The endogenous aspartate, glutamate and GABA content (pmoles/mg protein/5 min) of two successive unstimulated release samples was determined and compared (Table 12). In each case the amino acid content of the second sample was not significantly different from the first. The wash procedure outlined in the methods was therefore sufficient to allow basal endogenous amino acid release to stabilize and was used in all further studies.

ii) The effect of K^+ concentrations on the basal release of endogenous amino acids: dependency on calcium ions.

The chromatogram of a typical basal sample together with one collected in the presence of 50mM K^+, indicated that the principal amino acids released following chemical depolarization of the hippocampal synaptosomes were aspartate, glutamate and GABA (Fig. 23). At this K^+ concentration a small increase in the perfusate content of other amino acids was detected, in this case glutamine and taurine. This was less apparent at lower depolarizing K^+ concentrations. Therefore only the modulation of aspartate, glutamate and GABA release was determined in subsequent studies, although any unexpected changes in the concentrations of other amino acids were monitored.

Table 12 Measurement of basal endogenous amino acid content.

	pmoles/mg protein/5 min			
	Sample 1	Sample 2	n	
Aspartate	452.5 ± 46.3	447.5 ± 26.3	5	NSD
Glutamate	893.6 ± 74.4	747.0 ± 19.1	5	NSD
GABA	320.4 ± 38.5	324.5 ± 66.0	5	NSD

Rat hippocampal synaptosomes were washed with 10x2ml aliquots of warm, oxygenated Krebs-Henseleit solution. Basal endogenous aspartate, glutamate and GABA release was determined by incubation of the synaptosomes in two successive 500µl samples of Krebs-Henseleit solution for 5 min each. Amino acid content was determined by HPLC and expressed as pmoles/mg protein/5 min and compared in the two samples using Student's 2-tailed t-test.

NSD = no significant difference between sample 1 and sample 2 at p = 0.05.

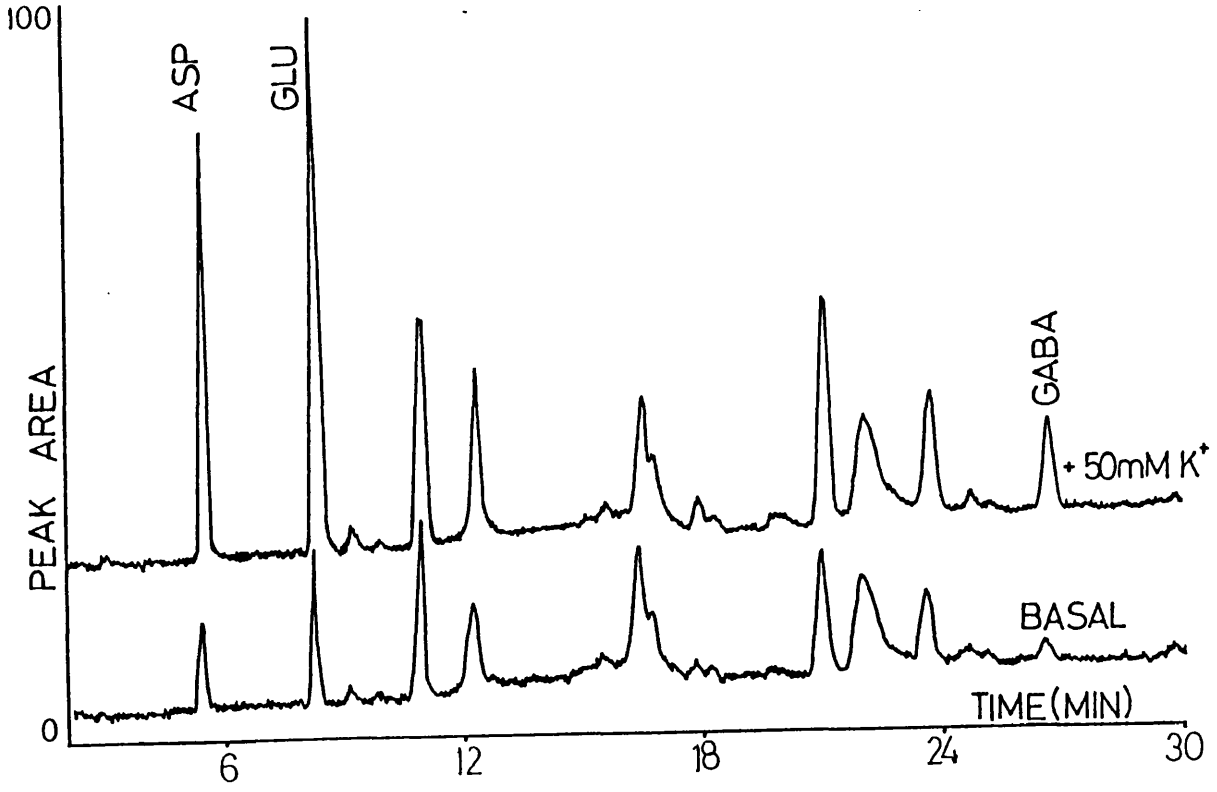


Fig. 23 Representative chromatogram of basal and evoked endogenous amino acid release from rat hippocampal synaptosomes.

Chromatogram from a basal perfusate sample (lower trace) and its associated 50mM K^+-stimulated sample (upper trace). Amino acid peaks were identified by retention time (abscissa) and concentrations determined from the peak areas (ordinate). Depolarization produced a relatively selective increase in aspartate, glutamate and GABA release.

Increasing K^+ concentrations (15mM, 25mM and 50mM) produced a dose-dependent increase in the amount of endogenous aspartate, glutamate and GABA released into the perfusate during the 5 min incubation compared to basal levels. Basal samples contained 500-600 pmoles/mg protein/5 min aspartate, 800-900 pmoles/mg protein/5 min glutamate and 400-500 pmoles/mg protein/5 min GABA. In the presence of 15mM, 25mM and 50mM KCl levels of aspartate were increased by 98%, 185% and 408% respectively; levels of glutamate were increased by 102%, 239% and 552% respectively; and levels of GABA were increased by 79%, 135% and 257% respectively (Table 13).

Experiments were repeated in the absence of calcium ions. Basal concentrations of amino acids measured in the absence of Ca^{2+} were not significantly different from those measured in the presence of 1.8mM $CaCl_2$ (Table 14). However, K^+-evoked release of aspartate, glutamate and GABA was reduced in the absence of Ca^{2+}. This was most striking for GABA release which was 80-85% Ca^{2+}-dependent at each K^+ concentration (Fig. 26). Evoked aspartate release was 45-60% Ca^{2+}-dependent (Fig. 24) and evoked glutamate release 26-43% Ca^{2+}-dependent (Fig. 25).

Table 13 The effect of increasing concentrations of K^+ on basal levels of endogenous aspartate, glutamate and GABA released from rat hippocampal synaptosomes.

	15mM K^+		25mM K^+		50mM K^+	
	Basal	Stim.	Basal	Stim.	Basal	Stim.
ASP	547±50	1081±170	512±52	1457±94	588±96	2984±231
GLU	887±76	1791±158	809±64	2739±145	827±79	5396±286
GABA	394±31	707±80	391±40	919±76	490±67	1749±208

Levels of endogenous aspartate (ASP), glutamate (GLU) and GABA were determined in the absence and presence of 15mM, 25mM and 50mM K^+ by HPLC. Amino acid concentration was expressed as the number of pmoles of amino acid released into 0.5ml incubation medium during a 5 min incubation period per mg of protein (pmole/mg/5 min). Values are the mean ± s.e.m. of 21-24 observations from 14 experiments.

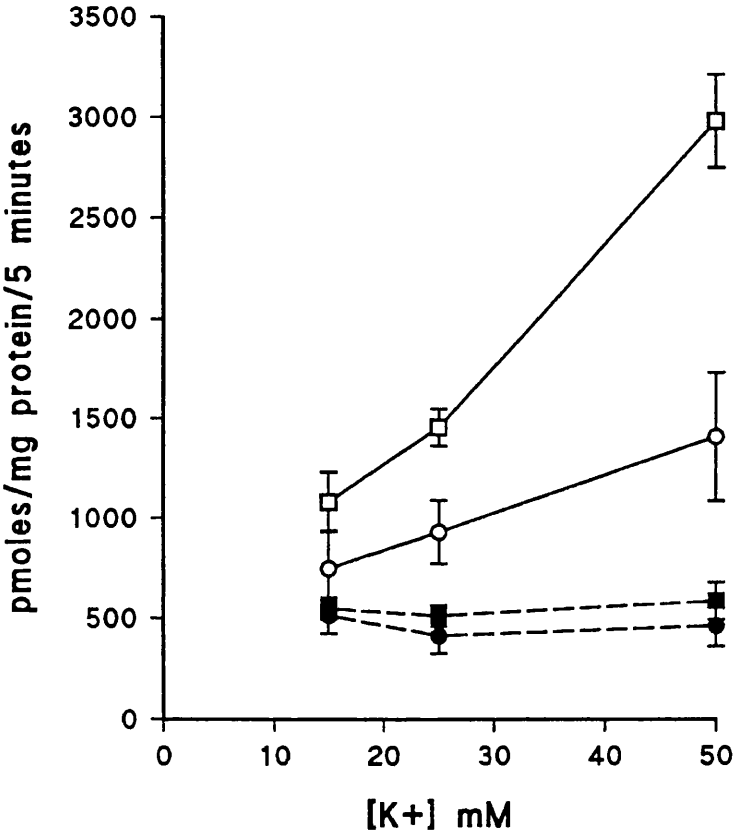
Table 14 The Ca^{2+}-independence of basal release of endogenous aspartate, glutamate and GABA from rat hippocampal synaptosomes.

	+ 1.8mM Ca^{2+} (n)	+ 0mM Ca^{2+} (n)	
ASP	540 ± 40 (72)	461 ± 48 (23)	NSD
GLU	841 ± 41 (72)	972 ± 94 (23)	NSD
GABA	427 ± 29 (68)	345 ± 34 (22)	NSD

Basal levels of endogenous amino acids were determined in hippocampal synaptosomes incubated for 5 min in warm, oxygenated Krebs-Henseleit solution containing either 0mM $CaCl_2$ or 1.8mM $CaCl_2$. At each calcium concentration basal levels, in pmoles/ mg protein/5 min, were compared using Student's 2-tailed t-test. Values are the mean ± s.e.m. of 22-72 observations from 4-14 experiments.

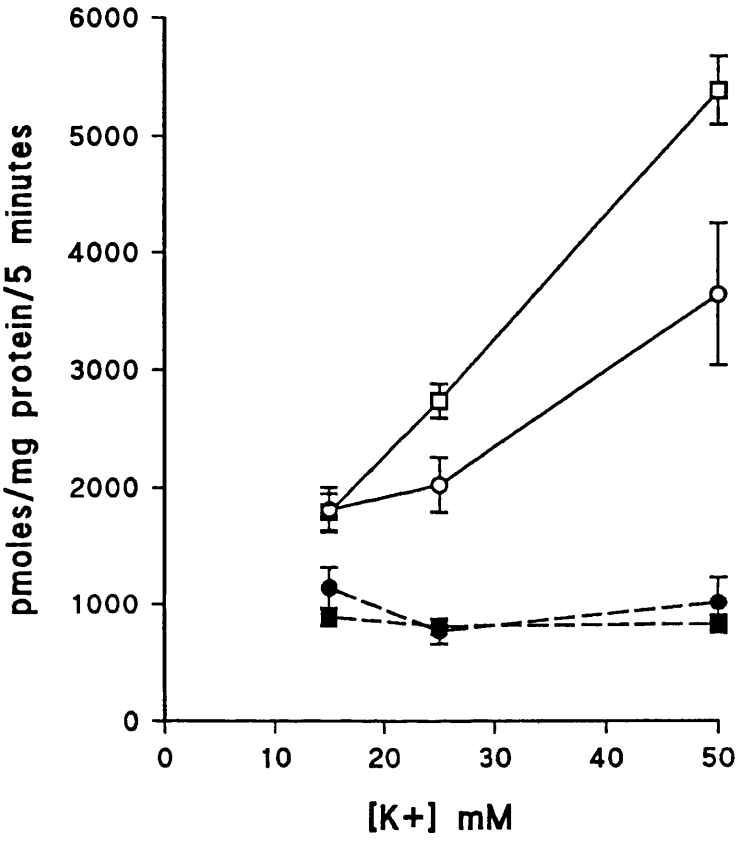
NSD = no significant difference at p = 0.05.

Fig. 24 K^+-Evoked release of endogenous aspartate from rat hippocampal synaptosomes in the absence and presence of external Ca^{2+} ions.



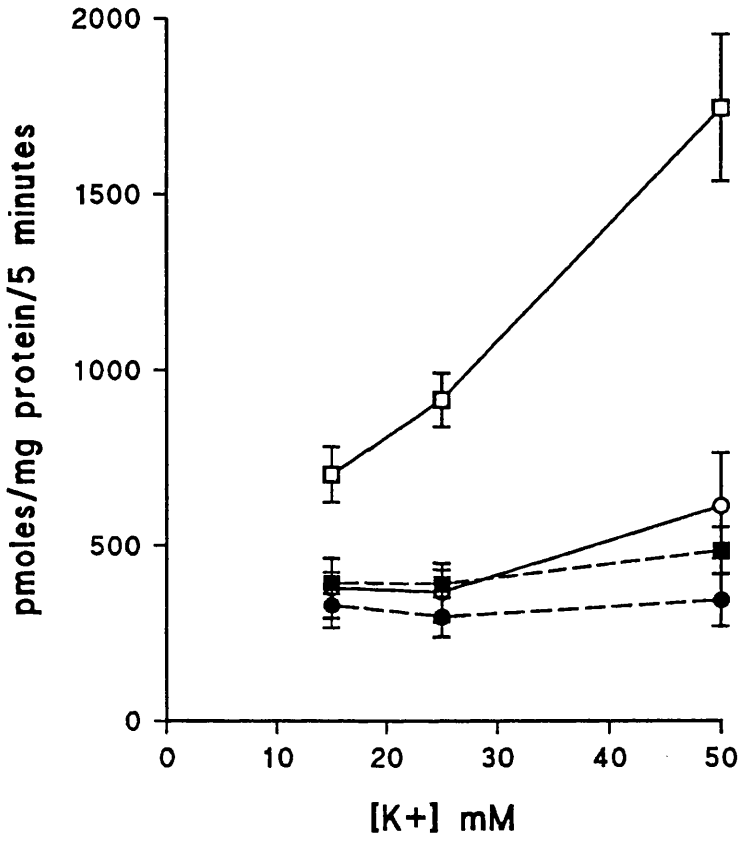
Elevated K^+ concentrations in the incubating medium produced a dose-dependent increase in endogenous aspartate content (pmoles/mg protein/5 min) in the presence of 1.8mM $CaCl_2$ (□). K^+-Evoked release was attenuated in the absence of external calcium by 45-60% (○). Basal levels of aspartate release were unaffected by the removal of calcium from the Krebs-Henseleit solution (dashed lines). Values are the mean ± s.e.m. of 7-24 observations from 5-15 experiments.

Fig. 25 K^+-Evoked release of endogenous glutamate from rat hippocampal synaptosomes in the absence and presence of external Ca^{2+} ions.



Elevated K^+ concentrations in the incubating medium produced a dose-dependent increase in endogenous glutamate content (pmoles/mg protein/5 min) in the presence of 1.8mM $CaCl_2$ (□). K^+-Evoked release was attenuated in the absence of external calcium by 45-60% (○). Basal levels of glutamate release were unaffected by the removal of calcium from the Krebs-Henseleit solution (dashed lines). Values are the mean ± s.e.m. of 7-24 observations from 5-15 experiments.

Fig. 26 K^+-Evoked release of endogenous GABA from rat hippocampal synaptosomes in the absence and presence of external Ca^{2+} ions.

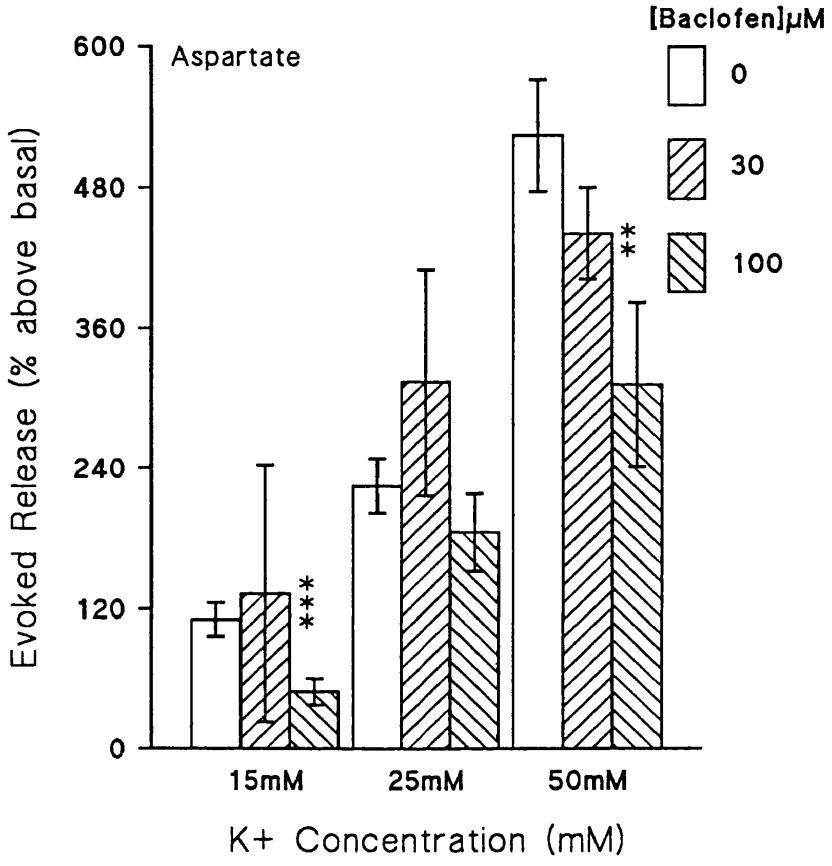


Elevated K^+ concentrations in the incubating medium produced a dose-dependent increase in endogenous GABA content (pmoles/mg protein/5 min) in the presence of 1.8mM $CaCl_2$ (□). K^+-Evoked release was attenuated in the absence of external calcium by 45-60% (○). Basal levels of GABA release were unaffected by the removal of calcium from the Krebs-Henseleit solution (dashed lines). Values are the mean ± s.e.m. of 7-24 observations from 5-15 experiments.

iii) The effect of (-)baclofen and 3-APA on K^+-stimulated release of endogenous aspartate, glutamate and GABA.

(-)Baclofen (30-100μM) produced a dose-dependent decrease in the release of endogenous aspartate (Fig 27), glutamate (Fig. 28) and GABA (Fig. 29) evoked by 50mM K^+ with more variable effects at the lower K^+ concentrations of 15mM and 25mM. Due to the variability in absolute values of pmoles/mg protein/5 min between different experiments the data for this study were also expressed as the % increase in endogenous amino acid content above basal level. (-)Baclofen (100μM) reduced the release of aspartate evoked by 50mM K^+ by 41% from 525±47% (n=24) to 312±70% (n=11), the release of glutamate by 48% from 688±53% (n=25) to 360±48% (n=11) and GABA release by 40% from 375±37% (n=23) to 225±25% (n=10). A comparable effect of 100μM (-) baclofen was observed against aspartate and glutamate release evoked by 15mM K^+, without any effect on GABA release. However, (-) baclofen (30-100μM) failed to attenuate release of all three amino acids evoked by 25mM K^+. These results differ from those of Bonanno et al (1989) who observed that the inhibitory action of (-)baclofen on K^+-evoked amino acid release from rat cortical synaptosomes decreased as the concentration of the K^+ stimulus was increased from 9-50mM.

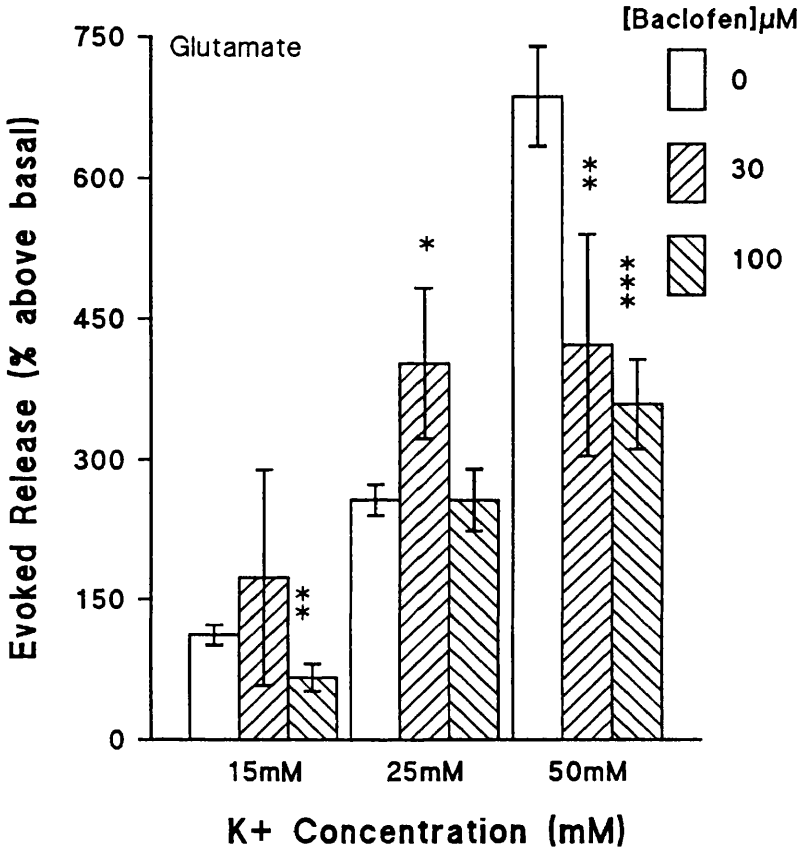
Fig. 27 The effect of (-)baclofen on K^+-evoked release of endogenous aspartate from rat hippocampal synaptosomes.



(-)Baclofen (30-100μM) produced a dose-dependent decrease in aspartate release evoked by 15mM and 50mM K^+, with no significant effect against 25mM K^+. Values are expressed as the % increase in aspartate release above basal levels and are the mean ± s.e.m. of 11-26 observations from 6-14 experiments. Effects of (-)baclofen were compared to control by Student's 2-tailed t-test.

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.005$

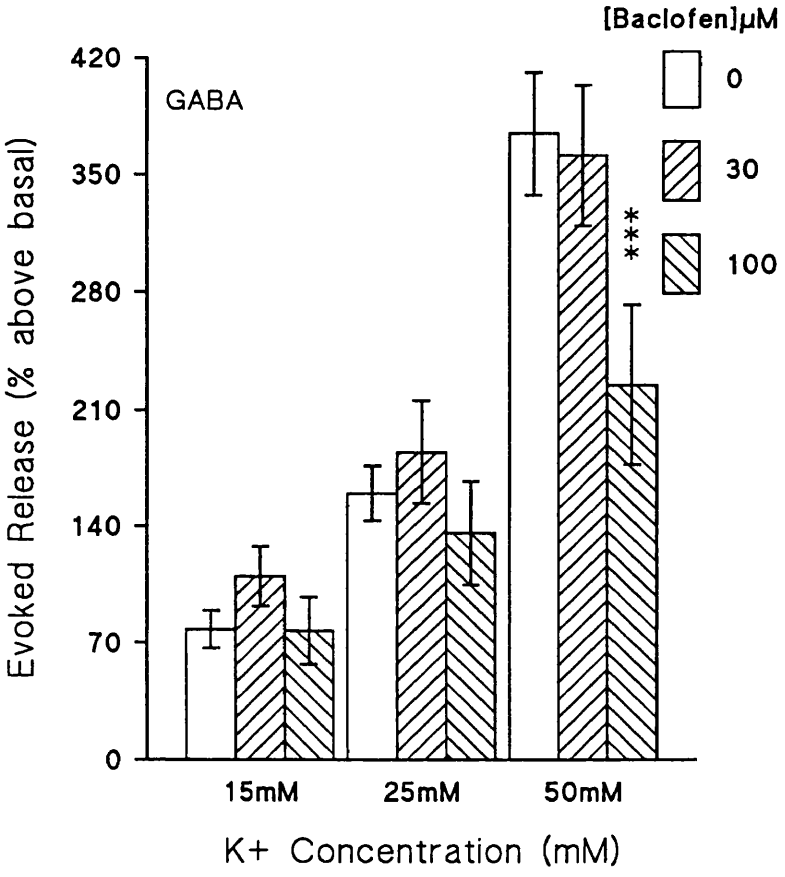
Fig. 28 The effect of (-)baclofen on K^+-evoked release of endogenous glutamate from rat hippocampal synaptosomes.



