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Presynaptic Actions of 4-Aminopyridine and γ-Aminobutyric Acid on Rat Sympathetic Ganglia in vitro

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Summary. Responses to bath-applications of 4-aminopyridine (4-AP) and γ-aminobutyric acid (GABA) were recorded intracellularly from neurones in the rat isolated superior cervical ganglion.

4-aminopyridine (0.1 – 1.0 mmol/l) usually induced spontaneous action potentials and excitatory postsynaptic potentials (EPSPs), which were blocked by hexamethonium. Membrane potential was unchanged; spike duration was slightly increased. Vagus nerve B-and C-fibre potentials were prolonged.

In 4-AP solution (0.1 – 0.3 mmol/l), GABA (0.1 mmol/l), 3-aminopropanesulphonic acid or muscimol evoked "bursts" of spikes and EPSPs in addition to a neuronal depolarization. These "bursts", which were not elicited by glycine, glutamate, taurine or (±)-baclofen, were completely antagonised by hexamethonium, tetrodotoxin or bicuculline methochloride.

It is concluded that: (a) 4-AP has a potent presynaptic action on sympathetic ganglia; (b) presynaptic actions of GABA can be recorded postsynaptically in the presence of 4-AP; and (c) the presynaptic GABA-receptors revealed in this condition are similar to those on the postsynaptic membrane.

Key words: 4-Aminopyridine – GABA – Sympathetic ganglion – Presynaptic receptors.

Introduction

Receptors for γ-aminobutyric acid (GABA), an inhibitory transmitter in the mammalian central nervous system, are also present on neurones in mammalian sympathetic ganglia (see Nistri and Constanti 1979 for a recent review). GABA receptors also exist on unmyelinated axons in the sympathetic preganglionic nerve (Brown and Marsh 1978). Additionally, it has recently been suggested that on the nerve terminals there exists a further population of GABA-receptors, the activation of which leads to a reduction in acetylcholine release (Brown and Higgins 1979). Such receptors on autonomic nerve terminals have been reported to be bicuculline-insensitive and to be activated by the anti-spastic drug baclofen (Bowery et al. 1979).

Efforts to demonstrate electrophysiologically presynaptic effects of GABA in intact ganglia are hampered by the strong postsynaptic inhibitory action of this compound. Kato and Kuba (1980) partially overcame this problem by carefully comparing the time courses of the GABA-induced postsynaptic conductance increase and depression of the excitatory postsynaptic potential amplitude in bullfrog sympathetic ganglia. In this paper, we report that in the presence of 4-aminopyridine (4-AP), presynaptic actions of GABA can be clearly observed whilst recording intracellularly from the postganglionic neurones. 4-aminopyridine, which is known to act as an axonal K^+-channel blocker (Schauf et al. 1976; Ulbricht and Wagner 1976; Yeh et al. 1976a; Meves and Pichon 1977), has been reported to greatly enhance presynaptic excitability, although the mechanism of this effect is, as yet, unclear (Lemeignan 1972, 1973).

The present experiments were performed with two questions in mind. First, to what extent does 4-AP affect pre- and postsynaptic function in isolated rat sympathetic ganglia? Second, what is the nature of the presynaptic GABA receptors?

Methods

Wistar rats, either sex (approximately 300 g) were anaesthetized with urethane (1.5 g/kg; i.p.) and the superior cervical ganglia were removed. After dissecting free the connective tissue sheath, ganglia were placed in a small Perspex bath and superfused with Krebs' solution at 5 – 10 ml/min, gassed with 95% O_2/5% CO_2. The pre- and

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postganglionic nerves were drawn into glass suction electrodes, which permitted orthodromic nervous stimulation (10 – 20 V, 0.1 ms) and also served to hold the preparation stable on the floor of the bath. The Krebs' solution contained (mmol/l): NaCl, 118; KCl, 4.8; CaCl, 2.5 $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1.2; D-glucose, 11. 16 experiments were performed at 30° C and 11 at 25° C; all drugs were applied via the superfusate.