(-)Baclofen (30-100μM) produced a dose-dependent decrease in glutamate release evoked by 15mM and 50mM K^+, with no significant effect against 25mM K^+. Values are expressed as the % increase in glutamate release above basal levels and are the mean ± s.e.m. of 11-25 observations from 6-14 experiments. Effects of (-)baclofen were compared to control by Student's 2-tailed t-test.

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.005$

Fig. 29 The effect of (-)baclofen on K^+-evoked release of endogenous GABA from rat hippocampal synaptosomes.



(-)Baclofen (30-100μM) produced a dose-dependent decrease in GABA release evoked by 50mM K^+, with no significant effect against 15mM and 25mM K^+. Values are expressed as the % increase in GABA release above basal levels and are the mean ± s.e.m. of 11-25 observations from 6-14 experiments. Effects of (-)baclofen were compared to control by Student's 2-tailed t-test.

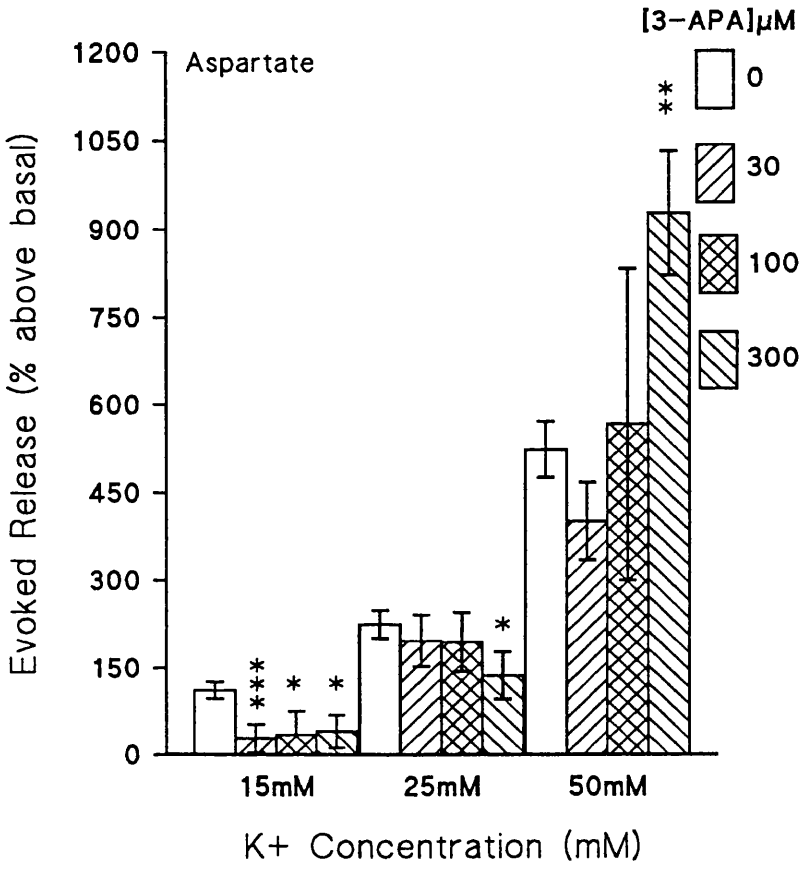
* $p < 0.05$ ** $p < 0.01$ *** $p < 0.005$

3-APA (30-300μM) produced a dose-dependent decrease in aspartate release evoked with 15mM and 25mM K^+. At 50mM K^+ there was no inhibitory effect of 3-APA (30-100μM) but a significant increase in evoked aspartate release was produced at 300μM (Fig. 30). Glutamate release was unaffected by 3-APA with the exception of a significant increase in release evoked by 50mM K^+ in the presence of 300μM 3-APA (Fig. 31). A similar effect was obtained for GABA although the enhancement of evoked GABA release was more profound, observed at all K^+ concentrations in the presence of 100-300μM 3-APA (Fig. 32). 3-APA, therefore, did not mimic the inhibitory effect of (-) baclofen on K^+-evoked release of endogenous amino acids from rat hippocampal synaptosomes.

iv) The effect of CGP 35348 on the inhibition of K^+-evoked release of endogenous aspartate, glutamate and GABA by (-)baclofen.

In this study the release of endogenous aspartate, glutamate and GABA was elicited by 50mM K^+. In some cases (-)baclofen (100μM), CGP 35348 (300μM) or both test compounds were included in the depolarizing medium. (-) Baclofen produced a 30% decrease in evoked amino acid release compared to control values, comparable with results from the earlier study (III.4.iii). CGP 35348 had no effect on evoked endogenous amino acid release nor did it modulate the (-)baclofen-mediated inhibition of aspartate or glutamate release. However CGP 35348 (300μM) completely antagonized the $GABA_B$-mediated inhibition of endogenous GABA release in this preparation (Fig. 33).

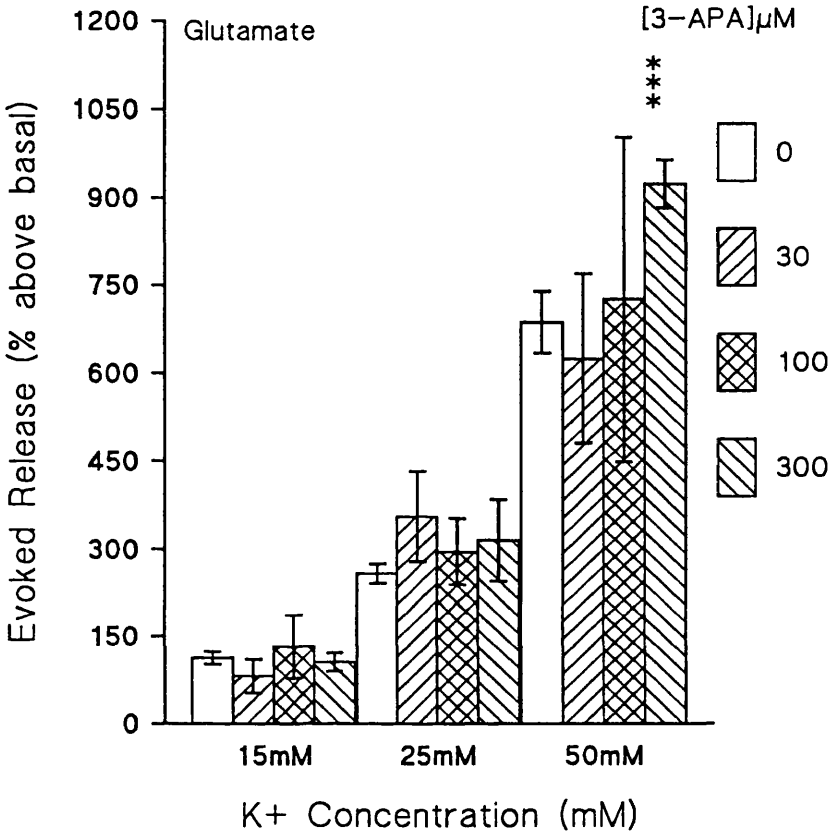
Fig. 30 The effect of 3-APA on the K^+-evoked release of endogenous aspartate from rat hippocampal synaptosomes.



3-APA (30-300μM) produced a dose-dependent decrease in aspartate release evoked by 15mM K^+. In all other cases aspartate release was either unaffected or significantly increased above control levels. Values are expressed as the % increase in aspartate release above control levels and are the mean ± s.e.m. of 5 observations from 2 experiments. Effects of 3-APA were compared to control using Student's 2-tailed t-test.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.005$

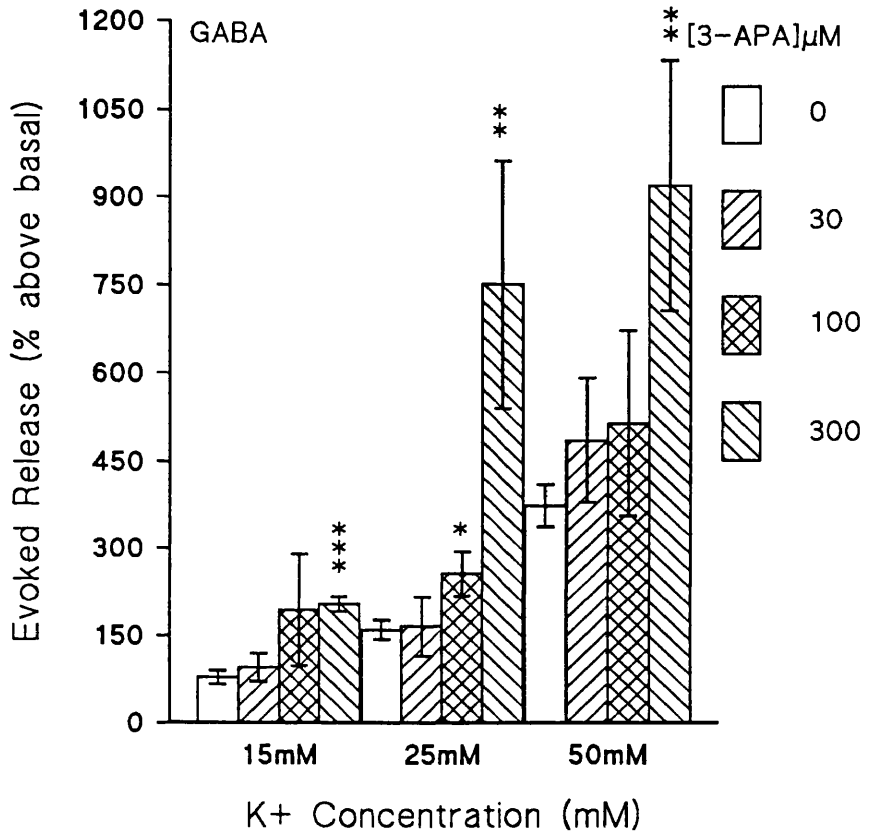
Fig. 31 The effect of 3-APA on the K^+-evoked release of endogenous glutamate from rat hippocampal synaptosomes.



3-APA was without effect on glutamate release except at 300μM with release evoked by 50mM K^+ when a significant enhancement above control levels was obtained. Values are expressed as the % increase in glutamate release above control levels and are the mean ± s.e.m. of 5 observations from 2 experiments. Effects of 3-APA were compared to control using Student's 2-tailed t-test.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.005$

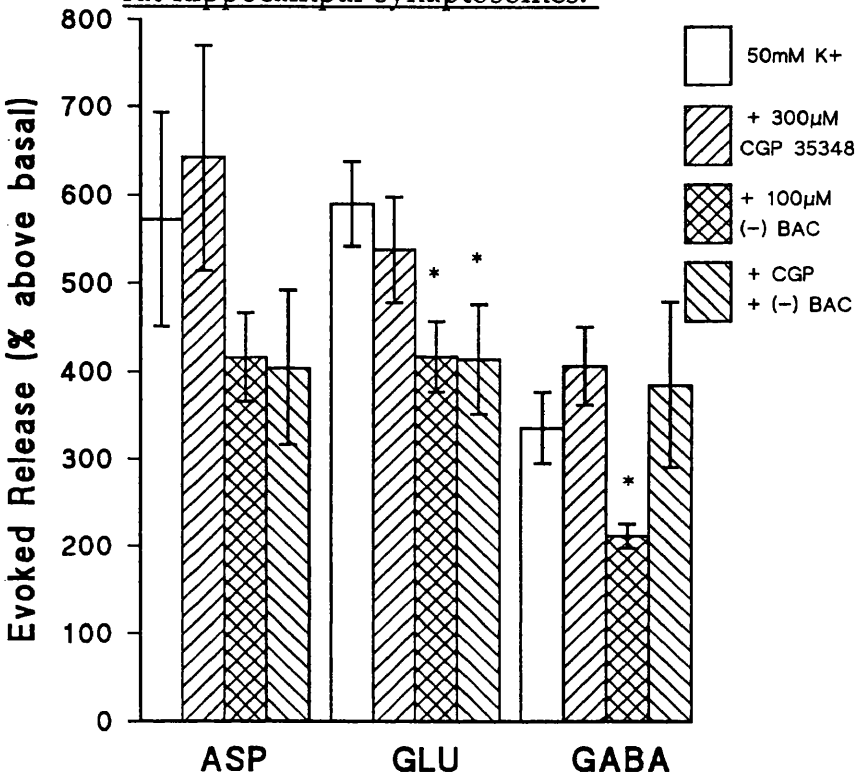
Fig. 32 The effect of 3-APA on the K^+-evoked release of endogenous GABA from rat hippocampal synaptosomes.



Evoked GABA release was either unaffected or significantly increased above control levels by 3-APA at each concentration of K^+. Values are expressed as the % increase in GABA release above control levels and are the mean ± s.e.m. of 5 observations from 2 experiments. Effects of 3-APA were compared to control using Student's 2-tailed t-test.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.005$

Fig. 33 The effect of CGP 35348 on (-)baclofen-mediated inhibition of K^+-evoked endogenous aspartate, glutamate and GABA release from rat hippocampal synaptosomes.



Basal amino acid release was determined by incubation of synaptosomes in 500µl warm oxygenated Krebs-Henseleit solution for 5 min. This was replaced with solution containing 50mM K^+ with or without (-) baclofen (100µM), CGP 35348 (300µM) or both compounds concomitantly. Evoked release was expressed as the % increase in amino acid content above basal levels and the effect of CGP 35348 on (-)baclofen inhibition was compared to the effect of (-) bclofen alone. (Student's 2-tailed t-test). * $p < 0.05$

5) Discussion.

i) Modulation of neurotransmitter release by $GABA_B$ agonists.

K^+-Evoked release of [^{3}H]-D-aspartate was demonstrated in both hippocampal slice and synaptosomal preparations, however only that from the tissue slices showed Ca^{2+}-dependence (up to 70%). It has been suggested that Ca^{2+}-independent release is derived from recently accumulated neurotransmitter pools whereas Ca^{2+}-dependent release is additionally derived from endogenous pools. Also transmitter release from cytoplasmic stores, brought about by the reversal of uptake mechanisms, is thought to be Ca^{2+}-independent whereas vesicular release requires an influx of calcium into the terminal to trigger it (see Adam-Vizi, 1992, for review). It may be possible that the longer incubation time used for the accumulation of [^{3}H]-D-aspartate by hippocampal slices (40 min compared to 10 min in synaptosomes) was sufficient for the tritiated amino acid to label 'endogenous pools' and hence exhibit a greater degree of Ca^{2+}-dependent release than K^+-evoked synaptosomal release. Though it should be noted that the uptake mechanism at the level of the synaptic vesicle is reportedly more specific than the plasma membrane carrier protein, having no affinity for aspartate, only glutamate (Naito and Ueda, 1985).

The inability of (-)baclofen to inhibit the K^+-evoked release of [^{3}H]-D-aspartate from either of these preparations contrasts with the findings of some other

groups. (-)Baclofen-mediated inhibition of [^{3}H]-D-aspartate release has been observed in guinea-pig cortical and striatal slices (Potashner and Gerard, 1983), rat cortical and cat spinal cord slices (Johnston et al., 1980) and cultured cerebellar granule cells (Zhu and Chuang, 1987). However in agreement with the current study, (-)baclofen had no effect on *in vivo* K^+-evoked [^{3}H]-D-aspartate release from rat hippocampus monitored by intracerebral microdialysis, although basal release was enhanced in a Ca^{2+}-dependent manner (Nielsen et al., 1989). A similar effect on the basal release of [^{3}H]-GABA from rat whole brain synaptosomes was reported by Roberts and co-workers in 1978.

In section 3, experiments were performed exactly according to a published method (Bonanno et al., 1989) using rat hippocampal synaptosomes. (-) Baclofen was shown to potently inhibit [^{3}H]-GABA release from rat cerebrocortical synaptosomes yet failed to have any effect when the studies were repeated with hippocampal synaptosomes labelled with either [^{3}H]-GABA or [^{3}H]-D-aspartate. This would suggest that the problem lies with the preparation rather than with the method or even the choice of label.

To complicate matters it was possible to use the same hippocampal preparation to study the effect of (-)baclofen on the K^+-evoked release of endogenous amino acids. In this case (-)baclofen produced a dose-dependent decrease in Ca^{2+}-dependent, K^+-evoked release of endogenous aspartate, glutamate and GABA that appeared to increase as the K^+-concentration was

increased from 15mM to 50mM. Comparable observations of $GABA_B$-mediated inhibition of endogenous aspartate and glutamate from rat cerebral cortical synaptosomes have recently been reported (Bonanno et al., 1992). These findings are not in agreement with those of Burke and Nadler (1988) who found no effect of (-) baclofen (100μM) on the release of endogenous aspartate, glutamate and GABA from hippocampal CA1 minislices evoked by 50mM K^+. The reason for this discrepancy is not clear, the only apparent difference being the use of a slice preparation in the latter study and synaptosomal preparations in the other two.

The lack of effect of 3-APA on evoked amino acid release from rat hippocampal synaptosomes was unexpected as this compound has been reported to be a potent and selective inhibitor of [^{3}H]-GABA binding to $GABA_B$ receptors in rat whole brain membranes (Pratt et al., 1989) and acts as a functional $GABA_B$ agonist in rat brain slices (Seabrook et al., 1990). However, in agreement with the present study discrepancies between the effects of (-) baclofen and 3-APA in CNS preparations have been reported by other workers. 3-APA (50μM) did not mimic (-) baclofen's ability to depress the K^+-evoked release of [^{3}H]-glutamate (prelabelled by [^{3}H]-glutamine) from rat cultured cerebellar granule cells (Huston et al.,1990). In rat neocortical slices, maintained in Mg^{2+}-free Krebs medium, (-)baclofen suppressed the spontaneous paroxysmal discharges and, occasionally, produced a slight hyperpolarization. Both responses were sensitive to the $GABA_B$ antagonist 2-OH-saclofen. The profile of activity of 3-APA was quite different. 3-APA

produced a prompt, 2-OH-saclofen-insensitive hyperpolarization with no effect on discharge frequency, although some reduction in discharge amplitude was observed that could be reversed by the antagonist (Ong et al., 1990b). However this group have also shown that 3-APA mimics the (-)baclofen-mediated attenuation of synaptic transmission in cultured rat hippocampal neurones (Ong et al., 1990a). They claim this effect is due to the activation of presynaptic $GABA_B$ receptors as cultured hippocampal neurones reportedly lack postsynaptic $GABA_B$ receptors (Harrison, 1990).

This inconsistency between receptor binding data and functional assays could be explained if sub-populations of $GABA_B$ receptors exist which are distinguished by 3-APA. Theoretically, in rat whole brain membranes a lack of affinity of 3-APA for a small sub-population of $GABA_B$ receptors may be masked by the ability of the compound to displace [^{3}H]-GABA from other brain regions. This theory is addressed in Chapter five.

ii) Modulation of $GABA_B$-mediated inhibition of neurotransmitter release by putative $GABA_B$ antagonists.

Dutar and Nicoll (1988b) reported that postsynaptic $GABA_B$ receptors in the rat hippocampus were sensitive to PTX and the weak antagonist phaclofen. The presynaptic $GABA_B$ receptor was apparently insensitive to both agents. This was the first published evidence for $GABA_B$ receptor heterogeneity. Given the low potency of phaclofen the authors felt that the use of more

potent $GABA_B$ antagonists, such as the recently available CGP 35348, was necessary to confirm their original observations.

CGP 35348 (300μM) was found to antagonize the (-)baclofen-mediated inhibition of K^+-evoked endogenous GABA release from rat hippocampal synaptosomes, without effect on the inhibition of aspartate and glutamate. This would suggest that this compound is selective for the $GABA_B$ autoreceptor present on GABAergic terminals within the hippocampus and without appreciable affinity for the presynaptic heteroreceptor which modulates the release of aspartate and glutamate. Though these results support the theory of $GABA_B$ receptor heterogeneity it may be that $GABA_B$ heteroreceptors are merely less sensitive to CGP 35348 than $GABA_B$ autoreceptors and that they may be antagonized at higher concentration (>1mM) or by a more potent compound. Similar studies carried out by other groups have produced contradictory data as outlined below:

a) $GABA_B$ antagonists do not distinguish between presynaptic $GABA_B$ autoreceptors and $GABA_B$ heteroreceptors in rat brain preparations.

Thompson and co-workers (1992) reported that the (-)baclofen-mediated depression of both pharmacologically isolated IPSPs and EPSPs in the rat hippocampus was reversed by CGP 35348. Although the two receptor populations were not distinguished by this antagonist they did differ in their sensitivity to PTX. The generation of EPSPs was unaffected by PTX whereas

IPSPs were fully blocked by the toxin pretreatment. These findings confirm those of Dutar and Nicoll (1988b) and Colmers and Williams (1988).

b) $GABA_B$ antagonists do distinguish between presynaptic $GABA_B$ autoreceptors and $GABA_B$ heteroreceptors in rat brain preparations.

The present study indicated that $GABA_B$ autoreceptors in rat hippocampus are CGP 35348-sensitive whilst $GABA_B$ heteroreceptors on glutaminergic terminals are not. This has been confirmed by recent observations. Paired pulse depression (an index of $GABA_B$ autoreceptor activity) was alleviated by CGP 35348 in both the rat hippocampus (Olpe et al., 1992) and rat neocortex (Deisz et al., 1992), although additional studies showed that if the pulse interval was increased from 200msec to 400msec the sensitivity to CGP 35348 was lost (Deisz and Zieglgansberger, 1992). Both phaclofen and CGP 35348 antagonized the (-)baclofen-mediated inhibition of electrically-evoked endogenous GABA release from rat cortical synaptosomes (Waldmeier and Baumann, 1990). However, both compounds enhanced basal release at the stimulus frequency used making it difficult to interpret the results fully.
In synaptosomes prepared from rat spinal cord the effect of GABA (10μM) at prejunctional autoreceptors was antagonized by CGP 35348 but was insensitive to phaclofen (1mM) (Raiteri, 1992).

In contrast, Bonanno et al. (1992), using rat cerebral cortical synaptosomes, found the $GABA_B$ autoreceptor to be phaclofen-sensitive and CGP 35348-

insensitive, the $GABA_B$ heteroreceptor regulating glutamate release to be phaclofen-insensitive and CGP 35348-sensitive and a third receptor modulating the release of somatostatin to be sensitive to both antagonists. The availability of more potent antagonists may help to resolve these discrepancies.

CHAPTER FOUR: RESULTS

CHARACTERIZATION OF PRESYNAPTIC $GABA_B$ RECEPTORS IN THE RAT ANOCOCCYGEUS MUSCLE

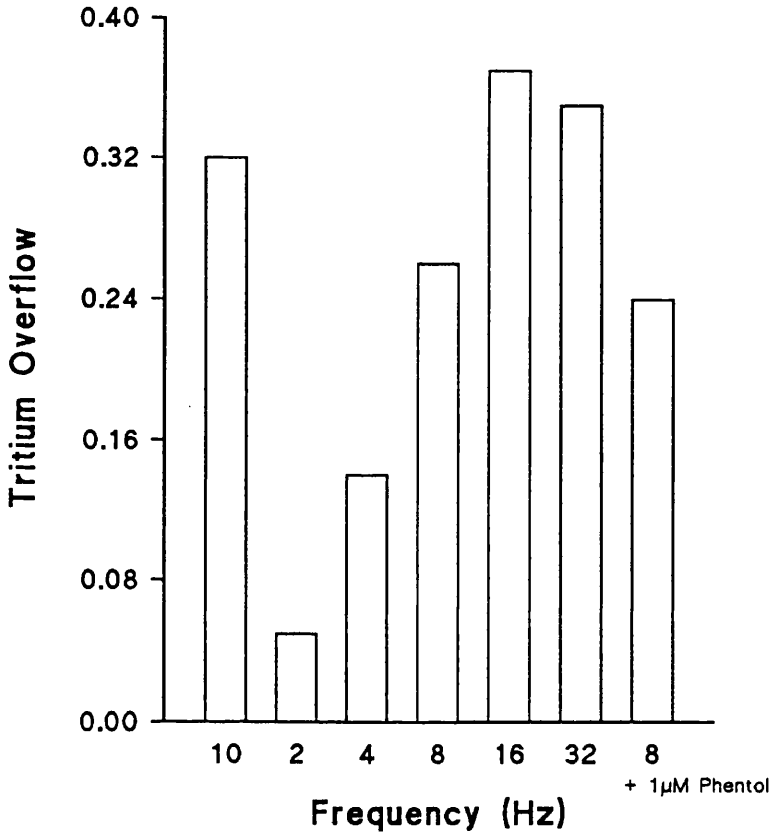
1) $GABA_B$ Receptor-Mediated Modulation of the Electrically-Evoked Release of [^{3}H]-Noradrenaline from the Rat Anococcygeus Muscle.

i) The dependency of the electrically-evoked release of [^{3}H]-noradrenaline on stimulus frequency.