Intracellular recordings were obtained from 36 neurones in 27 ganglia using a single resistance- and capacitance-compensated microelectrode coupled to an amplifier capable of delivering constant-current pulses. Electrodes were filled with neutralised 4 M potassium acetate (pH = 7.4; Brown and Constanti 1978) and had resistances between 60 and 120 MΩ. Only cells with stable resting potentials and action potentials ≥ 60 mV were selected for study. Potentials were displayed on an oscilloscope and a potentiometric chart recorder and also stored on magnetic tape for further analysis. The frequency of action potentials was measured "off line" using a Nicolet Med 80 computer. Extracellular d.c. recording from ganglia was performed at room temperature (≈ 20° C) using an "air gap" method (Brown and Marsh 1975).

In a number of experiments, instead of the superior cervical ganglion the cervical vagus nerve was removed (care being taken to exclude the nodose ganglion). After desheathing, both ends of the nerve were gently pulled into suction electrodes and stimulus parameters were selected to elicit either A and B fibre compound potentials or A, B and C fibre potentials (see Douglas and Ritchie 1957). Nerves were maintained in flowing Krebs' solution (see above) at 30° C. Compound action potentials were recorded with a d.c. coupled oscilloscope and averaged using a digital averager (Didac 800).

Drug sources were as follows: γ-aminobutyric acid, L-glutamate Na, glycine and tetrodotoxin (Sigma); taurine and hexamethonium bromide (Merck, Darmstadt, FRG); 4-aminopyridine (EGA-Chemie, Steinheim, FRG); 3-aminopropanesulphonic acid (K&K Laboratories). Biocuculline methochloride was kindly prepared from bicuculline by Dr. Klosa. Muscimol HCl was donated by Schering A.G., Berlin; baclofen was donated by Ciba-Geigy, Basel, Switzerland.

Results

Actions of 4-Aminopyridine

The most obvious effect of 4-aminopyridine (4-AP) on sympathetic neurones was a dramatic increase in spontaneous synaptic activity. Thus, after several minutes of superfusion with solutions containing 0.1 – 1 mmol/l 4-AP, 25 out of 36 cells exhibited spontaneous excitatory postsynaptic potentials (EPSPs) as well as action potentials (Fig. 1 A and B). Although the concentration-dependency of this effect was not systematically investigated, it was usually the case that 1 mmol/l 4-AP was more effective in eliciting spontaneous potentials. In the experiment illustrated in Fig. 1 C, addition of 1 mmol/l 4-AP caused the cell to discharge at a frequency approaching 3 Hz. This spontaneous firing, which was reversibly abolished by the nicotinic receptor antagonist hexamethonium (Fig. 1 C), ceased after about 12 min washing in normal Krebs' solution. In the presence of hexamethonium, action potentials could still be elicited by injecting depolarizing current across the cell membrane (see inset Fig. 1 C).

Table 1 summarizes the effects of 4-AP (0.1 – 1 mmol/l) on membrane potential, input resistance and action potential duration. No marked changes in resting potential were observed. Changes in input resistance (calculated from electrotonic potential amplitudes produced by constant-current pulses) were inconsistant; we observed either no change, increases or decreases of up to 25%. The action potential