Stimulation of the anococcygeus muscle with electrical pulses (10 second train, 0.5 msecond pulse width, supramaximal voltage) of successively greater frequency (2-32Hz) led to a frequency-dependent increase in tritium overflow (Fig. 34). Measurable tritium overflow, 0.05% of tissue content, was detectable at 2Hz and was maximal (>0.37%) at between 16 and 32Hz. So as to detect both decreases and increases in tritium overflow the submaximal stimulus frequency of 10Hz was chosen for all subsequent studies.

The twitch responses of the rat anococcygeus muscle increased in magnitude over the same frequency range (Fig. 35). This response was composed of two components; a fast initial twitch which peaked within 20 sec and a second much slower 'shoulder' with a duration of up to 4 min. The effect of pharmacological agents on both components was noted in ensuing experiments.

Fig. 34 The effect of increasing stimulus-frequency on tritium overflow from the rat anococcygeus muscle.



Electrical stimulation was applied via platinum ring electrodes to the rat anococcygeus muscle. The following stimulus parameters were employed: 10 sec train, 0.5msec pulse width, supramaximal voltage, train interval of 25 min. The frequency of stimulation was increased sequentially from 2 to 32 Hz following an initial pulse delivered at 10 Hz. The response to 8Hz was unaffected by the presence of 1 μM phentolamine.

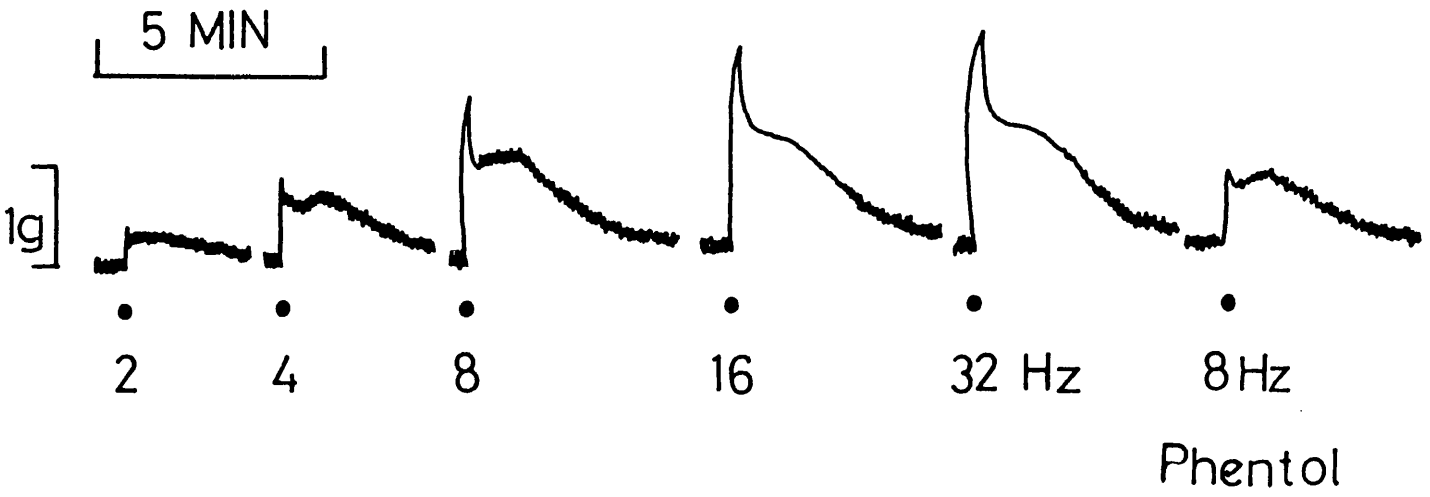


Fig. 35 The effect of increasing stimulus-frequency on the twitch response of the rat anococcygeus muscle.

The twitch response of the rat anococcygeus muscle to electrical stimulation was recorded concomitantly with tritium overflow shown in Fig. 34. The magnitude of the twitch response increased in parallel with tritium overflow, reaching maximal values between 16 and 32 Hz. Phentolamine distinguished the postsynaptically-mediated twitch response from presynaptic tritium release, by reducing only the former.

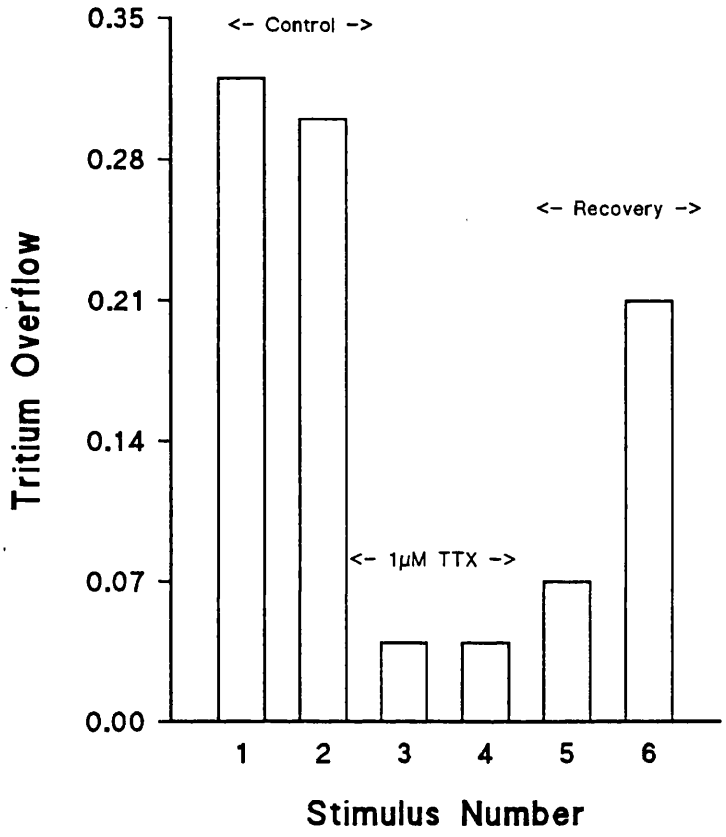
ii) The effect of TTX on electrically-evoked release of [^{3}H]-noradrenaline.

TTX (1μM) present in the perfusion medium during the third and fourth stimulation periods, completely depressed electrically-evoked tritium overflow (S_3 and S_4) compared to the control response (S_2) (Fig. 36). Overflow in the presence of the toxin comprised 0.04% of tissue tritium content, 87% less than under control conditions. The result suggested that the majority of the electrically-evoked [^{3}H]-noradrenaline overflow was released from presumed adrenergic neurones within the rat anococcygeus muscle preparation. Interestingly, the twitch response to the third stimulation, whilst reduced compared to the control response, was not affected to the same extent as the associated tritium overflow (Fig. 37). The fast twitch response to the fourth stimulus was completely abolished although the second component of the mechanical response was not suppressed by TTX.

iii) Ca^{2+}-dependency of the evoked release of [^{3}H]-noradrenaline.

A pair of anococcygeus muscles were used for this study one perfused continuously with normal Krebs-Henseleit solution containing 1.8mM $CaCl_2$ the other with Ca^{2+}-free medium, until after the third stimulus at which point Ca^{2+} was reintroduced. Under control conditions tritium overflow comprised 0.15 - 0.25% of tissue content, decreasing with successive periods of electrical stimulation (Fig. 38a). In the absence of Ca^{2+} tritium overflow was barely detectable, comprising no more than 0.04% of tissue content (Fig. 38b). On

Fig. 36 The effect of TTX on the electrically-evoked release of [^{3}H]-noradrenaline from the rat anococcygeus muscle.



Tritium overflow was calculated in response to successive periods of electrical stimulation delivered at 25 min intervals. TTX (1μM) was introduced into the perfusing medium 1 min prior to the 3rd stimulus and was present until immediately after the 4th stimulus. Tritium overflow was substantially reduced in the presence of the toxin and did not begin to recover until the 6th stimulus. Data are from one experiment.

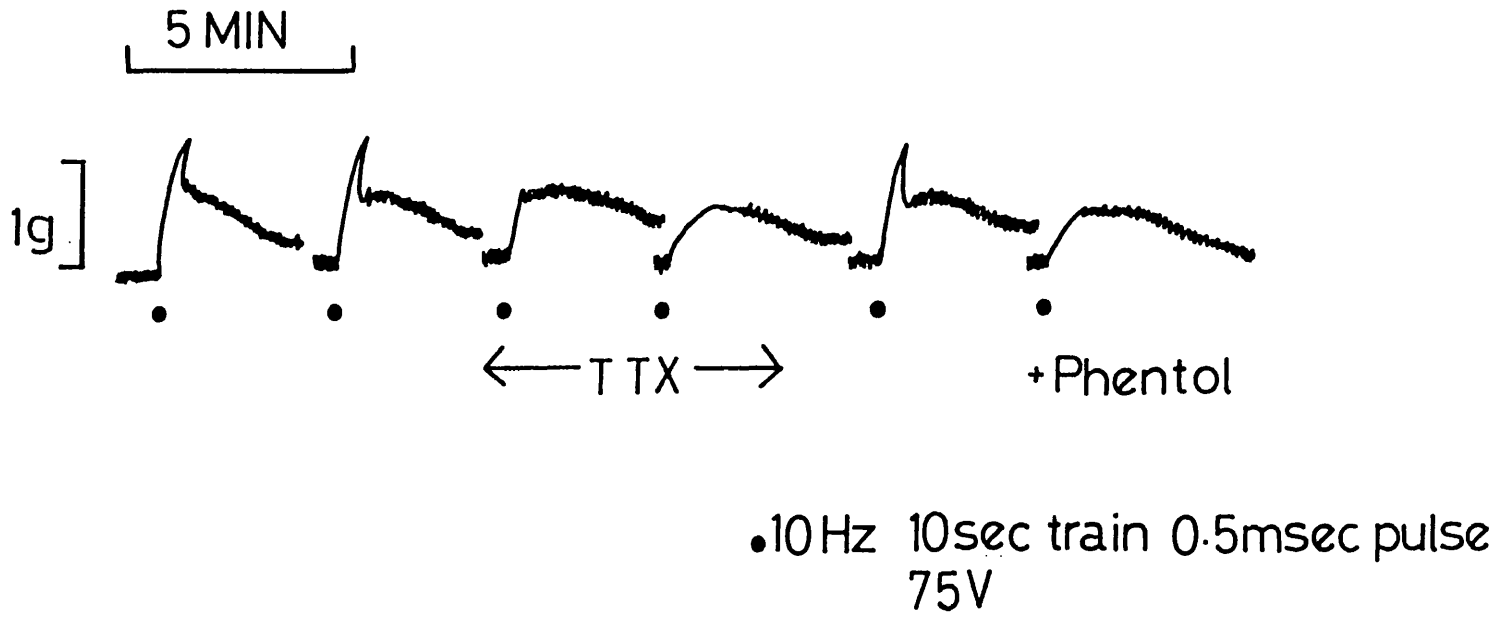
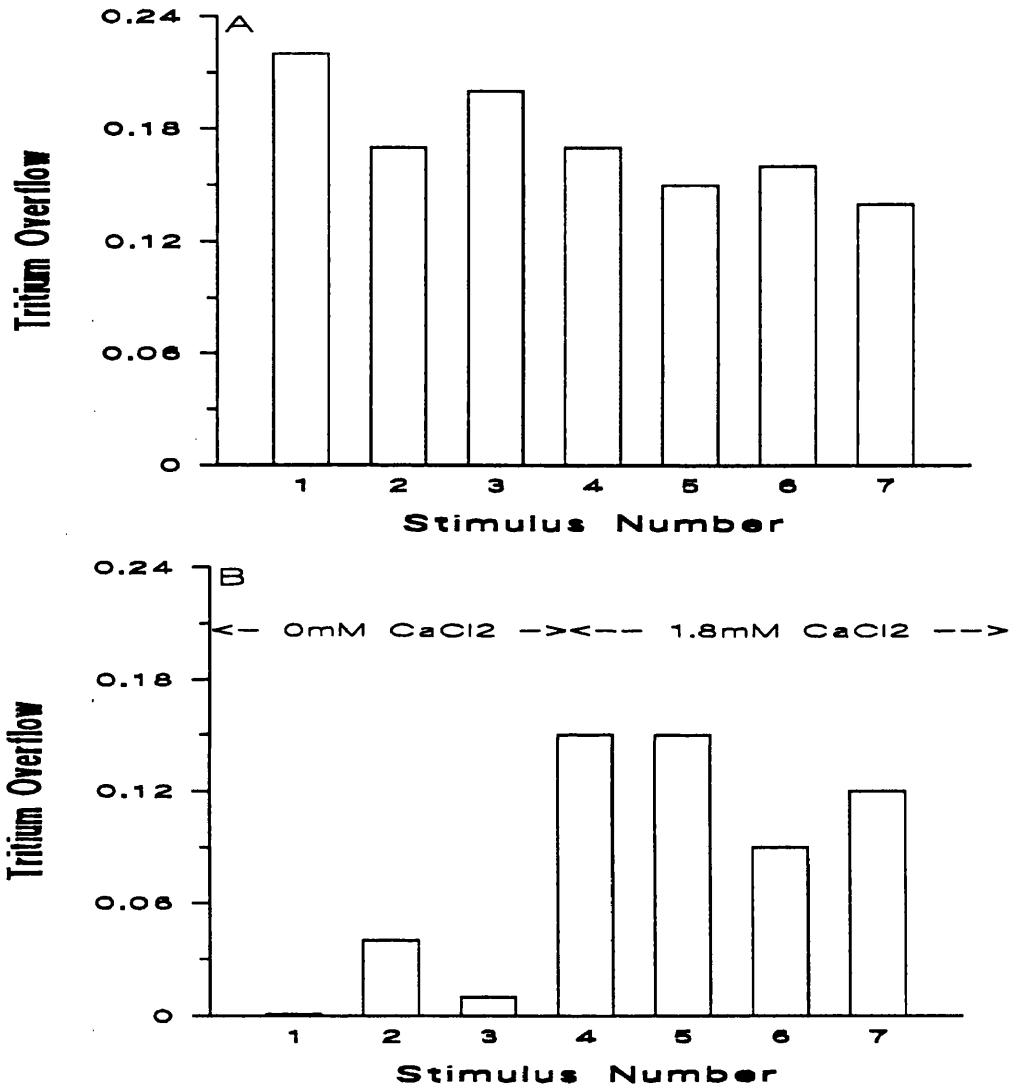


Fig. 37 The effect of TTX on the electrically-evoked twitch response of the rat anococcygeus muscle.

The mechanical twitch response of the rat anococcygeus muscle to electrical-stimulation was recorded concomitantly with tritium overflow measured in the experiment described in Fig. 36. The twitch response was depressed following the introduction of TTX (1μM) into the perfusing medium in parallel with reductions in tritium overflow.

Fig. 38 The Ca^{2+}-dependency of the electrically-evoked [^{3}H]-noradrenaline release from the rat anococcygeus muscle.



Preparations of rat anococcygeus muscle were transmurally stimulated in the presence (a) or absence (b) of Ca^{2+} and tritium overflow was compared. Ca^{2+} was reintroduced into the Ca^{2+}-free medium (b) immediately following the 3rd stimulation period. Electrically-evoked [^{3}H]-noradrenaline release exhibited a clear dependence on the presence of Ca^{2+} in the external medium. Data are from one experiment.

reintroduction of 1.8mM $CaCl_2$ into the perfusing medium tritium overflow for the remaining four stimulation periods was comparable to that in the control preparation. Therefore, almost 90% of electrically-evoked [^{3}H]-noradrenaline release was Ca^{2+}-dependent. The twitch response was equally sensitive to the lack of Ca^{2+}, although, as in the presence of TTX, the slow component remained unaffected.

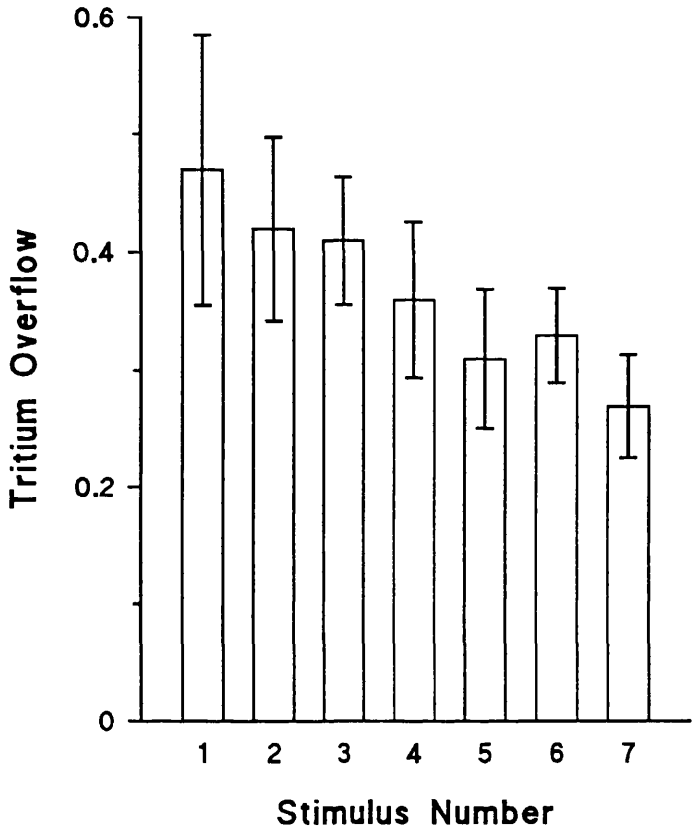
iv) The effect of successive periods of electrical stimulation on the magnitude of tritium overflow from the rat anococcygeus muscle.

Tritium overflow was calculated for each of the seven successive stimulation periods (S_1 - S_7) (Fig. 39). From this it was apparent that the magnitude of tritium overflow diminished with subsequent periods of electrical stimulation from 0.47 ± 0.12% (S_1) of tissue content to 0.27 ± 0.04% (S_7), a decrease of 43%. However, in subsequent studies the effect of test compounds on tritium overflow was compared to that evoked by the preceding control stimulation. Therefore the ratio of each of the seven control responses to the preceding response was calculated (S_{x+1}/S_x) (Fig. 40). Expressed in this way the ratio of each response to the preceding response was greater than 0.9 and provided a constant control response against which to determine the effect of test compounds.

v) The effect of $GABA_B$ agonists on evoked [^{3}H]-noradrenaline release.

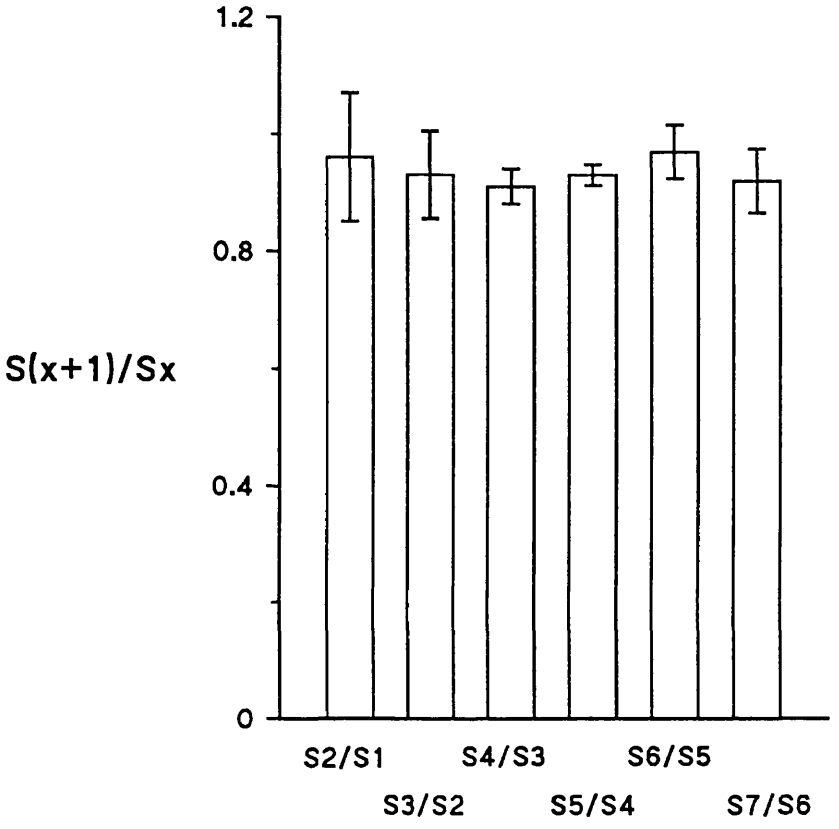
Preliminary experiments were carried out to show that the GABA-mediated inhibition of [^{3}H]-noradrenaline from this preparation was due to activation

Fig. 39 The effect of successive periods of electrical stimulation on the magnitude of tritium overflow.



Tritium overflow (S_1 - S_7) was calculated and found to decline gradually with successive periods of electrical stimulation. S_7 was reduced by 43% compared with S_1. Data are the mean ± s.e.m. of 5 observations from 3 experiments.

Fig. 40 The comparison of control responses to the preceding response.

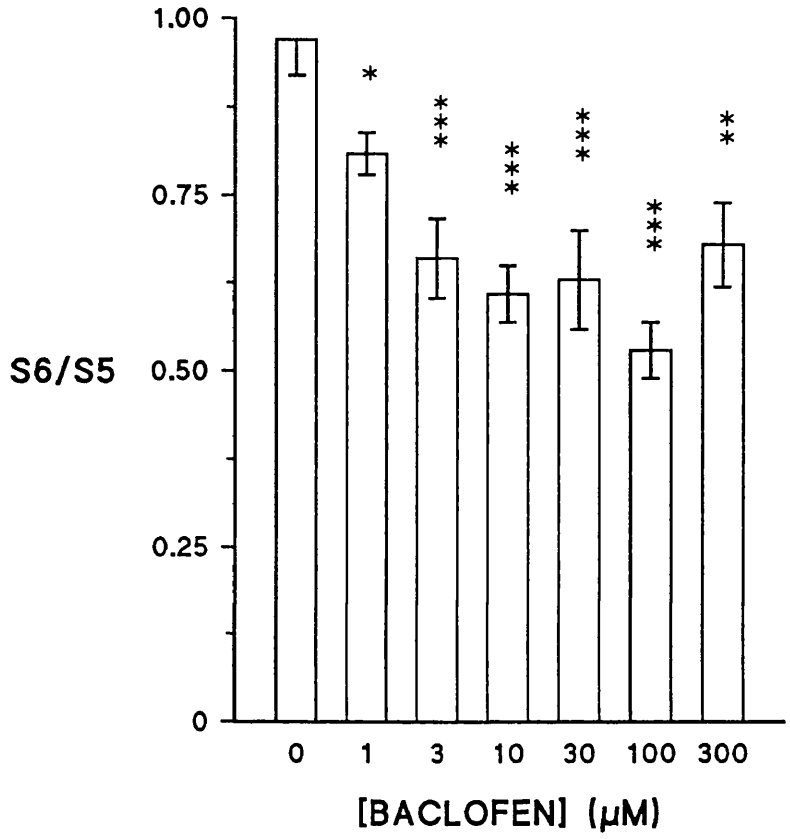


Tritium overflow elicited by successive periods of electrical stimulation in control preparations were each compared to the preceding response (S_{x+1}/S_x). Data are the mean ± s.e.m. of 5 observations from 3 experiments.

of $GABA_B$ receptors. GABA (30μM), in the presence of AOAA (50μM) and nipecotic acid (20μM), produced a 43% decrease in evoked release (S_2/S_1=0.57) whereas the $GABA_A$ agonist isoguvacine (30μM) was without effect (S_2/S_1=0.94). In both cases (-)baclofen (30μM) produced a 49% decrease in tritium release (S_6/S_5=0.51). As expected for a $GABA_B$-mediated response the effect of baclofen was stereoselective. In one preparation (+)baclofen (30μM) was inactive (S_2/S_1=0.93) whereas (-)baclofen (30μM) reduced [^{3}H]-noradrenaline release by 48% (S_2/S_1=0.52). (Data not shown)

(-)Baclofen (1-300μM), 3-APA (0.3-30μM) and SKF 97541 (0.3-30μM) produced dose-dependent decreases in electrically-evoked tritium overflow as determined by the S_6/S_5 ratios calculated in the presence of each dose of agonist and compared to the control ratio of 0.97 ± 0.05 (n=5). All three compounds produced a maximal depression of evoked tritium overflow of approximately 45-50 %. 3-APA (Fig. 42) and SKF 97541 (Fig. 43) were of similar potency, achieving maximal inhibition of the response at 10μM. (-) Baclofen (Fig. 41) was approximately 10 times less active than the other two compounds; 100μM of (-)baclofen was required to produce a 45% inhibition of evoked tritium overflow. A more quantitative estimate of the relative potencies of these $GABA_B$ agonists was determined against the electrically-evoked twitch response in the same preparation described below (IV.2.i).