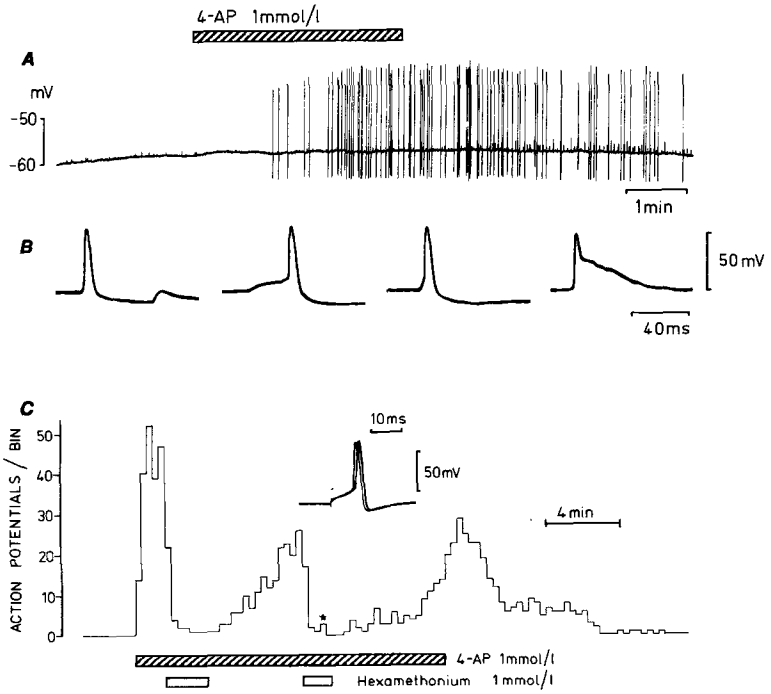

Fig. 1 A–C
Spontaneous synaptic activity recorded from sympathetic neurones during the action of 4-aminopyridine (4-AP). Part (**A**) shows the effect of a 3.5 min application of 1 mmol/l 4-AP (hatched bar) on the membrane potential. After a latency of about 1 min, during which there was virtually no change in resting potential, the cell began to fire spontaneously. Examples of spontaneous discharges are shown in (**B**). The amplitude of the action potentials in (**A**) was attenuated because of the chart recorder response time; temperature was 25° C. Part (**C**) illustrates the effect of hexamethonium (1 mmol/l; open bars) on 4-AP-induced neuronal action potentials. The frequency of intracellularly-recorded spontaneous action potentials is shown on the ordinates; bin width was 20 s. The inset shows three spikes, which were elicited during hexamethonium by injecting depolarizing current across the cell membrane; the asterisk indicates their occurrence in the histogram. Temperature was 30° C

Table 1. Effects of 4-aminopyridine on membrane potential (E_m), membrane resistance (R_m) and action potential duration (AP dur), measured 5–15 min after drug application

Concentration mmol/l	Cell No.[a]	E_m mV	ΔE_m mV	R_m[b] MΩ	ΔR_m %	AP[c] dur ms	Δ AP dur %
0.1	2	−50	0	40	0	2.0	+20
0.1	3	−60	+2	100	+10	1.6	+31
0.3	1	−50	−2	50	−20	1.8	+11
0.3	4	−55	+2	47	− 9	2.0	+20
0.3	5	−48	+3	19	− 5	1.9	+11
1.0	4	−60	−2	50	−16	2.1	+52
1.0	5	−47	−3	15	−13	2.1	+19
1.0	6	−50	+2	52	+23	1.9	+63

[a] Data analysis restricted to 6 cells with stable resting potential and input resistance
[b] Calculated from potential deflections produced by 0.4 nA hyperpolarizing constant-current pulses
[c] Measured as the duration at 50% of total action potential amplitude

duration was significantly increased by up to 63%, whereas action potential amplitude was usually unaffected. The increased duration was mainly due to a prolongation of the repolarizing phase of the spike (Fig. 2A; see also McAfee and Yarowsky 1979). The amplitude and overall duration of postsynaptic potentials induced by orthodromic nerve stimulation was unaffected by 100 μmol/l 4-AP (Fig. 2B).

Extracellular d.c. recordings revealed that 0.1–1.0 mmol/l of 4-AP either induced small ganglionic positivities or negativities. However, higher concentrations (3–10 mmol/l) always induced negativities suggesting a depolarization of the postganglionic neurones (see also Hue et al. 1978).

In addition to the experiments on sympathetic ganglia, the action of 100 μmol/l 4-AP was tested on the isolated vagus nerve (9 preparations). The following effects on the compound action potential were observed: (1) the A-fibre potential was unaltered (Fig. 2C). (2) The B-and C-fibre potentials were clearly increased in duration (Fig. 2D). These effects were observed within 2–5 min and were reversible by washing for about 30 min.

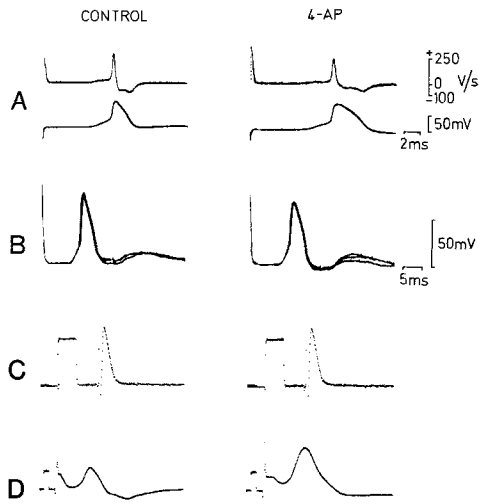

Fig. 2. Effects of 4-aminopyridine (4-AP) on (**A**) soma spike duration, (**B**) responses to orthodromic nerve stimulation, (**C**) and (**D**) the compound action potential of an isolated vagus nerve. The left panel in (**A**) shows the soma spike and its first time derivative (top trace). The right panel was recorded 15 min after addition of 1 mmol/l 4-AP; note the decreased rate of rise and marked prolongation of the repolarizing phase. Temperature was 30°C. In (**B**) (different ganglion) postsynaptic responses to orthodromic nerve stimulation are shown; control (left), and 10 min after addition of 100 μmol/l 4-AP (right). In each example, 3 responses are photographically superimposed. Resting potential was about −50 mV; temperature was 30°C. Parts (**C**) and (**D**) illustrate the effect of 100 μmol/l 4-AP on vagus nerve compound potentials. In each case 10 responses were averaged in control solution (left panel) and 5 min after application of 4-AP (right panel). The stimulation parameters were as follows: A-fibre potential (**C**), 0.5 Hz, 10 μs, supramaximal voltage; C-fibre potential (**D**), 0.5 Hz, 100 μs, supramaximal voltage. Calibration pulse: 2 mV, 1 ms

Actions of GABA in 4-Aminopyridine

Figure 3 illustrates the action of 100 μmol/l GABA in normal Krebs' solution and during superfusion with 100 μmol/l 4-AP. In the control (Fig. 3A), application of GABA resulted in a reversible neuronal depolarization as previously described (Adams and Brown 1975). The ganglion was then superfused with 100 μmol/l 4-AP for 10 min and GABA reapplied. In the 4-AP solution, GABA additionally elicited a "burst" of action potentials as well as some EPSPs. This effect was reversible after washing in normal Krebs' solution for 10 min (Fig. 3C). Examples of subthreshold potentials and action potentials (which were often preceded or followed by synaptic potentials) are shown in Fig. 3B2 and 3B3 respectively. In 8 other neurones, in all of which 4-AP alone produced no spontaneous activity, GABA had a similar effect to that illustrated in Fig. 3. In 13 out of 19 other cells, which exhibited spontaneous activity in 4-AP (100–300 μmol/l), GABA clearly increased the frequency of