Fig. 41 The effect of (-)baclofen on electrically-evoked [^{3}H]-noradrenaline release from the rat anococcygeus muscle.

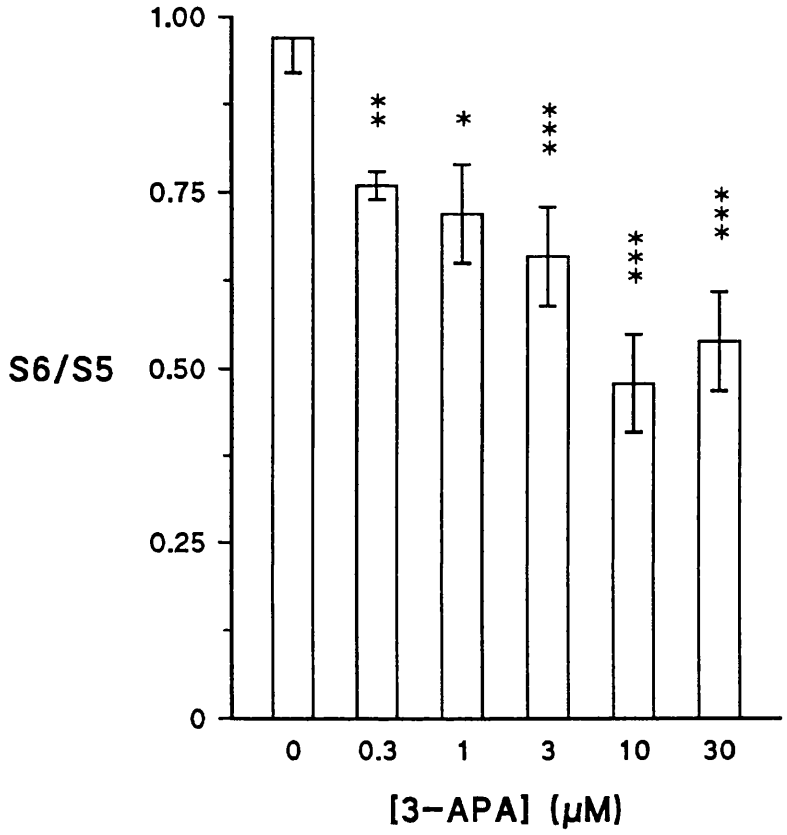


(-)Baclofen (1-300μM) was introduced into the perfusion medium 90 sec prior to the 6th stimulation period. The resulting S_6/S_5 ratios were compared to the control value of 0.97 ± 0.05 (n=5) using Student's 2-tailed t-test.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.005$

Data are the mean ± s.e.m. of 3 - 6 experiments per concentration.

Fig. 42 The effect of 3-APA on electrically-evoked [^{3}H]-noradrenaline release from the rat anococcygeus muscle.

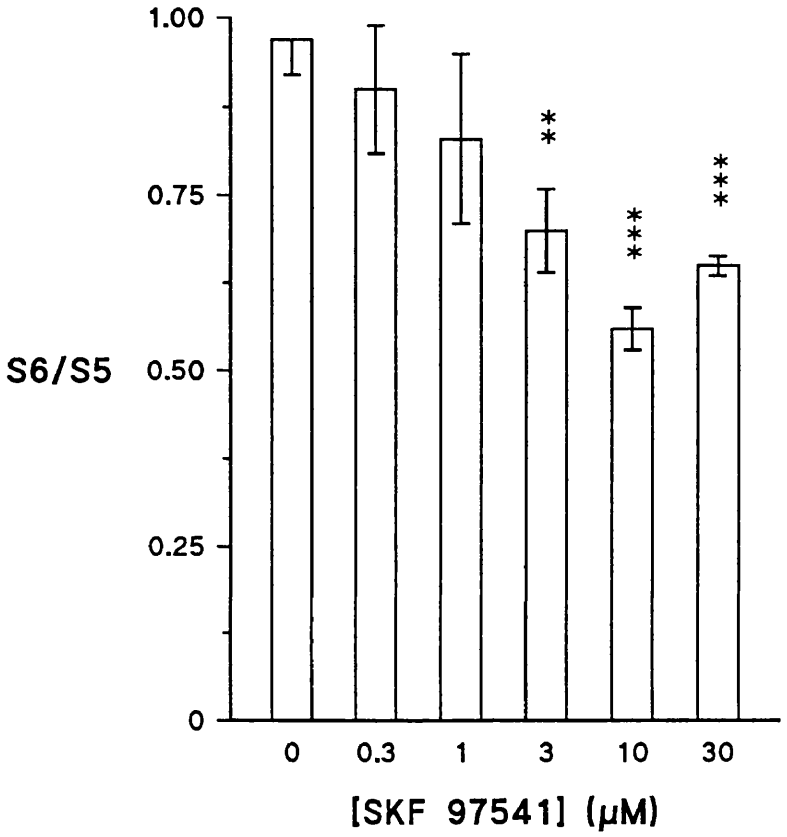


3-APA (0.3-30μM) was introduced into the perfusion medium 90 sec prior to the 6th stimulation period. The resulting S_6/S_5 ratios were compared to the control value of 0.97 ± 0.05 (n=5) using Student's 2-tailed t-test.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.005$

Data are the mean ± s.e.m. of 3 - 7 experiments per concentration.

Fig. 43 The effect of SKF 97541 on electrically-evoked [^{3}H]-noradrenaline release from the rat anococcygeus muscle.



SKF 97541 (0.3-30μM) was introduced into the perfusion medium 90 sec prior to the 6th stimulation period. The resulting S_6/S_5 ratios were compared to the control value of 0.97 ± 0.05 (n=5) by Student's 2-tailed t-test.

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.005$

Data are the mean ± s.e.m. of 4-6 experiments per concentration.

vi The effect of putative antagonists on the $GABA_B$-mediated inhibition of electrically-evoked release of [^{3}H]-noradrenaline.

Compounds were first tested alone to verify that they possessed no activity of their own in this system. Test compounds were introduced into the perfusion system immediately after the fifth stimulation period, allowing an equilibration period of 20 min. S_6/S_5 ratios were determined for CGP 35348 (300μM), CGP 36742 (300μM) and CGP 46381 (30μM, 100μM) and were compared to the control value obtained in the absence of test compounds (Table 15). The effect of each compound on the evoked tritium overflow was not significantly different from control in each case. These compounds therefore lack significant $GABA_B$ agonist activity and if they have appreciable antagonist actions then this observation may indicate a lack of intrinsic $GABA_B$ tone in this preparation.

In further studies putative antagonists were added separately to the perfusing medium 20 min prior to the sixth stimulation period, concomitantly with either 30μM (-)baclofen or 3μM 3-APA for the last 90 sec. S_6/S_5 Ratios were calculated for each concentration of antagonist and compared to both the effect of the agonists alone and to control values (Student's 2-tailed t-test). CGP 35348 (100-300μM) (Fig. 44) and CGP 36742 (100-300μM) (Fig. 45) dose-dependently reversed the inhibition of electrically-evoked tritium overflow induced by 30μM (-)baclofen. In both cases the S_6/S_5 ratio for the highest concentration of antagonist in the presence of (-) baclofen was significantly

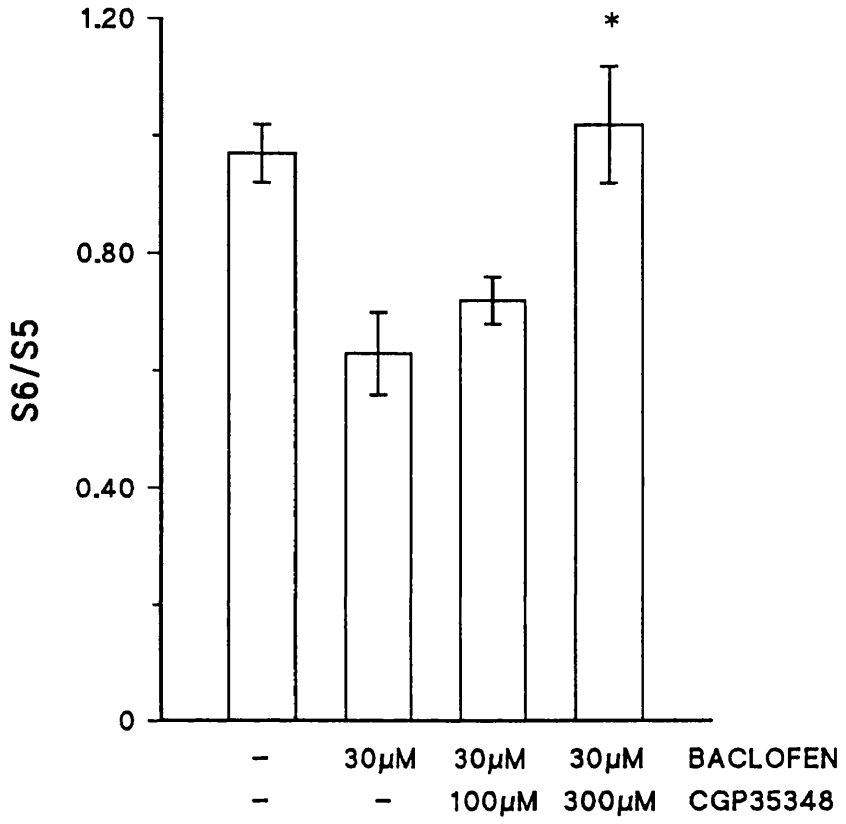
Table 15 The effect of putative $GABA_B$ antagonists on the electrically-evoked release of [^{3}H]-noradrenaline from the rat anococcygeus muscle.

Test Compound	S_6/S_5	n	
Control	0.97 ± 0.05	5	
300µM CGP 35348	0.98 ± 0.06	3	NSD
300µM CGP 36742	0.81 ± 0.12	4	NSD
30µM CGP 46381	0.97 ± 0.10	3	NSD
100µM CGP 46381	0.86 ± 0.12	2	NSD

Test compounds were introduced into the perfusion medium 20 min prior to the 6th stimulation period. Calculated S_6/S_5 ratios were compared control by Student's 2-tailed t-test.

NSD = no significant difference at $p < 0.05$

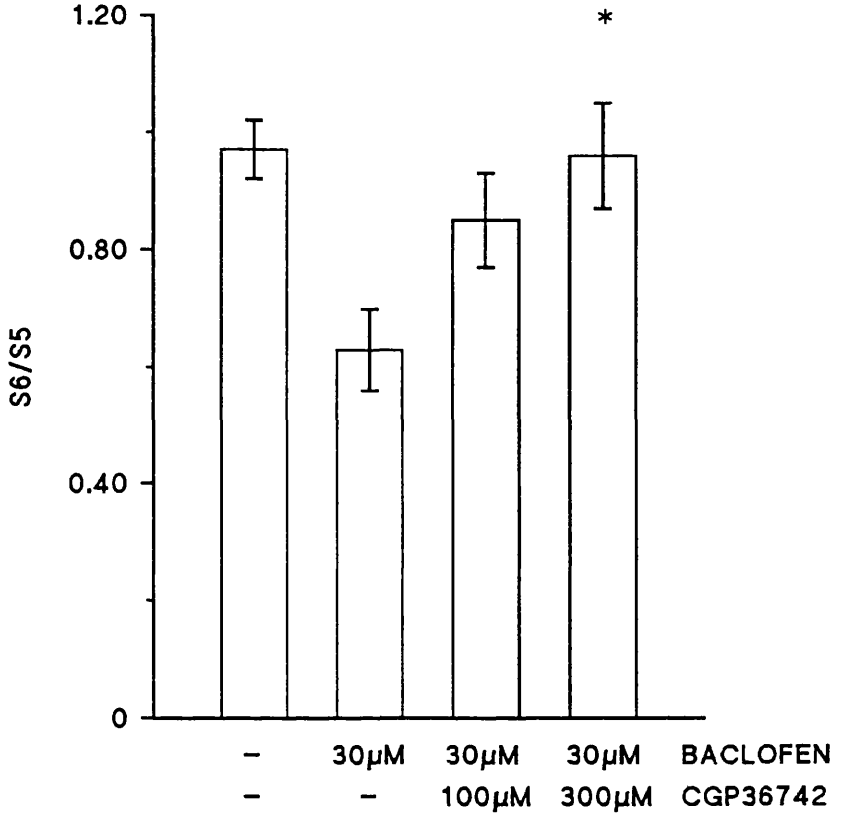
Fig. 44 Antagonism of (-)baclofen by CGP 35348 in the rat anococcygeus muscle.



CGP 35348 (100-300μM) was introduced into the perfusion medium 20 min prior to the 6th stimulation period, concomitantly with (-)baclofen (30μM) for the last 90 sec. S_6/S_5 ratios were compared to that obtained in the presence of (-)baclofen alone (Student's 2-tailed t-test).

* = $p < 0.05$ Data are from 4-5 experiments per concentration

Fig. 45 Antagonism of (-)baclofen by CGP 36742 in the rat anococcygeus muscle.



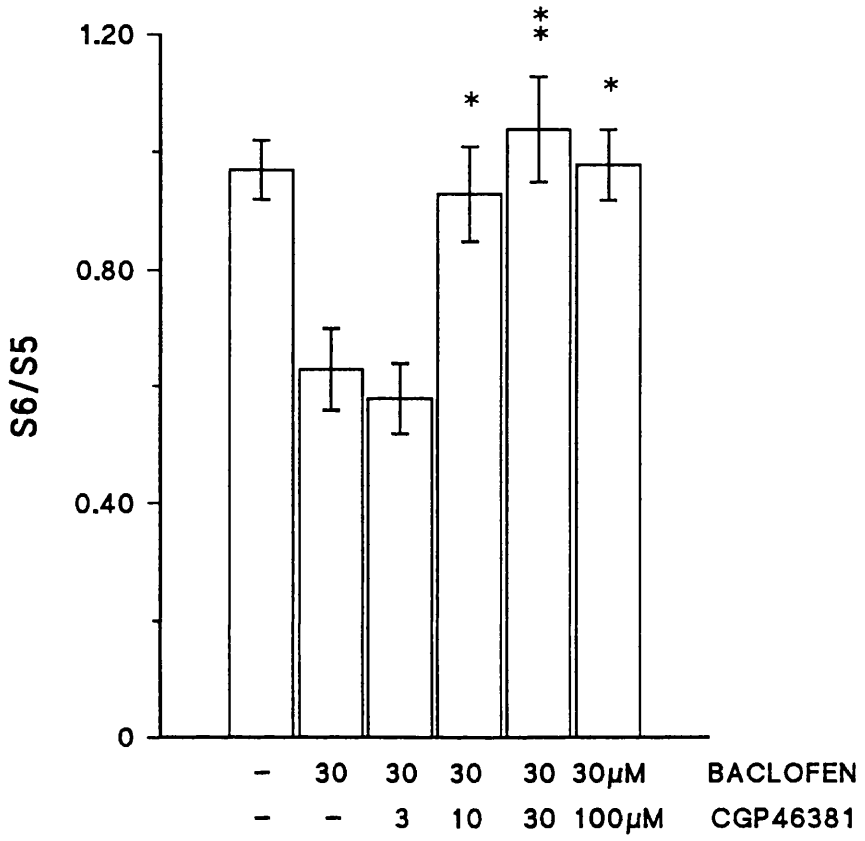
CGP 36742 (100-300μM) was introduced into the perfusion medium 20 min prior to the 6th stimulation period, concomitantly with (-)baclofen (30μM) for the last 90 sec. S_6/S_5 ratios were compared to that obtained in the presence of (-)baclofen alone. (Student's 2-tailed t-test).

* = $p < 0.05$ Data are from 4-6 experiments per concentration

different from that obtained with the agonist alone, but not from control. A similar result was obtained with CGP 46381 (3-100μM) but this compound was much more potent, the effect of (-)baclofen was fully reversed by the presence of 10μM CGP 46381 (Fig. 46).

The antagonists did not distinguish between the response to (-)baclofen and that to 3-APA. The depression of the evoked twitch response produced by 3μM 3-APA was completely antagonized by CGP 35348 (300μM), CGP 36742 (300μM) and CGP 46381 (30μM) (Table 16).

Fig. 46 Antagonism of (-)baclofen by CGP 46381 in the rat anococcygeus muscle.



CGP 46381 was introduced into the perfusion medium 20 min prior to the 6th stimulation period, concomitantly with (-)baclofen (30μM) for the last 90 sec. S_6/S_5 Ratios were compared to that obtained in the presence of (-) baclofen alone (Student's 2-tailed t-test).

* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.005$

Data are from 4-6 experiments per concentration.

Table 16 Antagonism of 3-APA by CGP 35348, CGP 36742 and CGP 46381 in the rat anococcygeus muscle.

Test Compound	S_6/S_6	n	
Control	0.97 ± 0.05	5	
3µM 3-APA	0.66 ± 0.07	7	
3µM 3-APA + 300µM CGP 35348	1.26 ± 0.18	5	*
3µM 3-APA + 300µM CGP 36742	1.15 ± 0.13	6	**
3µM 3-APA + 30µM CGP 46381	0.96 ± 0.05	3	**

CGP 35348 (300µM), CGP 36742 (300µM) or CGP 46381 (30µM) were introduced into the superfusion medium 20 min prior to the 6th stimulation period, and 3-APA (3µM) was also present for the last 90 sec. S_6/S_5 Ratios were compared to.that obtained in the presence of 3-APA alone (Student's 2-tailed t-test).

* = $p < 0.05$ ** = $p < 0.01$

Data are from 4-6 experiments per concentration.

2) $GABA_B$-Mediated Modulation of Electrically-Evoked Twitch Contractions in the Rat Anococcygeus Muscle.

i) The effect of $GABA_B$ agonists on the electrically-evoked twitch contraction.

(-)Baclofen (10^{-8}M-10^{-4}M), 3-APA (10^{-9}M-10^{-5}M) and SKF 97541 (10^{-9}M-10^{-5}M) produced dose-dependent decreases in the amplitude of the electrically-evoked twitch contraction of the rat anococcygeus muscle. A maximum depression of 70-90% of the control twitch height was achieved in each case and the order of potency obtained was :

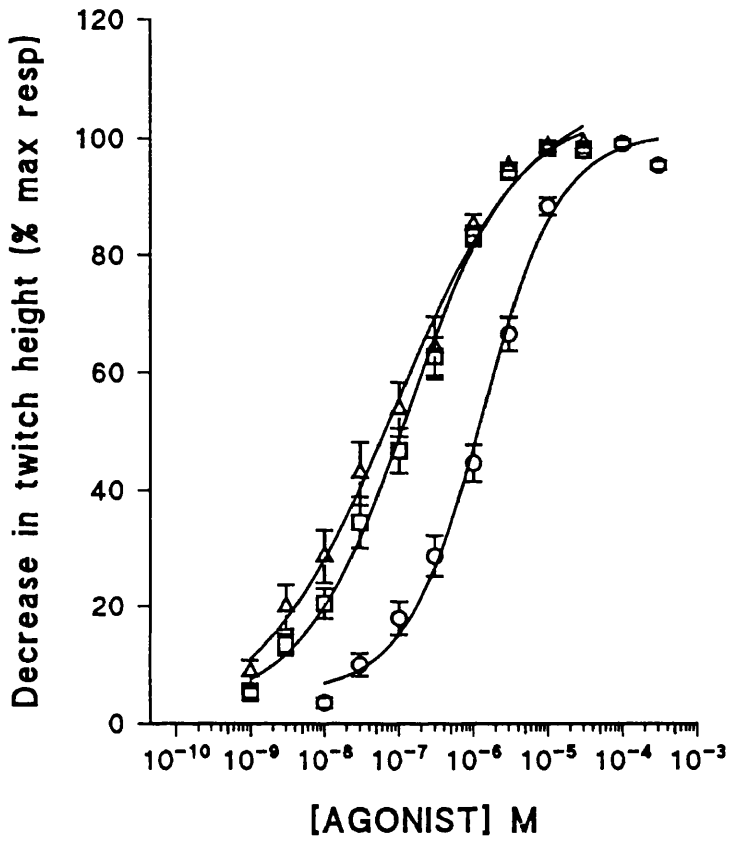
3-APA > SKF 97541 > (-)baclofen

3-APA was found to be twice as potent as its methyl derivative SKF 97541 which in turn exhibited a tenfold increase in potency over the prototypic $GABA_B$ agonist (-)baclofen (Fig. 47; Table 17).

ii) The effect of putative $GABA_B$ antagonists on $GABA_B$ receptor-mediated inhibition of the electrically-evoked twitch response.

Dose-response curves were constructed to (-)baclofen, 3-APA and SKF 97541 in the absence and presence of increasing concentrations of CGP 35348 (30, 100 and 300μM), CGP 36742 (30, 100 and 300μM) and CGP 46381 (3, 10, 30 and

Fig. 47 The effect of $GABA_B$ agonists on the electrically-evoked twitch response in the rat anococcygeus muscle.



Increasing concentrations of (-)baclofen (o), 3-APA (Δ) and SKF 97541 (□) were added cumulatively to the bathing medium and the depression in evoked twitch height recorded isometrically. Responses were expressed as a % of the maximum response achieved. Data are the mean ± s.e.m. of 18-21 experiments.

Table 17 The relative potencies of (-)baclofen, 3-APA and SKF 97541 as $GABA_B$ agonists in the rat anococcygeus muscle.

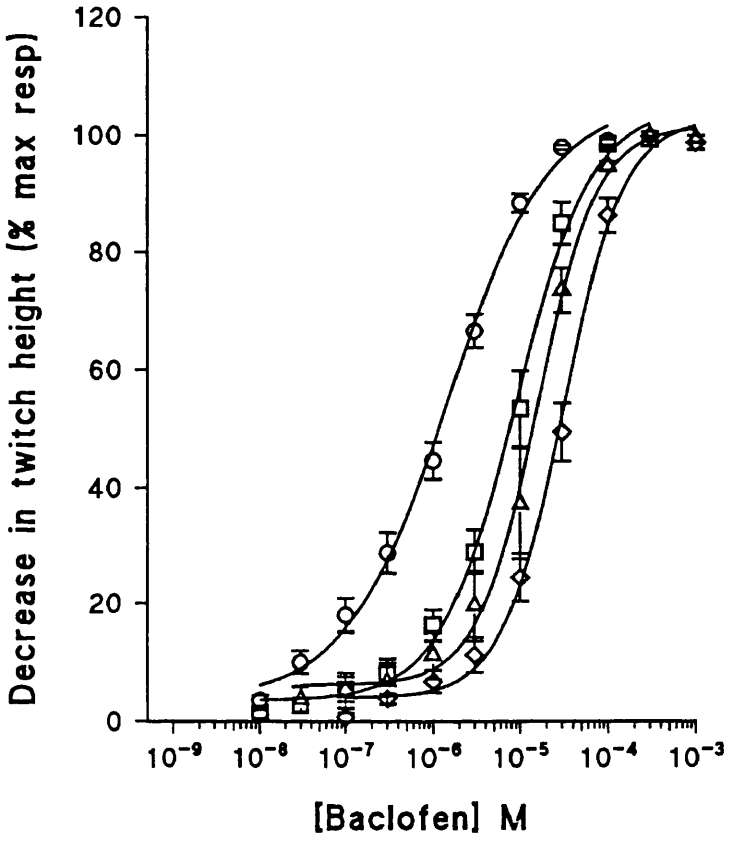
Agonist	Geometric Mean (range)	Rel. Potency	n
(-)Baclofen	1.02×10^{-6}M (1×10^{-7} - 4.8×10^{-6}M)	1	21
3-APA	4.63×10^{-8}M (2×10^{-9} - 5.5×10^{-7}M)	22	18
SKF 97541	1.03×10^{-7}M (9×10^{-9} - 5.0×10^{-7}M)	9.9	20

The values are the geometric mean and range of the EC_{50} values obtained for each of the agonists from 18-21 experiments.

100μM) which resulted in a progressive rightward shift of the agonist DRC's (Fig. 48, 49, 50, 52, 53, 54, 56, 57 and 58) with no apparent reduction in maximum response. For CGP 35348 the Schild regressions for each of the agonists had slope of unity and pK_B values of 4.98, 4.73 and 4.60 against (-) baclofen, 3-APA and SKF 97541 (Fig. 51) respectively. These data indicate that the agonists mediated their response through populations of $GABA_B$ receptors that could not be distinguished by CGP 35348. In addition the slope of unity implied that the antagonist was acting in a competitive manner at least over the concentration range tested.