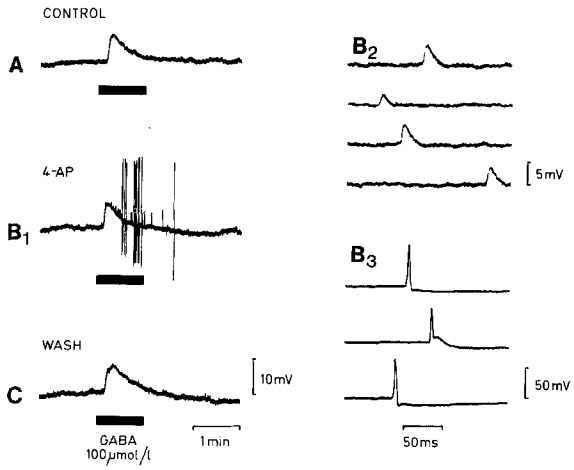

Fig. 3. Effect of 100 µmol/l GABA (solid bar) on the membrane potential of a ganglion cell (**A**) in control solution, (**B**) 10 min after addition of 100 µmol/l 4-aminopyridine (4-AP) and (**C**) 10 min after washing in normal solution. In the presence of 4-AP, GABA evoked a "burst" of subthreshold potentials and action potentials, examples of which are shown in B_2 and B_3 respectively. The amplitude of the action potentials in B_1 is attenuated by the chart recorder. Resting membrane potential of this cell was about −50 mV; temperature was 30° C

these events (see Fig. 5B). It was often observed that the maximum frequency of GABA-induced postsynaptic potentials occurred towards the end of, and immediately following GABA superfusion, rather than at the peak of the postsynaptic depolarization (see Fig. 3B1). Intracellular records showed that the amplitude of the GABA-induced soma depolarization was not significantly altered in 4-AP solution (Fig. 3). However, using extracellular d.c. recording, we found that the depolarizing action of 100 µmol/l GABA was slightly reduced in the presence of 300 µmol/l 4-AP (−10%; mean of 3 experiments). Experiments performed at 25° C were qualitatively similar to those at 30° C.

In order to determine the origin of the postsynaptic potentials elicited by GABA during 4-AP superfusion, we examined their sensitivity to hexamethonium and tetrodotoxin (TTX). In all 6 cells tested, hexamethonium (0.5−2 mmol/l) completely abolished action potentials and EPSPs induced by GABA in the presence of 4-AP (Fig. 4). Tetrodotoxin (350 nmol/l), a concentration sufficient to block Na^+-mediated action potentials, also abolished action potentials and, more