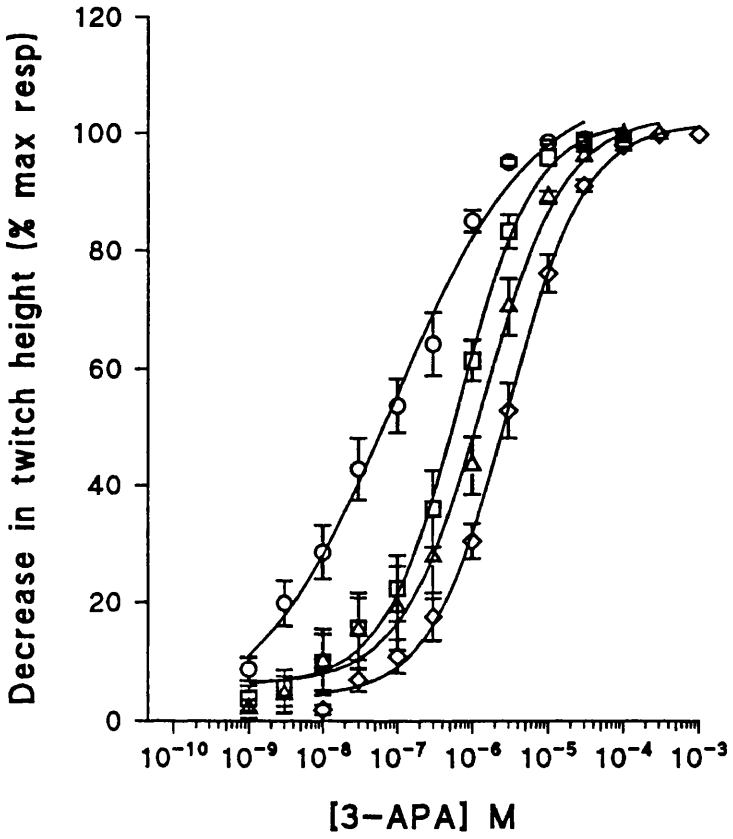
CGP 36742 was slightly more potent than CGP 35348. Again the slope of the Schild regressions were not significantly different from one suggesting a competitive interaction at the $GABA_B$ receptor and pK_B values of 5.07, 5.43 and 5.02 were obtained against (-)baclofen, 3-APA and SKF 97541 (Fig. 55) respectively. An significant improvement in potency was achieved with CGP 46381. This compound has the highest affinity for the $GABA_B$ receptor of any $GABA_B$ antagonist yet available and represents a significant breakthrough in $GABA_B$ receptor pharmacology. pK_B Values of 6.39, 5.70 and 5.71 were obtained against (-) baclofen, 3-APA and SKF 97541 respectively (Fig. 59). As before slopes were not significantly different from one.

Fig. 48 Antagonism of (-)baclofen by CGP 35348 in the rat anococcygeus muscle.



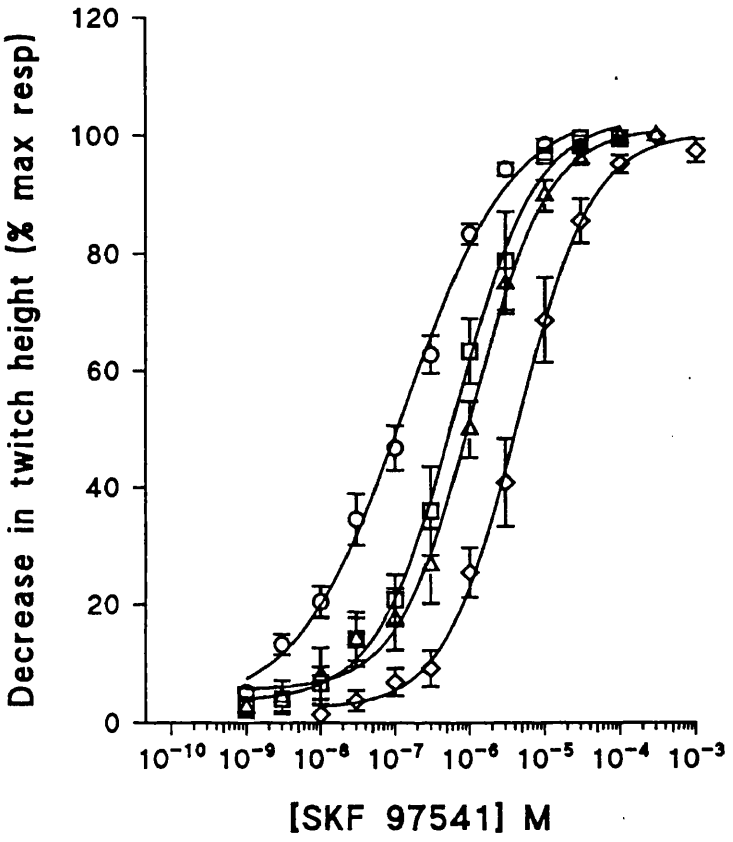
DRC's to (-)baclofen were constructed in the absence (○) and the presence of 30μM (□), 100μM (△) and 300μM (◇) of CGP 35348. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 4-21 experiments.

Fig. 49 Antagonism of 3-APA by CGP 35348 in the rat anococcygeus muscle.



DRC's to 3-APA were constructed in the absence (○) and the presence of 30μM (□), 100μM (△) and 300μM (◇) CGP 35348. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 4 - 18 experiments.

Fig. 50 Antagonism of SKF 97541 by CGP 35348 in the rat anococcygeus muscle.



DRC's were constructed in the absence (○) and presence of 30μM (□), 100μM (△) and 300μM (◇) CGP 35348. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 4 - 20 experiments.

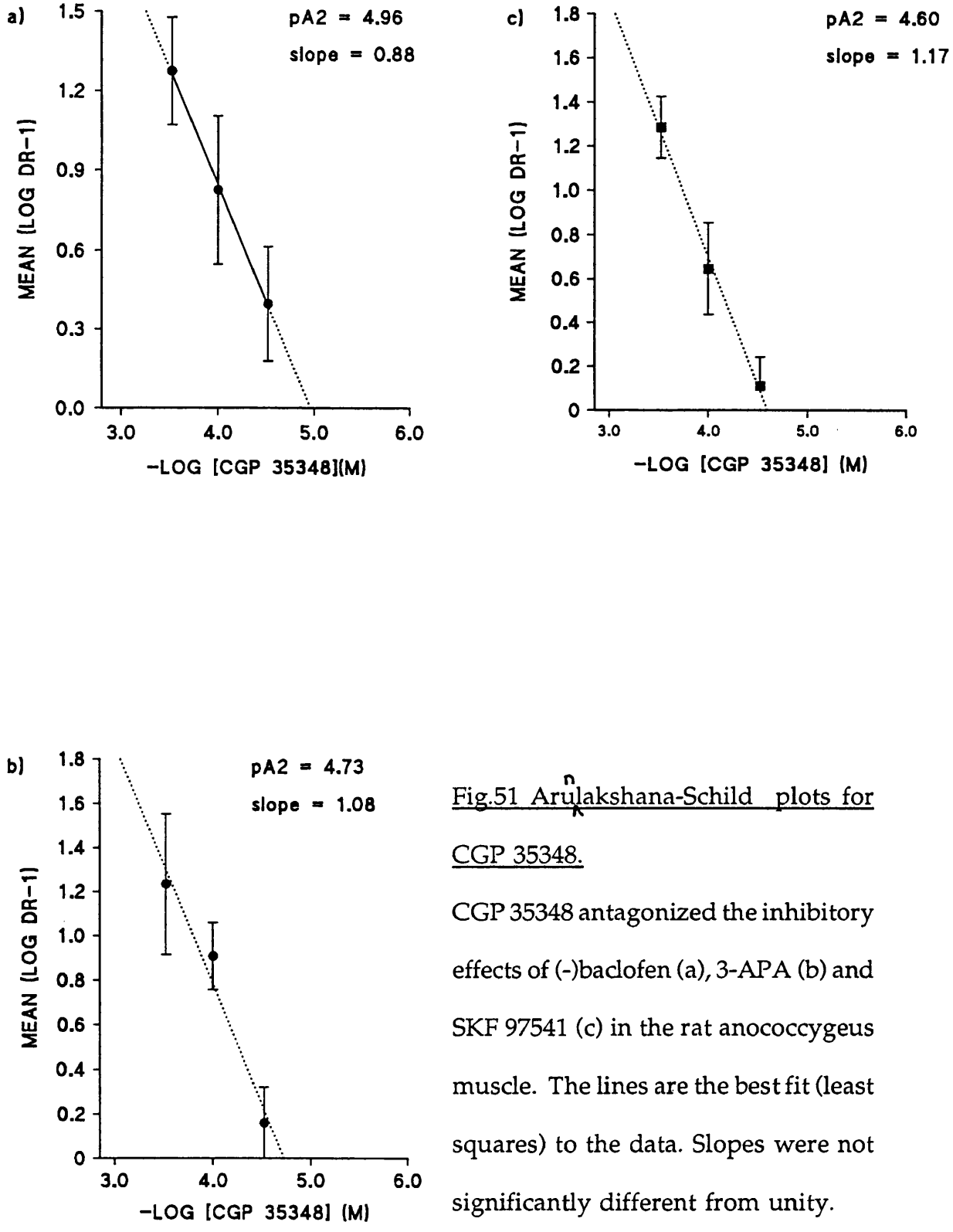
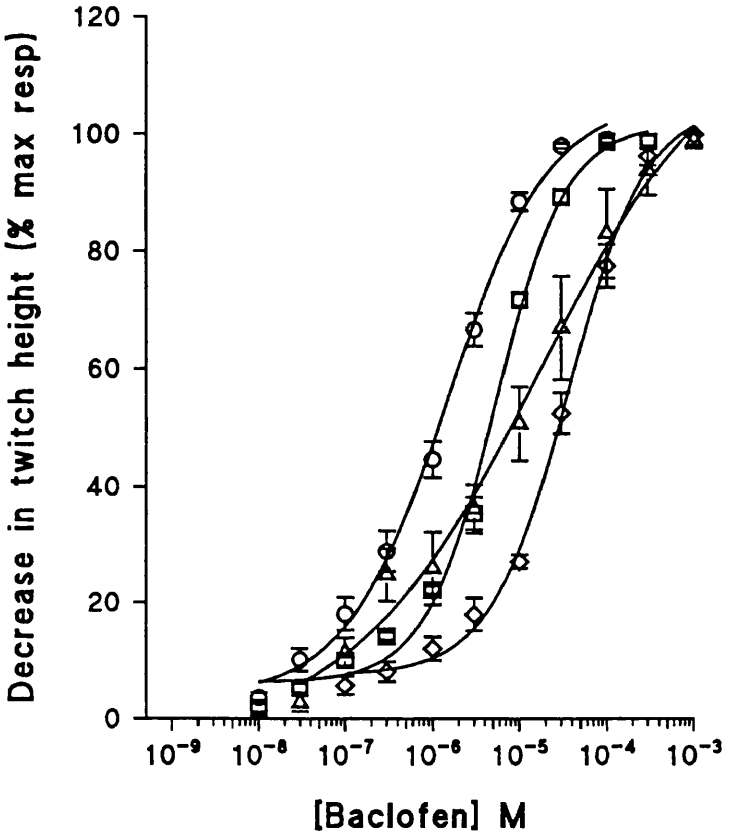


Fig.51 Arunlakshana-Schild plots for CGP 35348.

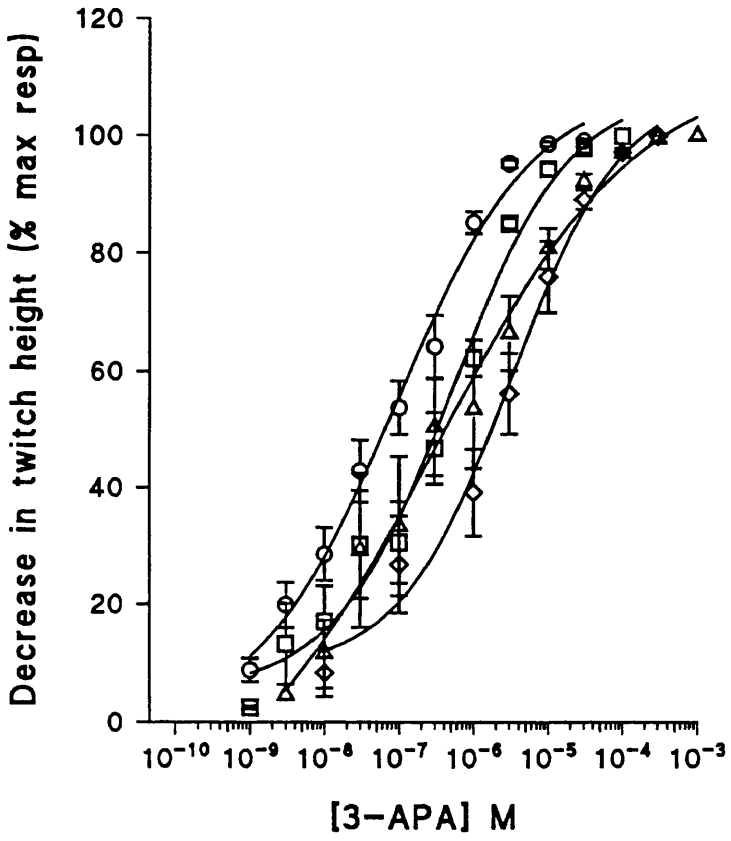
CGP 35348 antagonized the inhibitory effects of (-)baclofen (a), 3-APA (b) and SKF 97541 (c) in the rat anococcygeus muscle. The lines are the best fit (least squares) to the data. Slopes were not significantly different from unity.

Fig. 52 Antagonism of (-)baclofen by CGP 36742 in the rat anococcygeus muscle.



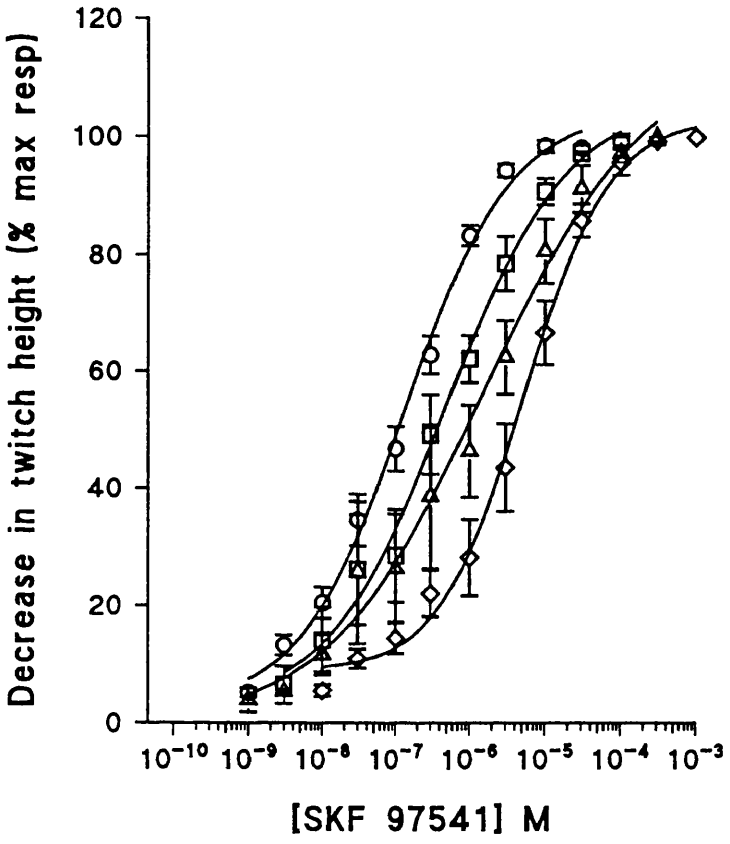
DRC's to (-)baclofen were constructed in the absence (○) and presence of 30μM (□), 100μM (△) and 300μM (◇) CGP 36742. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 5 - 21 experiments.

Fig. 53 Antagonism of 3-APA by CGP 36742 in the rat anococcygeus muscle.



DRC's to 3-APA were constructed in the absence (○) and the presence of 30μM (□), 100μM (Δ) and 300μM (◇) CGP 36742. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 4 - 18 experiments.

Fig. 54 Antagonism of SKF 97541 by CGP 36742 in the rat anococcygeus muscle.



DRC's to SKF 97541 were constructed in the absence (○) and presence of 30μM (□), 100μM (△) and 300μM (◇) CGP 36742. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 5 - 20 experiments.

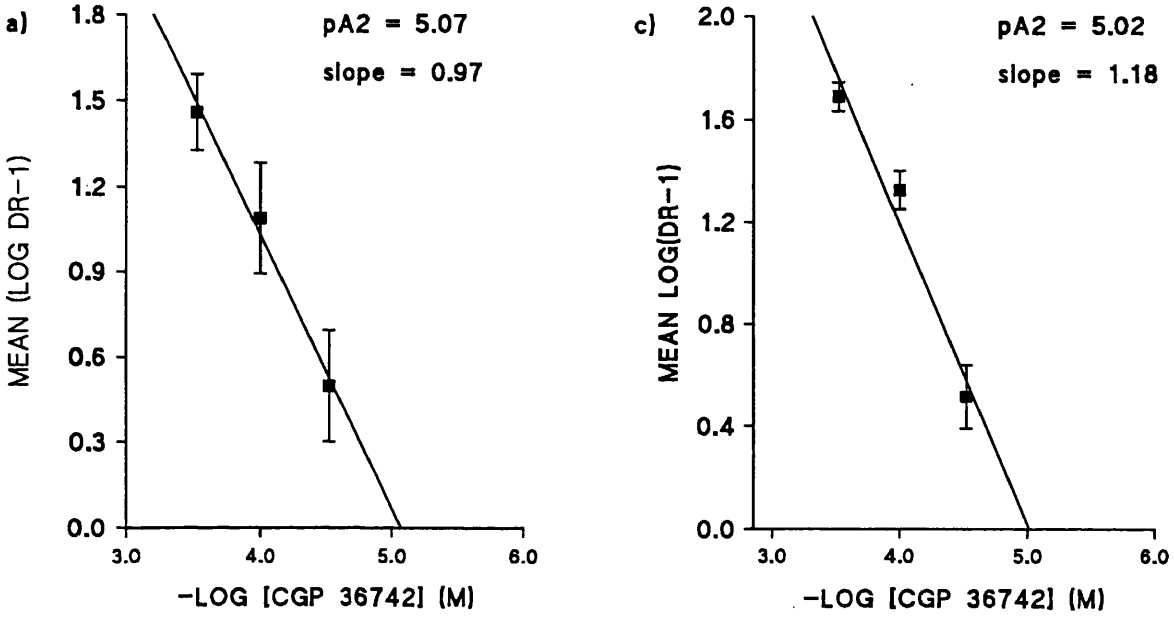
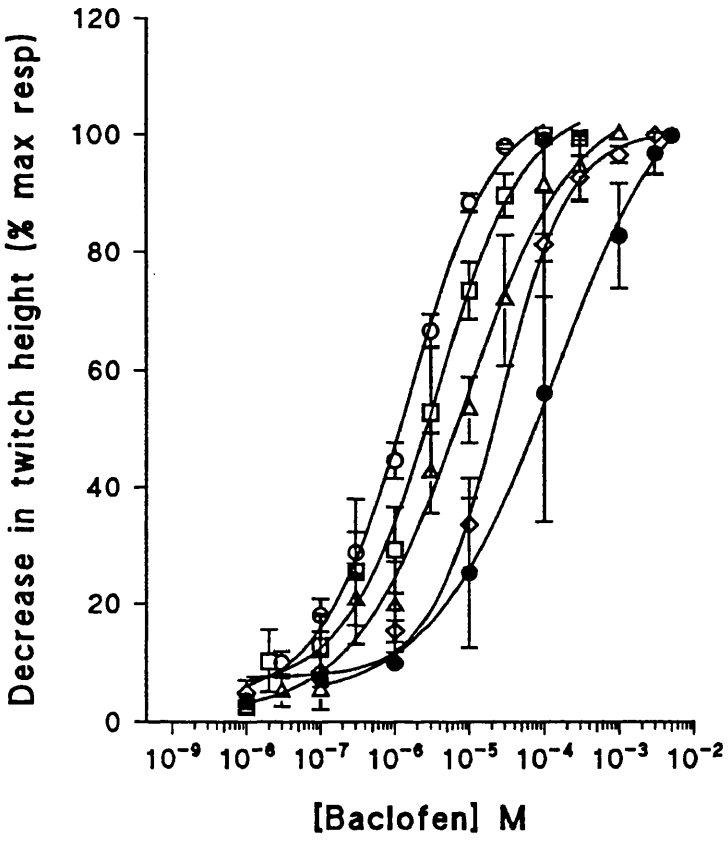


Fig.55 Arulakshana-Schild plots for CGP 36742.

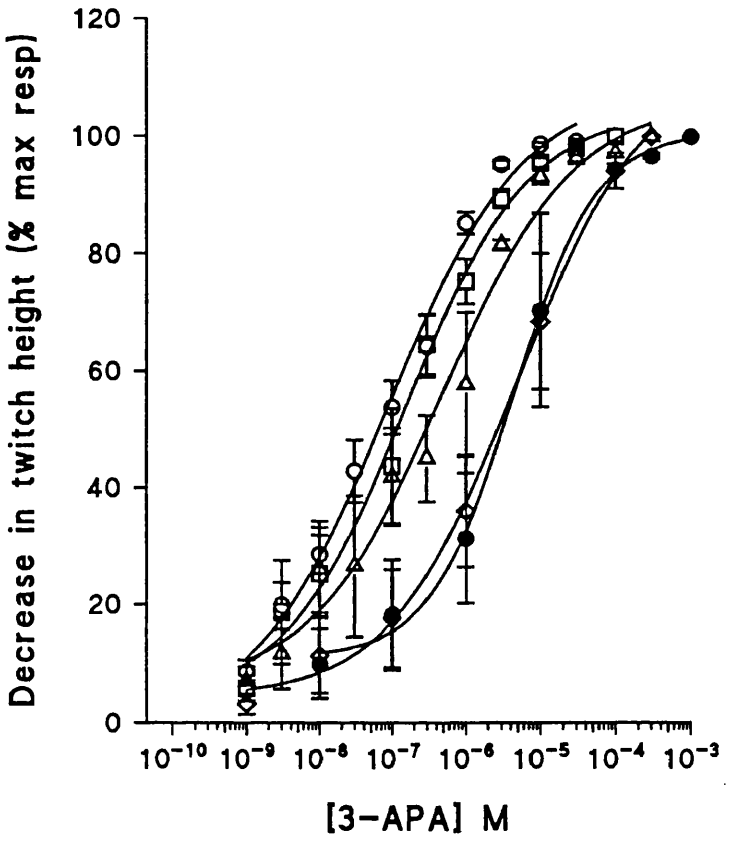
CGP 36742 antagonized the inhibitory effects of (-)baclofen (a), 3-APA (b) and SKF 97541 (c) in the rat anococcygeus muscle. The lines are the best fit (least squares) to the data. Slopes were not significantly different from unity.

Fig. 56 Antagonism of (-)baclofen by CGP 46381 in the rat anococcygeus muscle.



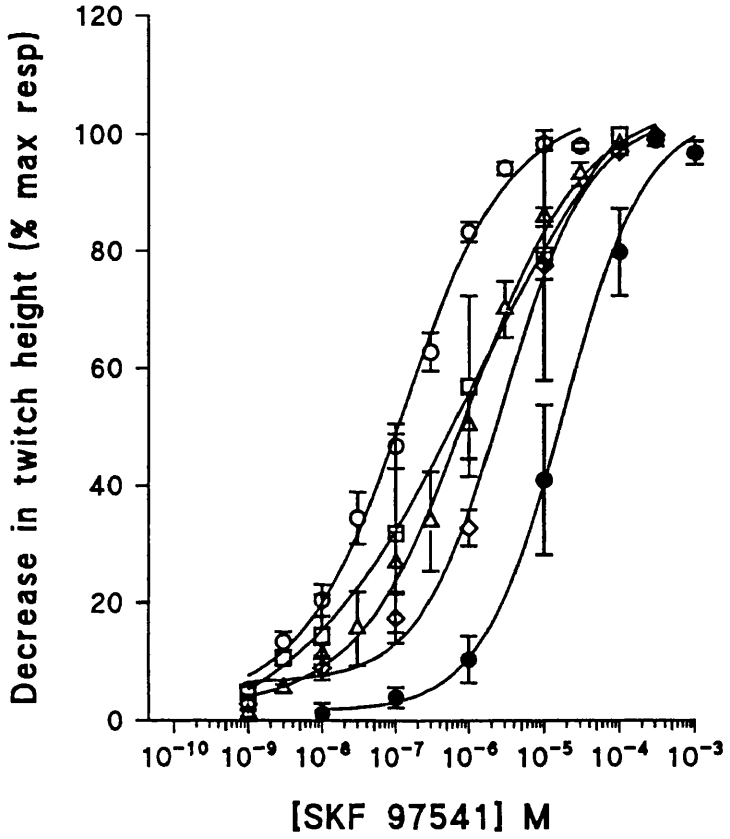
DRC's to (-)baclofen were constructed in the absence (○) and presence of 3μM (□), 10μM (△), 30μM (◇) and 100μM (●) CGP 46381. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 4 - 21 experiments.