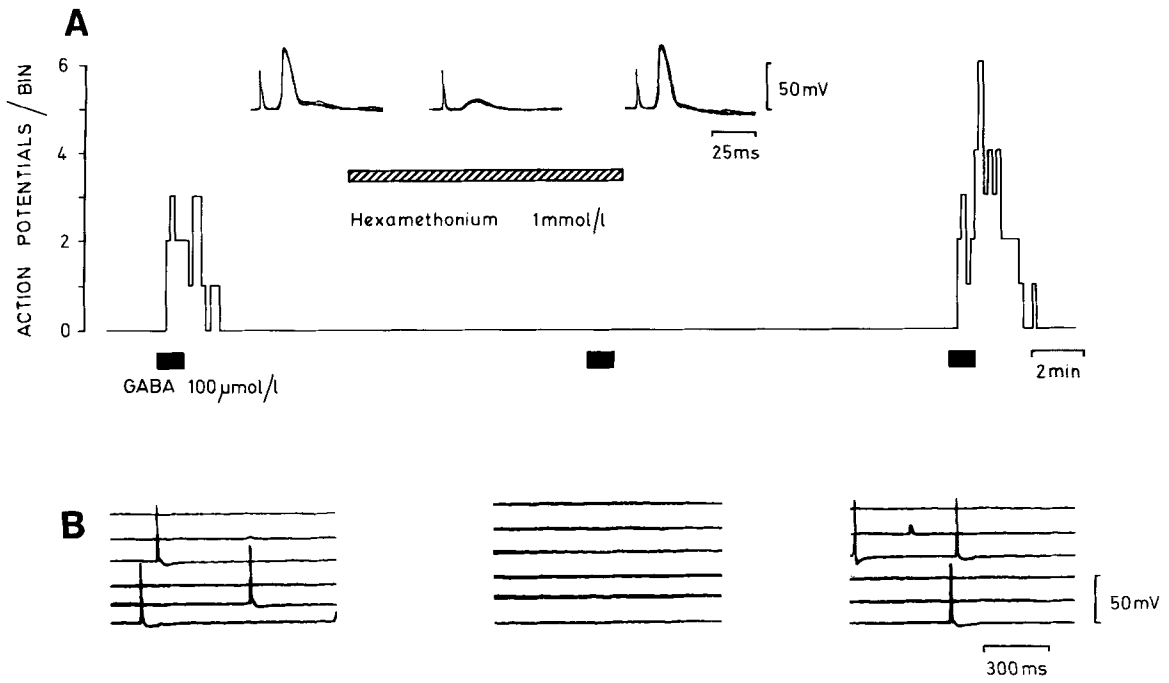

Fig. 4 A and B. Action of hexamethonium on the action potentials induced by GABA in 4-aminopyridine solution. The recording in (**A**), which started 8 min after addition of 4-AP (300 µmol/l), shows the computer plot of the frequency of action potentials (ordinate) recorded from the cell soma. The first application of GABA (solid bar) elicited a number of spikes; this effect was reversibly blocked by hexamethonium (hatched bar). The insets show responses to orthodromic stimulation (3 sweeps superimposed) recorded before, during and after hexamethonium and illustrate that synaptically-evoked action potentials could not be generated in the presence of hexamethonium. Bin width was 10 s. Part (**B**) shows examples of postsynaptic potentials evoked during the 3 GABA applications. Note that in addition to action potentials, there were some subthreshold potentials which were not counted and displayed in part (**A**). Resting potential of this cell was uncertain due to DC drifts; temperature was 30° C

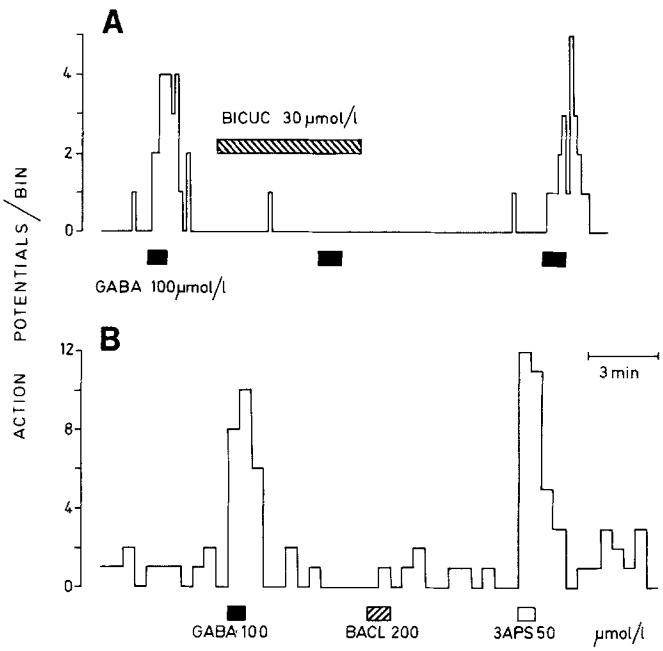

Fig. 5A and B. Actions of bicuculline, GABA and some GABA-mimetics in 4-aminopyridine solution. Computer plots of the occurrance of action potentials recorded intracellularly from two sympathetic neurones in different ganglia (both at 30° C). The ordinates indicate the frequency of action potentials; bin width in (**A**) was 10 s and in (**B**) 30 s. Note different scales in (**A**) and (**B**). The recording in **A** shows that the GABA-induced "bursts" of action potentials were reversibly blocked by application of 30 µmol/l bicuculline methochloride (hatched bar). The recording in (**B**) was obtained from a neurone that was spontaneously active in the presence of 4-aminopyridine (100 µmol/l). The section shown illustrates that brief applications of 100 µmol/l GABA (solid bar) or 50 µmol/l 3-aminopropanesulphonic acid (3-APS; open bar) greatly increased the firing frequency, whereas 200 µmol/l baclofen (BACL; hatched bar) had no such effect

important, subthreshold EPSPs induced by GABA in 4-AP (5 cells).