Fig. 57 Antagonism of 3-APA by CGP 46381 in the rat anococcygeus muscle.



DRC's to 3-APA were constructed in the absence (○) and presence of 3μM (□), 10μM (△), 30μM (◇) and 100μM (●) CGP 46381. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 4 - 18 experiments.

Fig. 58 Antagonism of SKF 97541 by CGP 46381 in the rat anococcygeus muscle.



DRC's to SKF 97541 were constructed in the absence (○) and presence of 3μM (□), 10μM (△), 30μM (◇) and 100μM (●) CGP 46381. Responses are expressed as a % of the maximum response achieved for each DRC. Data are from 3 - 20 experiments.

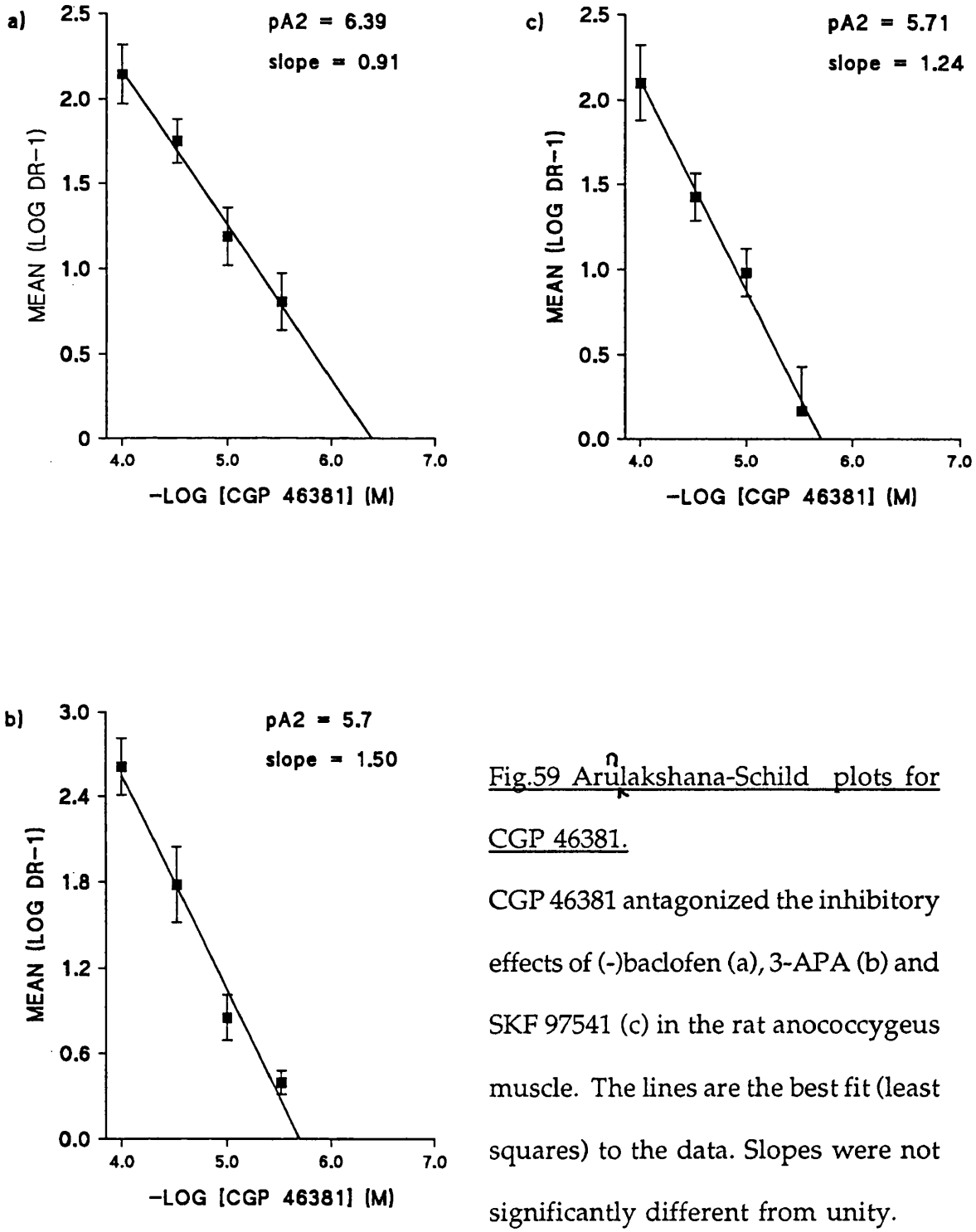


Fig.59 Arulakshana-Schild plots for CGP 46381.

CGP 46381 antagonized the inhibitory effects of (-)baclofen (a), 3-APA (b) and SKF 97541 (c) in the rat anococcygeus muscle. The lines are the best fit (least squares) to the data. Slopes were not significantly different from unity.

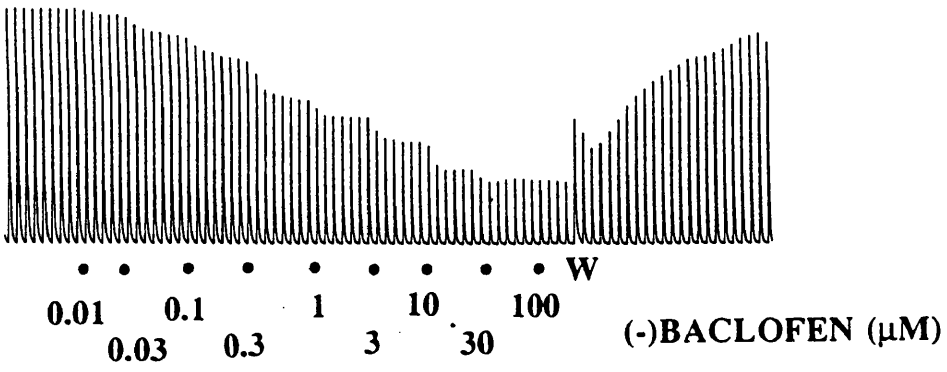
3) Discussion.

A neurotransmitter role for GABA in the peripheral nervous system has been much more difficult to establish than in the CNS due in part to the much lower concentrations of GABA present in the periphery (less than 1% of CNS levels in most tissues). However a possible physiological role has been postulated for GABA in the enteric nervous system for which evidence of a transmitter role for GABA is almost complete (Jessen et al., 1979; Taniguchi et al., 1982; Jessen et al., 1983; Kerr and Krantis, 1983; Miki et al., 1983; Davinger et al., 1987; Kerr et al., 1987). As GABA has no direct effect on the longitudinal and circular smooth muscle cells, its actions primarily involve the modulation of presynaptic motor innervation of these muscle layers. Thus, the activation of both $GABA_A$ and $GABA_B$ receptors appear to be involved in the modulation of intestinal motility (Ong and Kerr, 1983; Kerr and Ong, 1990). Some evidence for a neurotransmitter role for GABA is also available for the superior cervical ganglion (Bowery and Brown, 1974; Bertisson et al., 1976; Farkas et al., 1986; Happola et al., 1987), urinary bladder (Kusunoki et al., 1984; Maggi et al., 1985), gallbladder (Saito et al., 1985; Saito and Tanaka, 1986) and sinus node of the heart (Bowery et al., 1981; Taniyama et al., 1990). Whilst the physiological role of GABA in the periphery has yet to be fully elucidated isolated peripheral tissues such as the rat anococcygeus muscle (Muhyaddin et al., 1983; Hills et al., 1989), the rat or guinea-pig vas deferens (Kerr et al., 1990; Hills et al., 1991) and the guinea-pig ileum (Ong et al., 1987; Kerr et al., 1989) have been used extensively to assess the potency of potential $GABA_B$

agonists and antagonists.

In these preparations activation of presynaptic $GABA_B$ receptors (located on the terminals of adrenergic, NANC and cholinergic neurones respectively) mediates the inhibition of electrically-evoked neurotransmitter release usually measured as a decrease in evoked twitch height (Fig. 60).

Fig. 60 Electrically-evoked twitch responses in the rat anococcygeus.



(-)Baclofen-mediated inhibition of electrically-evoked noradrenaline release in the rat anococcygeus muscle.

(-)Baclofen, 3-APA and SKF 97541 caused a dose-dependent inhibition of the electrically evoked release of [^{3}H]-noradrenaline and of the twitch response in the rat anococcygeus muscle. In contrast to the twitch responses shown above (Fig. 60) the responses obtained to electrical stimulation in the release experiments (e.g. Fig 35) were biphasic. The second component of the mechanical response was not suppressed in the presence of TTX. Tetrodotoxin specifically binds to, and therefore blocks, voltage-dependent Na^+-channels such as those responsible for the generation of the neuronal action potential. Sensitivity of neurotransmitter release to TTX is therefore taken as evidence for release from neurones rather than, for example, from glial cells. These data therefore suggest that this component was not mediated by neuronally released noradrenaline or any co-transmitter that may be present. It is possible that this slow component was due to the direct stimulation of the smooth muscle cells caused by the very close proximity of the platinum electrodes to the preparation. Alternatively the lack of supporting bathing medium, which would be present in the usual organ bath system as used in this thesis, may have hindered the relaxation of the preparation following electrical stimulation and given rise to this sustained response. This is supported by the observation that the application of identical stimulation parameters to a preparation of rat anococcygeus muscle set up in a organ bath arrangement produces a fast, single component, response (not shown).

As predicted from binding data, 3-APA inhibited [^{3}H]-noradrenaline release and depressed the evoked twitch response with ten times greater potency than

(-)baclofen. SKF 97541 was slightly less potent than 3-APA in both experiments. These data are in agreement with the results of Hills and Howson (1992) who have published very similar EC_{50} values for the three agonists in the rat anococcygeus muscle and comparable results in the rat vas deferens and guinea-pig ileum (Hills and Howson, 1992). In the isolated guinea-pig trachea baclofen, 3-APA and SKF 97541 inhibited the electrically-stimulated contraction with the same relative potencies as observed in the rat anococcygeus muscle. It would appear therefore that in peripheral preparations $GABA_B$ receptors located presynaptically on adrenergic, cholinergic and NANC neurones are not distinguishable with the available $GABA_B$ agonists.

The two new compounds, CGP 36742 and CGP 46381 compared favourably with the $GABA_B$ antagonist CGP 35348 in the rat anococcygeus muscle. All three compounds had no effect on either evoked release or twitch response indicating the absence of GABAergic tone in this preparation. However these compounds fully reversed the inhibition of evoked [^{3}H]-noradrenaline release mediated by (-) baclofen and 3-APA, with CGP 46381 an order of magnitude more potent than the other two. More quantitative data were obtained from the Schild regressions for antagonism of (-)baclofen, 3-APA and SKF 97541 in the rat anococcygeus. In these experiments all three compounds produced rightward shifts of the agonist DRC's without apparent reduction in maximum response and accordingly the slope of the Schild regression lines were not significantly different from unity. This suggested that the antagonism was

competitive over the concentration range tested and therefore differed from the findings of Hills and coworkers (Hills et al., 1991; Hills and Howson, 1992). They reported that CGP 35348 antagonized the effect of (±)baclofen in the rat vas deferens (pK_B 5.0) and 3-APA in the rat anococcygeus muscle (pK_B 5.4) but that the slope of the Schild plot in both cases was less than 1 (0.6 and 0.7 respectively). They did not discount the possibility that CGP 35348 was either a non-competitive antagonist at the peripheral presynaptic $GABA_B$ receptor or acting at more than one $GABA_B$ receptor subtype. This is not supported by the findings of this thesis.

The pA_2 obtained in the rat anococcygeus muscle for CGP 35348, CGP 36742 and CGP 46381 are in good agreement with their ability to inhibit [^{3}H]-3-APA from rat brain membranes (IC_{50} values of 34μM, 35μM and 5μM respectively) (Klebs et al., 1992) and this may reflect their improved tissue penetrability over phaclofen, saclofen and (-)baclofen.

Interestingly, in the studies, discussed more fully in chapter III, (-)baclofen inhibited K^+-evoked release of endogenous aspartate, glutamate and GABA from rat hippocampal synaptosomes but its effect was not mimicked by 3-APA. This would suggest that peripheral presynaptic $GABA_B$ receptors on adrenergic terminals in the rat anococcygeus muscle do not appear to have the same pharmacological profile as central presynaptic $GABA_B$ receptors in the rat hippocampus and supports the concept of $GABA_B$ receptor heterogeneity.

CHAPTER FIVE: RESULTS

[^{3}H]-GABA DISPLACEMENT STUDIES IN RAT WHOLE BRAIN SLICES

1) Displacement of [^{3}H]-GABA from $GABA_A$ and $GABA_B$ Binding Sites in Whole Rat Brain Slices by Putative $GABA_B$ Ligands.

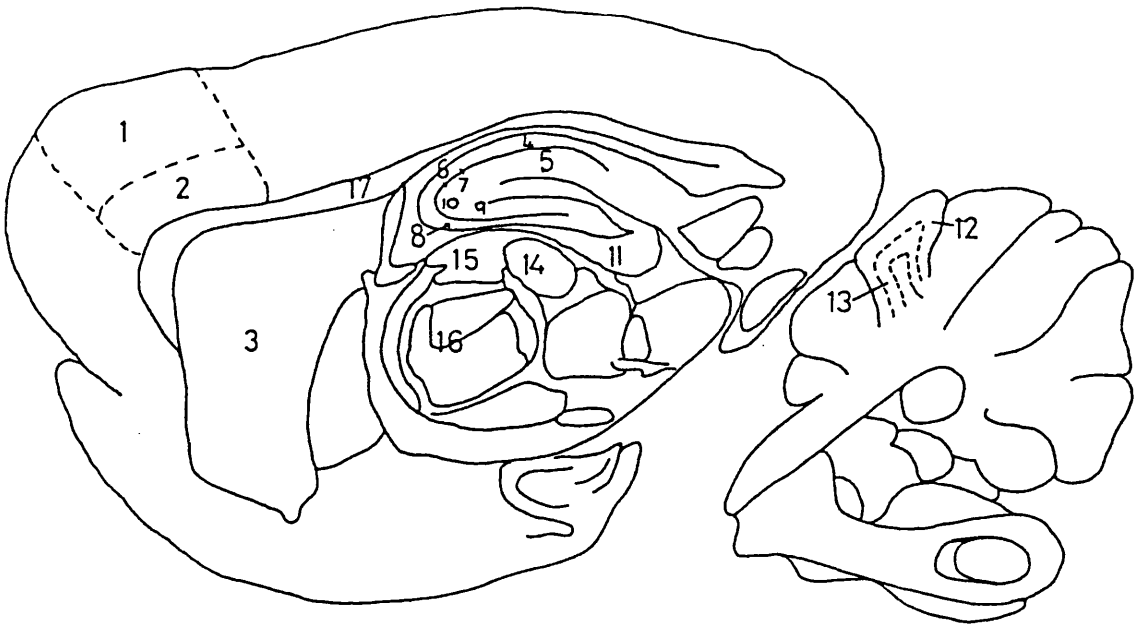
i) $GABA_A$ binding assay.

The ability of putative $GABA_B$ ligands to displace [^{3}H]-GABA from $GABA_A$ binding sites was assessed in rat whole brain slices. Specific $GABA_A$ binding accounted for 89.6% of total [^{3}H]-GABA binding and was essentially unaffected by the addition of 100μM (-)baclofen, SKF 97541, CGP 35348, CGP 36742, CGP 46381 to the incubation medium (data not shown). In contrast, 3-APA (0.1-100μM) produced a dose-dependent decrease in specific $GABA_A$ binding, of up to 92%, with an IC_{50} value of 5.5μM.

ii) $GABA_B$ binding assay.

A study was made of $GABA_B$ binding site distribution throughout rat brain (Fig. 61) using autoradiographic techniques (Fig. 62). Seventeen brain regions were investigated, and of these the highest density of $GABA_B$ sites were found in the cerebellar molecular layer (136±4 fmoles/mg), the dorsolateral geniculate, lateral dorsal thalamic and ventroposterolateral/ ventroposteromedial thalamic nuclei (94-123 fmoles/mg) and the outer layers of the cerebral cortex (112±4 fmoles/mg). Intermediate levels of binding (31-67 fmoles/mg) were present throughout the inner cerebral cortical layers, the corpus striatum, the hippocampal CA1, CA2, CA3 regions (with binding

Fig. 61 Diagram of parasagittal section through the rat brain.

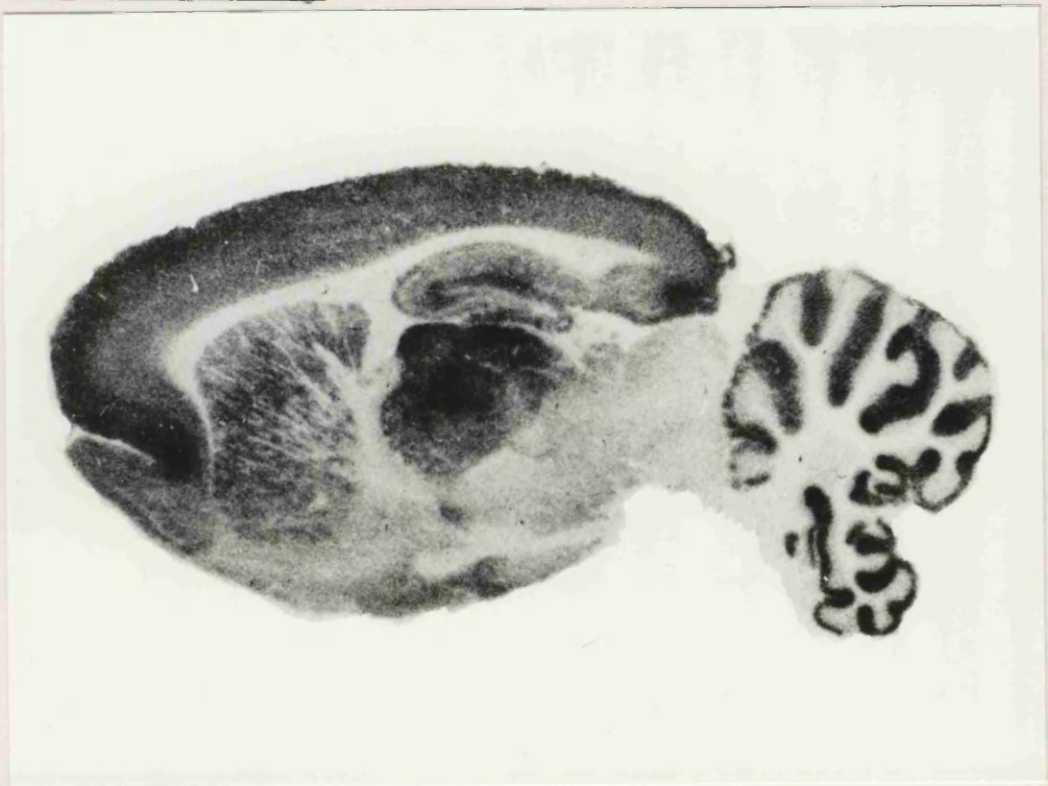


10µm Sections were cut from frozen rat brain and used for the autoradiographic visualization of $GABA_B$ binding sites. Seventeen brain regions were studied as follows:

1) CxO Outer layers of cerebral cortex
2) CxI Inner layers of cerebral cortex
3) St Corpus striatum
4) CA1 O Hippocampus CA1 oriens
5) CA1 R Hippocampus CA1 radiatum
6) CA2 O Hippocampus CA2 oriens
7) CA2 R Hippocampus CA2 radiatum
8) CA3 O Hippocampus CA3 oriens
9) CA3 R Hippocampus CA3 radiatum
10) ML Moleculare lacunosum
11) DG Dentate gyrus
12) CbM Cerebellar molecular cell layer
13) CbG Cerebellar granule cell layer
14) DLG Dorsolateral geniculate nucleus
15) LD Lateral dorsal thalamic nucleus
16) VPM Ventral posteromedial/ventral posterolateral thalamic nuclei
17) cc corpus callosum

Fig. 62 Autoradiograms of [^{3}H]-GABA binding to $GABA_B$ receptors in rat brain sections.

Total $GABA_B$ Binding.

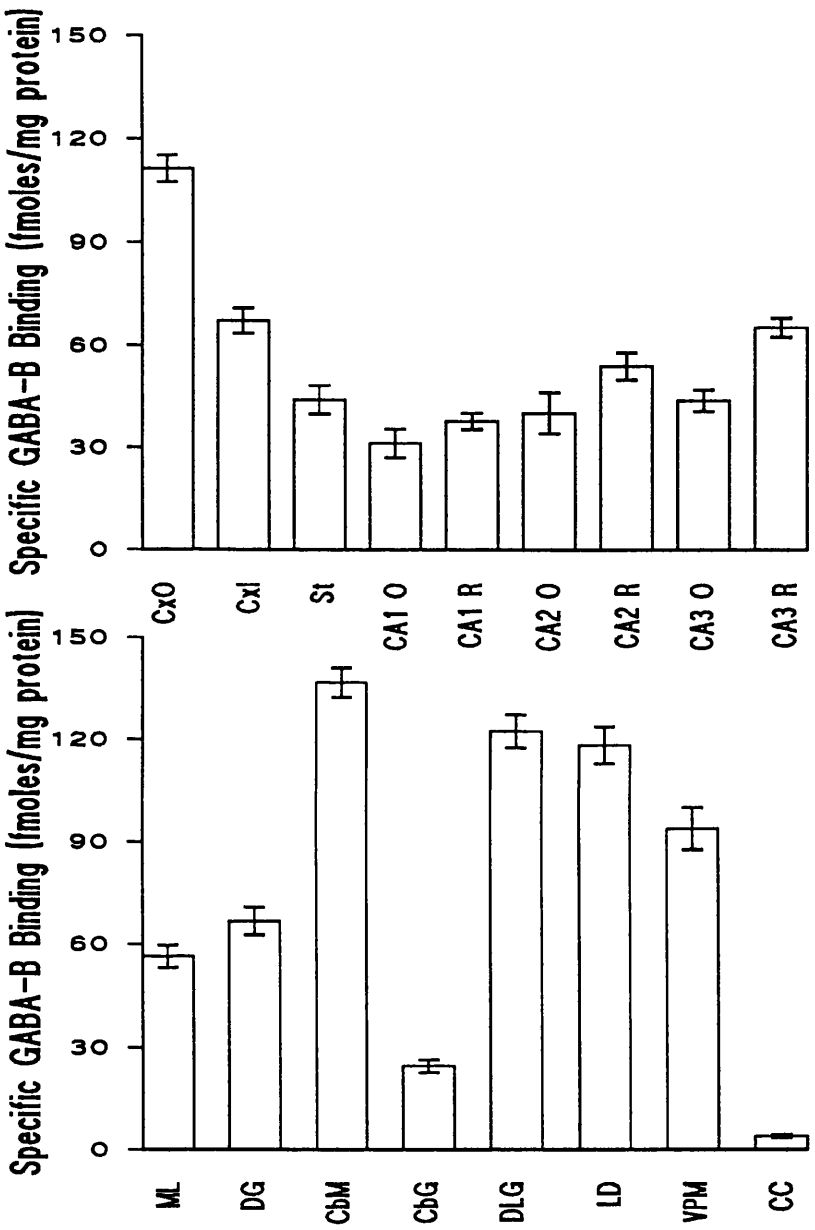


Non-specific Binding.

density significantly higher in the s.radiatum compared to the s.oriens), lacunosum moleculare and dentate gyrus. With the exception of the corpus callosum, which was assumedto represent background levels, the lowest density of $GABA_B$ binding was observed in the cerebellar granule cell layer (25±2 fmoles/mg) (Fig. 63).

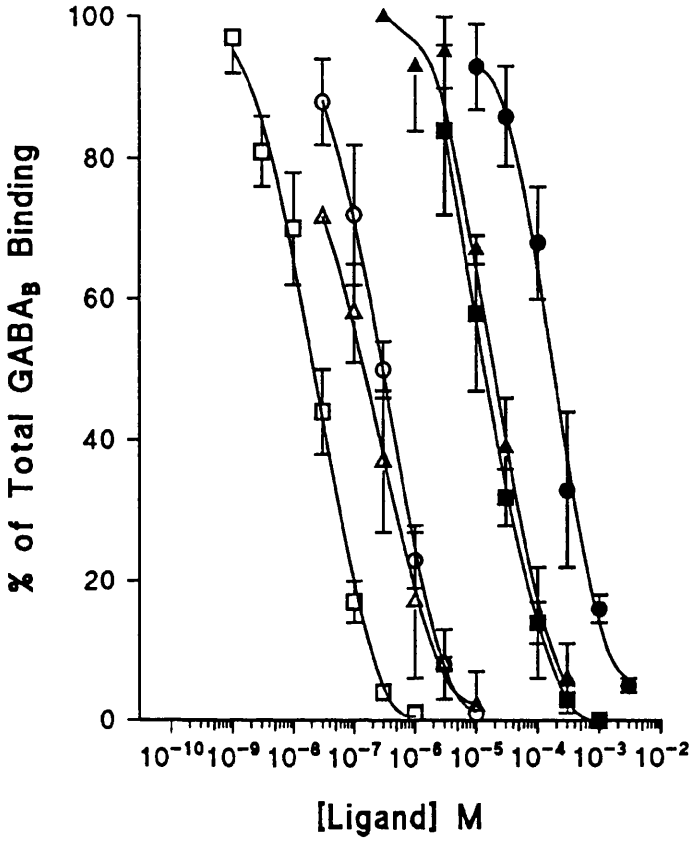
(-)Baclofen (10^{-9}M-10^{-5}M), 3-APA (10^{-10}M-10^{-6}M), SKF 97541 ($3x10^{-9}$M-10^{-5}M), CGP 35348 ($3x10^{-6}$M-$3x10^{-3}$M), CGP 36742 ($3x10^{-6}$M-10^{-3}M) and CGP 46381 (10^{-7}M-$3x10^{-4}$M) produced dose-dependent inhibitions of specific $GABA_B$ binding in all the brain regions studied (cortex Fig. 64; striatum Fig. 65; hippocampus Fig. 66; cerebellum molecular layer Fig. 67). Complete displacement (100%) was achieved at the highest concentrations used for each compound. 3-APA was the most potent displacer of [^{3}H]-GABA with IC_{50} values of between 12.7nM (CA1 oriens) and 29.9nM (lateral dorsal thalamic nucleus). SKF 97541 and (-) baclofen were 10-20 times less potent than 3-APA throughout the brain, with SKF 97541 slightly, though not significantly, more potent than (-)baclofen in 11 of the 16 brain areas (Table 18). Of the putative $GABA_B$ antagonists CGP 46381 (regional IC_{50} values 6.4-24.2μM) and CGP 36742 (IC_{50} values 6.6-20.2μM) were 10-15 times more potent than CGP 35348 (IC_{50} values 63.2-303.4μM) (Table 19). It was difficult to ascertain whether potency differences between the brain areas for each compound reflected anything more than experimental variability, as no consistent pattern emerged.

Fig. 63 Distribution of $GABA_B$ binding sites in rat brain.



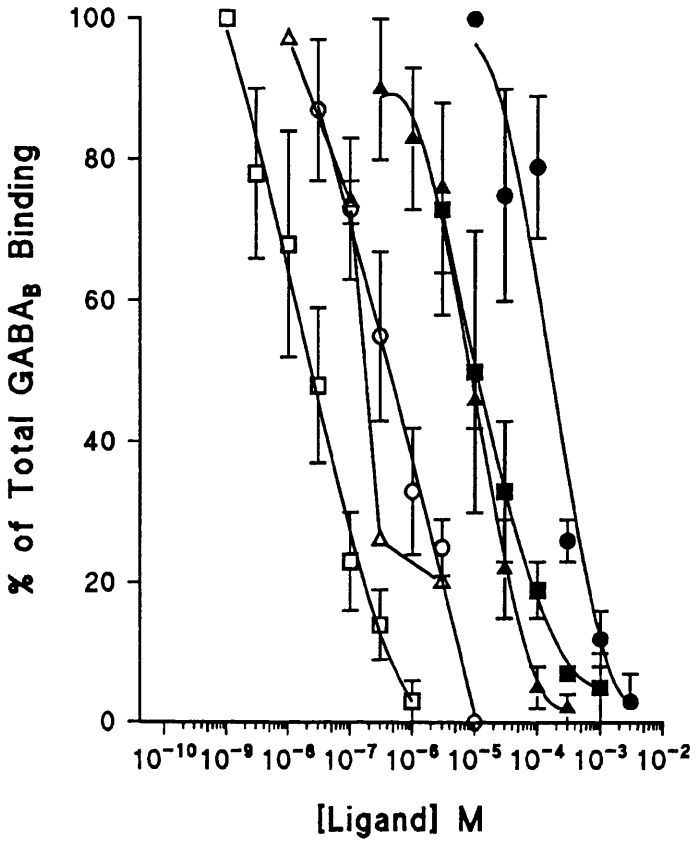
The density of $GABA_B$ binding was determined throughout the rat brain using quantitative autoradiographic techniques. Values are the mean ± s.e.m. from 4 experiments performed in triplicate. Abbreviations as for Fig. 61.

Fig. 64 Displacement of [^{3}H]-GABA from cerebrocortical $GABA_B$ binding sites in rat brain.



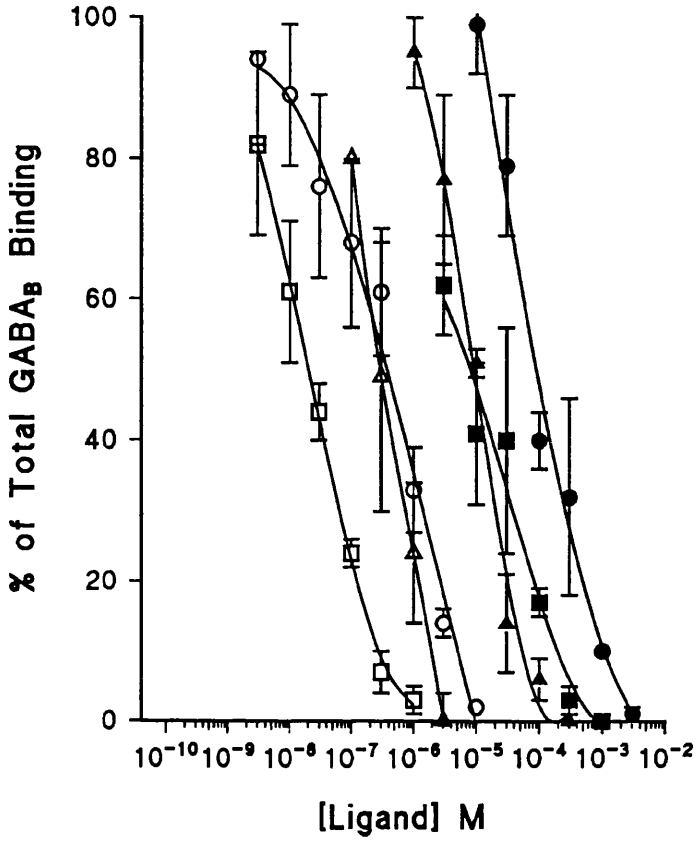
Rat brain slices were incubated for 20 minutes with [^{3}H]-GABA (30nM) in the presence of isoguvacine (40μM) and $CaCl_2$ (2.5mM) with or without the addition of increasing concentrations of the following displacing compounds: (-)baclofen (○); 3-APA (□); SKF 97541 (△); CGP 35348 (●); CGP 36742 (■); CGP 46381 (▲). Non-specific binding was defined with 100μM (-)baclofen. Cortical $GABA_B$ binding densities (fmoles/mg protein) were determined by autoradiographic techniques. The reduction in specific $GABA_B$ binding at each concentration of displacing ligand was expressed as a % reduction in total binding density. 3-4 experiments were performed in triplicate.

Fig. 65 Displacement of [^{3}H]-GABA from striatal $GABA_B$ binding sites in rat brain.



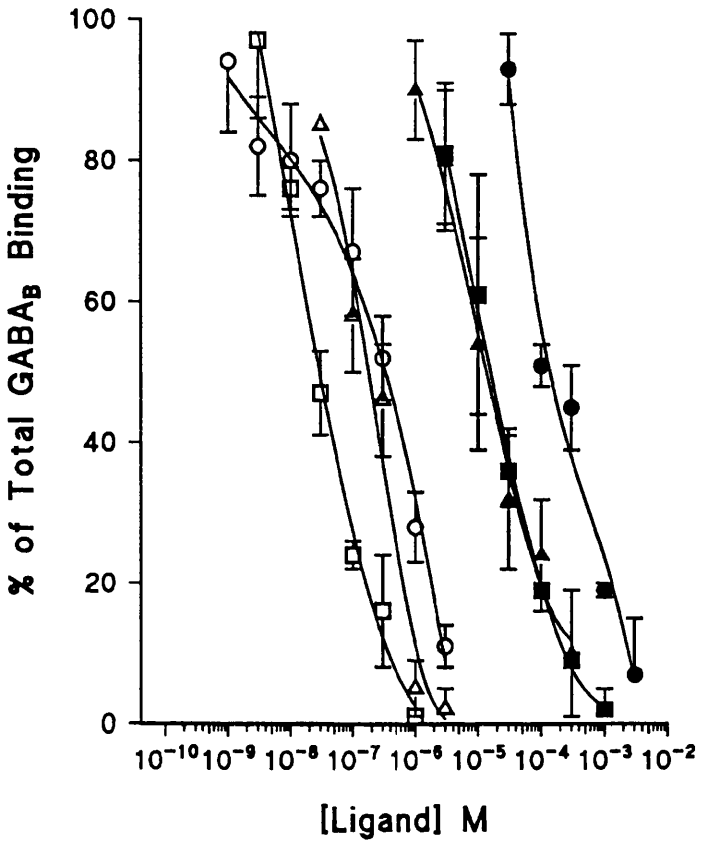
Rat brain slices were incubated for 20 minutes with [^{3}H]-GABA (30nM) in the presence of isoguvacine (40µM) and $CaCl_2$ (2.5mM) with or without the addition of increasing concentrations of the following displacing compounds: (-)baclofen (○); 3-APA (□); SKF 97541 (△); CGP 35348 (●); CGP 36742 (■); CGP 46381 (▲). Non-specific binding was defined with 100µM (-)baclofen. Striatal $GABA_B$ binding densities (fmoles/mg protein) were determined by autoradiographic techniques. The reduction in specific $GABA_B$ binding at each concentration of displacing ligand was expressed as a % reduction in total binding density. 3-4 experiments were performed in triplicate.

Fig. 66 Displacement of [^{3}H]-GABA from hippocampal $GABA_B$ binding sites in rat brain.



Rat brain slices were incubated for 20 minutes with [^{3}H]-GABA (30nM) in the presence of isoguvacine (40μM) and $CaCl_2$ (2.5mM) with or without the addition of increasing concentrations of the following displacing compounds: (-)baclofen (○); 3-APA (□); SKF 97541 (△); CGP 35348 (●); CGP 36742 (■); CGP 46381 (▲). Non-specific binding was defined with 100μM (-)baclofen. Hippocampal $GABA_B$ binding densities (fmoles/mg protein) were determined by autoradiographic techniques. The reduction in specific $GABA_B$ binding at each concentration of displacing ligand was expressed as a % reduction in total binding density. 3-4 experiments were performed in triplicate.

Fig. 67 Displacement of [^{3}H]-GABA from cerebellar $GABA_B$ binding sites in rat brain.



Rat brain slices were incubated for 20 minutes with [^{3}H]-GABA (30nM) in the presence of isoguvacine (40μM) and $CaCl_2$ (2.5mM) with or without the addition of increasing concentrations of the following displacing compounds: (-)baclofen (○); 3-APA (□); SKF 97541 (△); CGP 35348 (●); CGP 36742 (■); CGP 46381 (▲). Non-specific binding was defined with 100μM (-)baclofen. Cerebellar $GABA_B$ binding densities (fmoles/mg protein) were determined by autoradiographic techniques. The reduction in specific $GABA_B$ binding at each concentration of displacing ligand was expressed as a % reduction in total binding density. 3-4 experiments were performed in triplicate.

Table 18 EC_{50} Values for the displacement of [^{3}H]-GABA from $GABA_B$ binding sites in rat brain by (-)baclofen, 3-APA and SKF 97541.

Brain Region	(-)Baclofen (nM)	3-APA (nM)	SKF 97541 (nM)
CxO	189 (87-430)	19 (12-27)	221 (150-450)
CxI	150 (34-400)	17 (4-42)	290 (240-350)
St	196 (20-1300)	22 (4-60)	130 (30-410)
CA1 O	164 (73-860)	13 (3-37)	193 (135-2200)
CA1 R	234 (54-1350)	16 (9-30)	412 (175-1200)
CA2 O	366 (80-1300)	20 (7-80)	225 (70-900)
CA2 R	338 (65-1250)	24 (8-56)	641 (300-730)
CA3 O	489 (250-1500)	24 (9-95)	261 (120-570)
CA3 R	407 (100-1800)	28 (13-56)	255 (120-430)
ML	559 (410-840)	18 (10-66)	139 (33-310)
DG	408 (115-2200)	18 (8-29)	208 (150-260)
CbM	267 (130-460)	17 (14-23)	208 (80-390)
CbG	326 (95-860)	15 (7-51)	107 (27-420)
DLG	403 (165-1250)	24 (13-69)	195 (110-420)
LD	185 (48-600)	30 (22-38)	111 (20-450)
VPM	337 (89-850)	19 (9-42)	306 (200-420)

IC_{50} Values are the geometric mean (and range) of 3-4 experiments performed in triplicate. Abbreviations as outlined in Fig. 61.

Table 19 EC_{50} Values for the displacement of [^{3}H]-GABA from $GABA_B$ binding sites in rat brain by CGP 35348, CGP 36742 and CGP 46381.

Brain Region	CGP 35348 (μM)	CGP 36742 (μM)	CGP 46381 (μM)
CxO	162 (93-350)	12 (9-22)	21 (15-27)
CxI	238 (140-420)	11 (4-53)	14 (7-26)
St	56 (40-82)	6 (3-10)	8 (2-23)
CA1 O	123 (60-134)	10 (3-46)	10 (3-30)
CA1 R	297 (180-690)	17 (8-69)	21 (11-58)
CA2 O	76 (27-225)	7 (3-11)	7 (2-63)
CA2 R	94 (68-175)	8 (5-16)	7 (4-23)
CA3 O	81 (49-110)	12 (7-24)	6 (3-25)
CA3 R	82 (45-110)	11 (5-28)	9 (3-35)
ML	195 (115-400)	14 (8-20)	10 (5-25)
DG	73 (28-120)	16 (7-47)	8 (3-38)
CbM	127 (89-210)	11 (7-28)	10 (6-24)
CbG	86 (29-380)	13 (8-27)	23 (8-42)
DLG	156 (110-190)	17 (7-42)	13 (3-80)
LD	122 (80-140)	19 (9-68)	20 (2-95)
VPM	117 (75-195)	10 (1-57)	16 (2-64)

IC_{50} Values are the geometric mean (and range) of 3 experiments performed in triplicate. Abbreviations as outlined in Fig. 61.

2) Discussion.

Of the six compounds tested only 3-APA had appreciable affinity for $GABA_A$ receptors. In this study 3-APA displaced [^{3}H]-GABA from $GABA_B$ binding sites (IC_{50} 13-30nM) with 183-433 times greater potency than from $GABA_A$ binding sites (IC_{50} 5.5μM) in rat brain. This margin of selectivity for $GABA_B$ over $GABA_A$ sites was at least one order of magnitude less than previously reported (Pratt et al., 1989) using similar experimental conditions. These results indicate that whilst 3-APA is selective for $GABA_B$ receptors it is by no means specific and, unless experimental conditions are controlled to exclude $GABA_A$ receptor activation (as in binding studies), results obtained using this compound should be interpreted cautiously. Since 3-APA potently displaced [^{3}H]-GABA from $GABA_B$ binding sites throughout the rat brain, including all the hippocampal areas investigated, this contradicts the observation made in Chapter 3 in which 3-APA failed to inhibit the K^+-evoked release of endogenous amino acids from rat hippocampal synaptosomes. Possible reasons for this discrepancy are:

Presynaptic $GABA_A$ receptors are found in the rat hippocampus (Andrade et al., 1986; Dutar and Nicoll, 1988b; Colmers and Pittman, 1989). The observed effect of 3-APA on the K^+-stimulated endogenous amino acid release from rat hippocampal synaptosomes was therefore presumably the net response to simultaneous activation of both $GABA_A$ and $GABA_B$ receptors. Enhancement

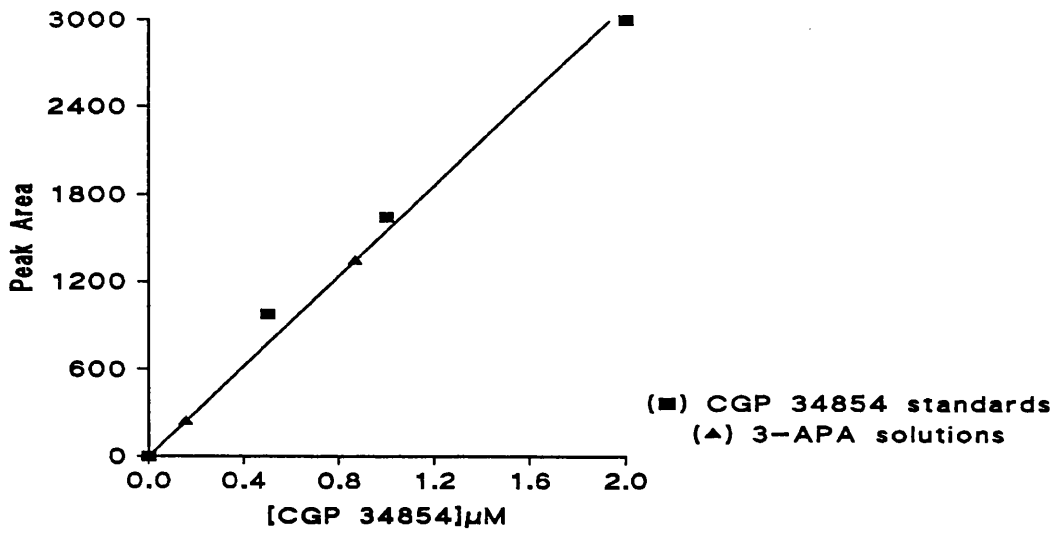
of both basal (Docherty and Bradford, 1987; Fung and Fillenz, 1985) and stimulated (Demeneix et al., 1986; Gervais, 1987; Levi and Gallo, 1981) neurotransmitter release by muscimol or isoguvacine has been described and may be explained by the presence of an outwardly directed electrochemical gradient for Cl^-. Thus $GABA_A$ receptor activation can lead to terminal depolarization (as in the dorsal root ganglion) resulting in neurotransmitter release. The effect of 3-APA on neurotransmitter release in the hippocampus may therefore be determined by the net movement of ions due to the $GABA_A$ receptor-mediated opening of chloride channels and the $GABA_B$ receptor-mediated opening of potassium channels/or closing of calcium channels. Preliminary experiments indicated that isoguvacine did indeed enhance the K^+-stimulated release of endogenous aspartate, glutamate and GABA from rat hippocampal synaptosomes (data not shown) but further studies are required to determine whether or not 3-APA will inhibit such release in the presence of bicuculline. There is no evidence that $GABA_A$ receptors are present on adrenergic terminals in the rat anococcygeus muscle and therefore the GABAergic effect of 3-APA in this preparation can be assumed to be due to presynaptic $GABA_B$ receptor activation.

In addition to possible $GABA_A$ effects it was noted that in release samples obtained in the presence of 3-APA (III.4.iii) two large additional amino acid peaks were present at peak retention times of approximately 19 and 22 min. The peak areas increased with increasing concentrations of 3-APA tested in the experiment and the first peak was smaller than the second. That 3-APA

should be detected by HPLC was not surprising as this compound is a close GABA analogue, but the occurrence of two peaks indicated the presence of a possible contaminant. A sample of a 3-APA oxidation product, CGP 34854, was kindly provided by Ciba-Geigy chemists for analysis by HPLC.

Solutions containing 10μM 3-APA or a combination of 10μM 3-APA and 50μM CGP 34854 were prepared and analyzed by HPLC. 3-APA alone produced two peaks with retention times of 18.8 and 22.1 min and peak areas of 1086.8 and 8366.8 (ratio 1:8) respectively. The combination of 3-APA and CGP 34854 also eluted as two peaks at 19.8 min (slightly later than for 3-APA alone because of the increased broadness of the peak) and 22 min. It is not unreasonable to assume from this that the smaller of the two peaks produced by 3-APA alone is due to the presence of the oxidation product CGP 34854. To determine to what extent 3-APA is contaminated by CGP 34854 a standard curve was obtained from samples of CGP 34854 (0.5-2μM) from which the amount of this product in a 1μM and a 10μM solution of 3-APA was determined. From the calibration curve (Fig. 68) 1μM 3-APA contained 0.16μM CGP 34854 and 10μM 3-APA contained almost 1μM CGP 34854. This particular batch would therefore seem to contain approximately 10% of the oxidation product though this is likely to vary with each batch.

Fig. 68 Calibration curve for CGP 34854.



In displacement studies using whole rat brain slices, 100μM CGP 34854 displaced 27% of specific $GABA_A$ binding and 50% of specific $GABA_B$ binding. Reports from Ciba Geigy (personal communication to Prof. N.G. Bowery) indicate that this compound exhibits weak $GABA_B$ antagonist activity in a number of experimental systems. Therefore 3-APA, described in many studies as a potent, selective $GABA_B$ agonist is actually a compound that is selective for $GABA_B$ receptors but has low μM affinity for $GABA_A$ receptors and is susceptible to oxidation, the oxidation product possessing weak $GABA_B$ antagonistic activity of its own. Thus it is perhaps not surprising that such conflicting data have been obtained using this compound.

The order of potency of the three $GABA_B$ agonists as displacers of [^{3}H]-GABA from $GABA_B$ receptors assessed in whole rat brain sections was essentially the same for each of the brain regions studied as follows:

3-APA >> SKF 97541 > (-)Baclofen

and for the three antagonists:

CGP 46381 > CGP 36742 >> CGP 35348

In each case the order of potency was the same as that observed in the rat anococcygeus preparation but the relative potencies of the compound was different. In the periphery 3-APA was only slightly more potent than SKF 97541 and these two were 10-20 times more potent than (-)baclofen. In the rat brain slice preparation 3-APA was 10-20 times more potent than either SKF 97541 or (-) baclofen, with SKF 97541 slightly more potent than (-)baclofen in most of the brain regions. Likewise, in the rat anococcygeus muscle CGP 46381 was at least 10 times more potent than CGP 36742 which was slightly more potent than CGP 35348. In the rat brain CGP 46381 and CGP 36742 were equipotent and 10-15 times more potent than CGP 35348. It is not easy to determine whether the observed differences in relative potencies of these compounds in the rat peripheral and central nervous systems reflect $GABA_B$ receptor heterogeneity. Experiments using a much larger range of $GABA_B$ ligands are required to provide more conclusive evidence to support this possibility.

CHAPTER SIX: RESULTS

THE EFFECT OF PERTUSSIS TOXIN ON REGIONAL $GABA_B$ BINDING IN RAT BRAIN SYNAPTIC MEMBRANES

1) Regional and age-related variations in the sensitivity of $GABA_B$ binding in rat brain to pertussis toxin.

It has been shown that unilateral injection of PTX(4μg) into the dentate gyrus produces a significant reduction in $GABA_B$ but not $GABA_A$ binding throughout the hippocampus on the injected side but not the contralateral side of the rat brain (Bowery et al., 1990). Despite a loss of $GABA_B$ binding density in the ventral hippocampus, an area remote from the site of injection, binding in other brain regions was unaffected. Results from further investigations indicated that the localized effect of PTX was probably due to the poor tissue penetration properties of this toxin. $GABA_B$ binding was quantified autoradiographically in rat brain sections cut from blocks of tissue which had been incubated for up to 24 hours in preactivated PTX or its vehicle. PTX reduced the density of $GABA_B$ binding by 20-50% throughout the rat brain including cortex, hippocampus, geniculate nuclei and cerebellum but with the surprising exception of the corpus striatum which was unaffected (Knott et al., 1992). These experiments were designed to avoid regional differences in the diffusion of PTX, therefore the possibility that $GABA_B$ receptors are heterogenous in their sensitivity to this toxin could not be discounted.

To address this possibility the effect of PTX on *in vitro* $GABA_B$ binding was investigated in synaptic membranes prepared from the cortex, hippocampus, cerebellum (three areas previously found to be responsive to PTX pretreatment) and corpus striatum of rat brain.

Three groups of male Wistar rats were studied as follows:

Group A Sexually mature rats, 10-12 weeks postnatal (270-320g)

Group B Young adult rats, 7-9 weeks postnatal (160-220g)

Group C Sexually immature rats, 5-6 weeks postnatal (80-150g)

A preliminary study was carried out to verify that the incubation conditions used did not adversely affect $GABA_B$ binding. Levels of $GABA_B$ binding were not significantly different in rat brain membranes incubated for 30 minutes at 29°C compared to membranes incubated at 18°C (data not shown).

i) Group A.

In membranes prepared from the brains of sexually mature male, Wistar rats the amount of [^{3}H]-GABA specifically bound to $GABA_B$ sites (fmoles/mg protein) was 332±21 in cortex, 94±9 in striatum, 104±15 in hippocampus and 239±31 in cerebellum. Following incubation of membranes with PTX, specific $GABA_B$ binding was significantly reduced by 32±6% in cortex, 41±7% in hippocampus and 31±9% in cerebellum. In agreement with the previous findings by Knott and colleagues (1992) striatal $GABA_B$ binding was unaffected by PTX (Table 20).

Table 20 The effect of PTX on $GABA_B$ binding in rat brain membranes: Group A.

	Specific $GABA_B$ Binding (fmoles/mg protein)			
	Cortex	Striatum	Hippocampus	Cerebellum
Control	332±21 (57%)	94±9 (37%)	104±15 (43%)	239±31 (54%)
+ PTX	221±21***	94±9 NSD	60±10*	170±17*

Rat brain membranes were prepared from the cortices, striata, hippocampi and cerebella of adult male Wistar rats (10-12 weeks postnatal, 270-320g). $GABA_B$ binding (fmoles/mg protein) was determined following incubation of membranes with either pre-activated PTX or toxin vehicle (control) for 30 min at 29°C (n=6). $GABA_B$ binding in the presence of PTX was compared to control (Student's paired t-test ,2-tailed).

NSD no significant difference at $p = 0.05$ * $p < 0.05$ *** $p < 0.005$

Specific $GABA_B$ binding as a % of total [^{3}H]-GABA binding is given in parentheses.

ii) Group B.

In membranes prepared from the brains of young adult rats specific $GABA_B$ binding (fmoles/mg protein) did not differ significantly from that of group A in any of the four brain regions studied. Incubation with PTX significantly reduced both hippocampal and striatal $GABA_B$ binding by 31%. Although binding was inhibited in cortical and cerebellar toxin-treated membranes, by 27±13% and 23±5% respectively, these effects were not significantly different from control (Table 21).

iii) Group C.

Specific $GABA_B$ binding (fmoles/mg protein) in membranes prepared from the brains of sexually immature rats was not significantly different from that of either of the older age groups. PTX was without effect on cortical and cerebellar $GABA_B$ binding (reductions of 2±11% and 12±15% respectively) but significantly reduced specific $GABA_B$ binding in the striatum by 40±9% and hippocampus by 50±5% (Table 22).

Addition of GTPγS (2μM) to the binding assay reduced $GABA_B$ binding by 80-93% in all four brain regions in each of the age three groups (Fig. 69).

Table 21 The effect of PTX on $GABA_B$ binding in rat brain membranes: Group B.

	Specific $GABA_B$ binding (fmoles/mg protein)			
	Cortex	Striatum	Hippocampus	Cerebellum
Control	342±26 (76%)	88±22 (43%)	134±24 (48%)	338±51 (69%)
+ PTX	245±29 NSD	63±20*	94±20*	254±26 NSD

Rat brain membranes were prepared from the cortices, striata, hippocampi and cerebella of young adult male Wistar rats (7-9 weeks postnatal, 160-220g). $GABA_B$ binding (fmoles/mg protein) was determined following incubation of membranes with either pre-activated PTX or toxin vehicle (control) for 30 minutes at 29°C (n=4). $GABA_B$ binding in the presence of PTX was compared to control (Student's paired t-test, 2-tailed).

NSD no significant difference at $p = 0.05$ * $p < 0.05$

Specific $GABA_B$ binding as a % of total [^{3}H]-GABA binding is given in parentheses.

Table 22 The effect of PTX on $GABA_B$ binding in rat brain membranes: Group C.

	Specific $GABA_B$ binding (fmoles/mg protein)			
	Cortex	Striatum	Hippocampus	Cerebellum
Control	312±30 (71%)	140±24(53%)	159±32 (51%)	415±76 (74%)
+ PTX	313±52 NSD	92±28***	82±17**	335±18 NSD

Rat brain membranes were prepared from the cortices, striata, hippocampi and cerebella of immature male Wistar rats (5-6 weeks postnatal, 80-150g). $GABA_B$ binding (fmoles/mg protein) was determined following incubation of membranes with either pre-activated PTX or toxin vehicle (control) for 30 min at 29°C (n=6). $GABA_B$ binding in the presence of PTX was compared to control (Student's paired t-test, 2-tailed).

NSD no significant difference at $p = 0.05$ ** $p < 0.01$ *** $p < 0.005$

Specific $GABA_B$ binding as a % of total [^{3}H]-GABA binding is given in parentheses.

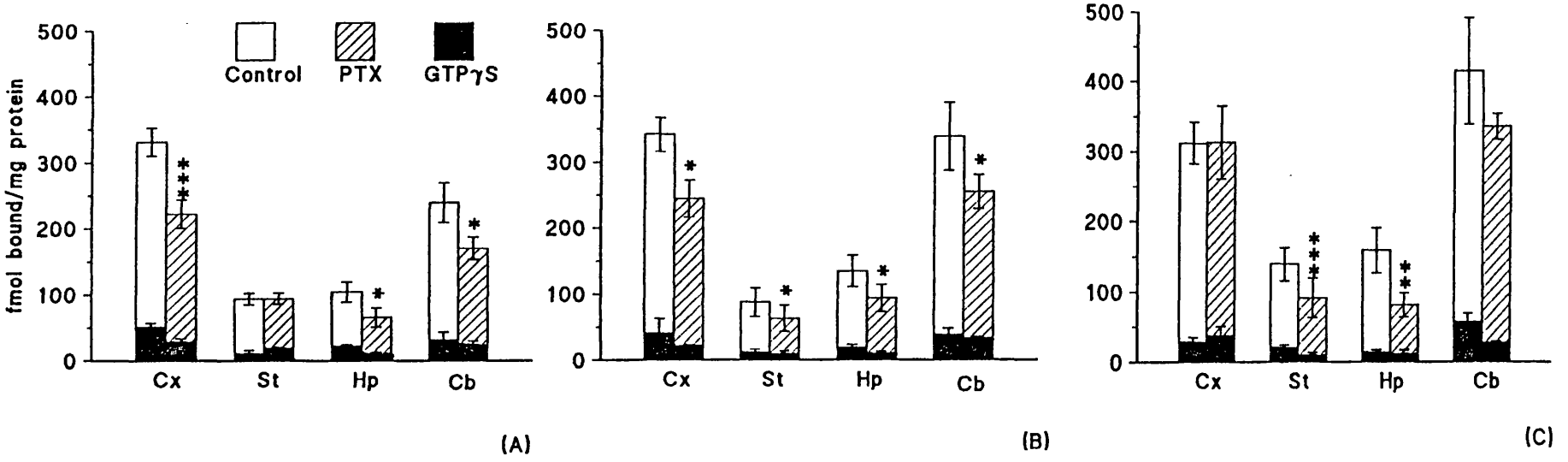


Fig. 69 Regional and age-related variations in the PTX-sensitivity of $GABA_B$ binding in rat brain membranes.

$GABA_B$ binding density was determined in the absence (open bars) and the presence (hatched bars) of PTX (7-15μg/mg protein) in membranes prepared from the cortex, corpus striatum, hippocampus and cerebellum of rats studied at 10-12weeks (A), 7-9 weeks (B) and 5-6 weeks (C) postnatally. GTPγS (2μM) was added to triplicate tubes in each experiment as a marker of $GABA_B$ receptor-G-protein association (solid bars). Binding density in the absence and presence of PTX was compared using Student's paired t-test, 2-tailed. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.005$

iv) Age-related changes in PTX sensitivity.

The changes in sensitivity to PTX with age were more obvious if specific $GABA_B$ binding density remaining in the presence of the toxin was expressed as a ratio of that determined in its absence (Table 23).

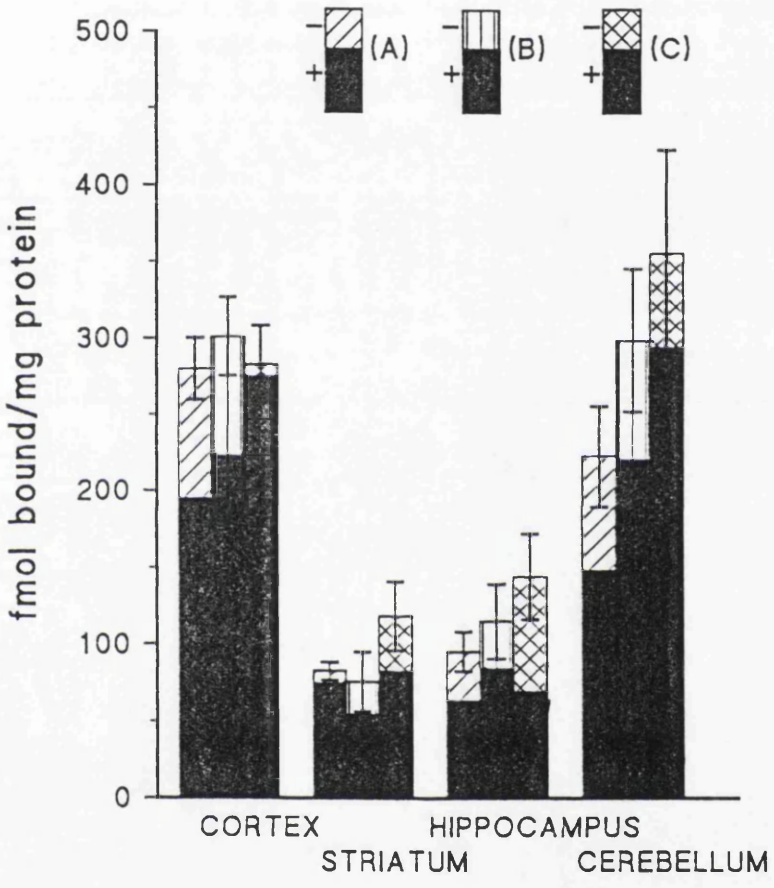
Table 23 Ratio of specific $GABA_B$ binding in the presence and absence of PTX: regional and age related variations.

	Ratio of Specific $GABA_B$ Binding PTX:Control			
Postnatal Age	Cortex	Striatum	Hippocampus	Cerebellum
10-12wks (A)	0.68±0.06	1.06±0.14	0.59±0.07	0.69±0.09
7-9wks (B)	0.74±0.13	0.70±0.11	0.69±0.06	0.77±0.05
5-6wks (C)	0.98±0.13	0.60±0.08	0.49±0.05	0.89±0.15

From these data it was apparent that whilst hippocampal $GABA_B$ binding retained its sensitivity (30-50%) to PTX at all stages of development (6-12 weeks postnatal), both it, and to a much greater extent, the striatum became progressively less sensitive to PTX with age. In contrast the cortex and cerebellum increased in responsiveness to PTX pretreatment as the animals matured.

If only high affinity (i.e. GTPγS-sensitive) $GABA_B$ binding was considered then some interesting trends emerged. Specific high affinity $GABA_B$ binding fell by approximately 35% in both the striatum and hippocampus from 6-12 weeks, yet the PTX-resistant portion of $GABA_B$ binding in these areas remained constant (Fig. 70). Therefore the increasing insensitivity to PTX observed in these regions appeared to be due to a progressive loss of the PTX-sensitive $GABA_B$ component. High affinity $GABA_B$ binding density was also reduced by the same amount in the cerebellum with maturity. However in this area PTX-resistant $GABA_B$ binding also fell by 50% as age increased and so, if anything, a small increase in the PTX-sensitive portion is observed (from 57 fmoles/mg in group C to 75 fmoles/mg in group A). Cortical high affinity $GABA_B$ binding density did not alter from 6-12 weeks so that the apparent reduction in the PTX-insensitivity is paralleled by an increase in the PTX-sensitive binding component (Fig. 70).

Fig 70 GTPγS-Sensitive $GABA_B$ binding in the presence and absence of PTX.



GTPγS-Sensitive $GABA_B$ binding (fmol/mg) was determined in the absence (hatched bars) and presence (solid bars) of PTX.

2) Discussion.

$GABA_B$ binding can be detected in rat brain at postnatal day 1, increases to maximum levels at day 14 and declines to stabilize at adult levels by 21-28 days after birth (Al-Dahan and Thalmann, 1989; Dr. C. Knott personal communication). This coincides with changes in the activity of both the high affinity GABA uptake system (Balcar and Johnston, 1989) and glutamate decarboxylase (Coyle and Enna, 1976) and with increases in the concentration of the G_o α-subunit measured by immunoassay in rat brain (Asano et al., 1988) all of which reach adult levels after 4 weeks. Animals used in this present study ranged in age from 6 to 12 weeks and therefore the changes in $GABA_B$ binding observed with age and PTX pretreatment occurred after the development of the GABAergic system in the rat brain.

Binding of [^{3}H]-GABA to $GABA_B$ sites may be influenced by the presence of specific endogenous inhibitors (Toffano et al., 1978; Kuroda et al., 1982; Yamada et al., 1987), the concentrations of which may vary regionally and reportedly decline during ontogeny (Skerritt and Johnston, 1982). For this reason the rat brain membrane preparations used in these studies were extensively washed during preparation and samples were analyzed by HPLC. The GABA content of washed tissue samples from the four brain regions in adult rats (group A) was found to be below the level of detection (sub-pmoles: data not shown). This indicated that the washing procedure successfully removed almost all endogenous GABA that may possibly have interfered with

$GABA_B$ binding.

The activity of PTX is both temperature and time dependent (Katada and Ui, 1982; Ui, 1984). However prolonged incubation of rat brain membranes at temperatures above 30°C has a deleterious effect on total $GABA_B$ binding, presumably due to the denaturation of the receptor protein (Asano et al., 1985). Therefore to study the effect of PTX, tissue preincubation conditions were chosen under which $GABA_B$ binding remains stable and the concentration of PTX used (7-20µg/mg protein) was comparable or greater than used by other researchers. Using these experimental conditions PTX did not inhibit $GABA_B$ binding by more than 50%. However this is not dissimilar to the findings from other studies in which incubation times of up to 24 hours were used (Asano et al., 1985; Xu and Wojcik, 1986; Knott et al., 1992). Therefore, although the biological activity of PTX can vary greatly, the degree of effect of the toxin observed in these experiments is unlikely to be limited by either the concentration of toxin or the preincubation conditions.

It is possible that the observed regional and age-associated variations of $GABA_B$ binding in rat brain to PTX were related to the extent of endogenous ADP-ribosylation activity. Endogenous ribosylation of G_s has been reported and was suggested to be mediated via the $\beta\gamma$ subunits released from activated $G_{i/o}$ (Jacquemin et al., 1986). More recently mono-ADP ribosylation of a 39kDa - presumably G_o-like - protein stimulated by nitric oxide liberating compounds such as sodium nitroprusside and 3-morpholinosydnonimine was

observed in human platelets and rat brain and heart cytosolic fractions. Haemoglobin reversed the effects of sodium nitroprusside in the platelet preparation (Brune and Lapetina, 1989). As cGMP, its analogues 8-bromo cGMP and dibutyryl cGMP and agents which increase cytosolic cGMP concentrations (hydroxylamine and aniline) failed to modify this ADP-ribosylation the effect of nitric oxide would appear to be a novel one independent of the activation of the guanyl cyclase system (Brune and Lapetina, 1989). Such endogenous ADP-ribosylation has been demonstrated to show regional heterogeneity, for G_s-like proteins at least (Duman et al., 1991) with highest levels of activity in the hippocampus and cortex and lower levels in striatum and cerebellum. Whether a different pattern of activity exists for $G_{i/o}$ proteins remains to be seen.

Hormonal influences on both G-proteins and $GABA_B$ receptors may also be responsible for the heterogeneous response to PTX observed in rat brain. Endogenous ADP-ribosylation is modulated by circulating levels of glucocorticoids. Chronic administration of corticosterone increased levels of $G_s\alpha$ immunoreactivity, mRNA and ADP ribosylation and decreased $G_i\alpha$ immunoreactivity and mRNA in rat brain. $G_o\alpha$ appeared to unaffected (Saito et al., 1989; Duman et al., 1991). Whilst this may be of relevance in pathological states in which glucocorticoids are produced excessively (e.g. Cushing's syndrome or severe stress) or following long term synthetic steroid treatment, the influence of the sex hormones on $GABA_B$ function may be of greater physiological importance.

Gonadal steroids, known to modulate other neurotransmitter systems (Bigeon et al., 1983; Hruska, 1986), may also influence $GABA_B$ receptor activity as sexual maturation occurs. Effects of oestrogen on both $GABA_A$ (Perez et al., 1986; Schumacher et al., 1989) and $GABA_B$ (Francois-Bellan et al., 1989) receptors have been reported in the literature: $GABA_A$ binding is up-regulated by oestrogen in the corpus striatum, but not cortex or cerebellum, of castrated male rats and in the hippocampus of ovariectomized female rats; in contrast $GABA_B$ binding in corpus striatum was reduced following chronic administration of oestrogen to adult female rats. Levels of the male hormone testosterone, which is metabolized to the active steroids 5α-dihydrotestosterone and oestrogen, are reportedly higher in sexually mature male rats compared to younger animals (6-7 weeks old) (Dohler and Wuttke, 1975). This increase in oestrogen with age may, at least in part, explain the reduced PTX-sensitivity of $GABA_B$ binding observed in the striatum with maturation.

As a stable analogue of GTP, GTPγS binds to the α subunit of the G_i/G_o moiety resulting in the loss of high affinity $GABA_B$ binding. Therefore the lack of sensitivity to PTX did not imply the presence of $GABA_B$ receptors not coupled to inhibitory G-proteins. Rather it suggested that both PTX-sensitive and PTX-insensitive $GABA_B$ receptor-linked inhibitory G-proteins exist, the proportions of which vary regionally and with age. As yet a functional PTX-insensitive (but $G_{i/o}$-protein linked) $GABA_B$-mediated response has not been described in the rat brain and so the physiological relevance of the current findings is unclear, although evidence for PTX-insensitive (G_x) G-protein

mediated responses exits for other systems (Evans et al., 1985; Pobiner et al., 1985; Schlegel et al ., 1985). Perhaps the importance of these observations, at this stage, may be to highlight the difficulty in comparing the effects of PTX in biochemical and functional $GABA_B$ assays carried out using tissue from animals at differing stages of sexual maturity.

FUTURE DIRECTIONS

Future Directions.

The rat hippocampus has formed the focus of the experiments described in Chapter 3. Information on the effects of $GABA_B$ agonists and antagonists in *in vitro* hippocampal preparations is invaluable given the possible involvement of $GABA_B$ receptor activation in learning and memory processes. Administration of baclofen impairs memory in rats (Schwartzwelder et al., 1987) whilst CGP 35348 facilitation of long term potentiation has been demonstrated in the rat hippocampal slice (Olpe and Karlsson, 1990) and the novel $GABA_B$ antagonist CGP 36742 has positive effects on cognitive function in a number of animal experimental paradigms (Bittiger et al., 1992; Mondadori et al., 1992). In the present investigation although (-)baclofen attenuated the release of the excitatory amino acids glutamate and aspartate and that of GABA in hippocampal synaptosomes, only the latter effect was antagonized by CGP 35348. It will be of interest to determine whether the hippocampal presynaptic $GABA_B$ heteroreceptor is affected by more potent $GABA_B$ antagonists such as CGP 46381 or CGP 55845, a compound with affinity for $GABA_B$ binding sites in rat cortical membranes of 7nM (Froestl et al., 1992). If not, it may be that the improvement in cognitive function obtained with $GABA_B$ antagonists is due to the selective augmentation of GABA release mediated via tonically-activated $GABA_B$ autoreceptors.

An injection of tetanus toxin into the hippocampus produces a reduction in GABA release and results in local neuronal degeneration which can be

prevented by the lesioning of excitatory afferents or pretreatment with NMDA receptor antagonists (Bagetta et al., 1990; Bagetta et al., 1991). Extrapolation of these findings has led to the suggestion that antagonists acting at $GABA_B$ autoreceptors may be neuroprotective, and therefore novel drugs may be of possible use in stroke (Bowery, in press). Evidence to support this theory is as yet unavailable.

It was fortunate that Bowery and Hudson (1979) had the foresight to investigate the effects of baclofen on sympathetic outflow in peripheral tissues. Without this piece of crucial research it is a matter for speculation as to when $GABA_B$ receptors would have eventually been discovered. Since the early 1980's isolated peripheral preparations have been used to assess the functional activity and relative potencies of new $GABA_B$ receptor compounds as illustrated in Chapter 4 of this thesis. It is usually assumed that such preparations are a means to providing purely quantitative information with regards to a new ligand and are not in themselves of any particular physiological relevance (e.g. the somewhat esoteric rat anococcygeus muscle). However, the functional significance of GABA and $GABA_B$ receptors in the peripheral nervous system is likely to assume greater importance in future as a target for clinically useful drugs. The possibility that $GABA_B$ agonists have anti-asthmatic properties is already under investigation. Baclofen depresses the following; cholinergic receptor-mediated contractions of the guinea-pig trachea (Tamaoki et al., 1987; Chapman et al., 1991), evoked acetylcholine (Shirakawa et al., 1987) and tachykinin (Ray et al., 1991) release from

pulmonary tissues and vagally- (Chapman et al., 1992) or tachykinin (Belvisi et al., 1991) -induced bronchoconstrictor responses in guinea-pigs. In the light of the recent concern over the safety of available β_2-adrenergic agents, novel anti-asthma therapies or would be advantageous.

The thalamus is believed to be of paramount importance in the generation of the spike and wave discharges (SWD) observed in the rodent model of absences (Vergnes et al., 1987; Vergnes et al., 1990) and in patients with petit mal (absence) epilepsy (Williams, 1953). The density of $GABA_B$ binding is moderately high in this brain region as described in Chapter 5 and is displaced by (-) baclofen, 3-APA, SKF 97541, CGP 35348, CGP 36742 and CGP 46381. Contrary to the postulation that $GABA_B$ agonists may be anti-epileptic (presumably due to the depression of excitatory neurotransmission) systemic baclofen increases SWD in the genetic absence epilepsy rat (GAER's; Strasbourg) (Vergnes et al., 1990). The most exciting recent development in $GABA_B$ receptor pharmacology has been the observation that $GABA_B$ antagonists are effective in these models of absence seizures (Hosford et al., 1992; Marescaux et al., 1992; Snead et al., 1992). CGP 36742 which is active orally and is able to enter the brain from the circulation has been put forward as a candidate for clinical studies. As preliminary investigations indicate that this compound is well tolerated in human volunteers and produces few overt behavioural adverse effects (Bittiger et al., 1992) it is to be hoped that CGP 36742 will be the first therapeutically useful $GABA_B$ antagonist, not only in petit mal epilepsy but also in conditions of cognitive impairment.

Data provided in Chapter 6 indicated that whilst $GABA_B$ receptors in rat brain are linked to inhibitory G-proteins, the sensitivity of $GABA_B$ binding to PTX altered regionally and with age. A possible influence of gonadal steroids on $GABA_B$ receptors may be evaluated by an investigation of regional $GABA_B$ binding in castrated male rats of between 5 and 12 weeks old. If a similar pattern of PTX sensitivity is observed following this procedure then it is unlikely that sex steroids play a major role in the regulation of $GABA_B$ receptor-$G_{i/o}$-protein interactions by either stimulation of endogenous ADP-ribosylation or at the level of genes controlling G-protein synthesis. As there are experimental difficulties inherent in the use of PTX, investigations into alterations in $GABA_B$ receptor-inhibitory G-protein coupling using antibodies to specific $G_{i\alpha}$ subunits may be more informative.

As baclofen reaches its 21st birthday the prospect that $GABA_B$ agonists and antagonists will be of clinical use in a wide variety of pathophysiological conditions is tremendously exciting to researchers in the field. $GABA_B$ receptors have at last 'come of age'.

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