Effects of Bicuculline and some GABA-Mimetics

Figure 5 illustrates the results of experiments performed to characterize the GABA receptor responsible for the effects described. For simplicity, only the computer counts of postsynaptic action potentials are shown.

Part A was recorded 45 min after addition of 300 µmol/l 4-AP to the superfusate. The first application of GABA clearly increased the frequency of spikes, an effect which was reversibly antagonised by 30 µmol/l bicuculline methochloride (4 cells).

Part B (different ganglion) was recorded 50 min after addition of 100 µmol/l 4-AP and shows that the action of GABA was powerfully replicated by 3-aminopropanesulphonic acid (3-APS) but not by baclofen. From a number of such experiments, it was observed that the action of 100 µmol/l GABA in 4-AP was mimicked by 3-APS (30 – 50 µmol/l; 9 cells tested) and muscimol (50 µmol/l; 3 cells), but not by baclofen (100 – 200 µmol/l; 8 cells), glutamate, glycine or taurine (all 1 mmol/l; 2 cells). The latter 4 compounds did not change the neuronal resting membrane potential.

Discussion

a) 4-Aminopyridine

4-aminopyridine has been reported to have several powerful effects on synaptic function. These include (a) an enhancement of stimulus-induced transmitter release in the cat spinal cord (Lemeignan 1972, 1973; Jankowska et al. 1977), rat neuromuscular junction (Lundh 1978) and squid giant synapse (Llinás et al. 1976). (b) An induction of spontaneous transmitter release at the neuromuscular junction (Bowman et al. 1977; Marshall et al. 1979; however, see also Lundh 1978). (c) An increase in stimulus-induced changes of extracellular K^+ and Ca^{2+} activities in cat cerebellum (Nicholson et al. 1976). (d) A marked increase in the number of vesicle attachment sites in freeze fracture preparations of rat motoneurones (Tokunaga et al. 1979; see also Heuser et al. 1979).

A further effect of 4-AP, observed in squid axons, is the appearance of spontaneous membrane potential oscillations and action potentials (Llinás et al. 1976; Yeh et al. 1976b; Golenhofen and Mandrek 1978). In the present experiments on rat sympathetic neurones, the induction of spontaneous action potentials, as well as subthreshold EPSPs were also observed. Since these effects were not accompanied by a marked change in membrane potential, and were blocked by hexamethonium, they most likely arise as a result of acetylcholine release from presynaptic nerve terminals. The fact that these potentials were suppressed by TTX suggests that the acetylcholine release was action-potential-dependent (see also Yanagisawa et al. 1978). However, it should be noted that electrotonic depolarization of nerve terminals is sufficient to release neurotransmitter (Katz and Miledi 1969; Lundh and Thesleff 1977). Thus it is possible that axonal membrane potential oscillations alone could lead to acetylcholine release. In squid axons, 4-AP-induced oscillations were partially blocked by tetrodotoxin and for this reason it cannot be firmly concluded that the acetylcholine release we observed was dependent on the invasion of action potentials into the synaptic terminals.

In agreement with McAfee and Yarowski (1979), we found small increases in the duration of the repolarizing phase of the action potential, suggesting that the outward K^+ current was partially blocked. In

addition, 100 µmol/l 4-AP clearly enhanced the duration of the B- and C-fibre compound potentials in the isolated vagus nerve. The lack of effect on the vagus A-fibre potential may be explained by an absence of repolarizing potassium currents in these fibres as has been shown for single, rabbit (Chiu et al. 1979) and rat (Brismar 1980) myelinated axons.

b) Effects of GABA in 4-Aminopyridine

The postsynaptic actions of GABA on rat sympathetic neurones in vitro have been described in detail (Adams and Brown 1975; Brown and Constanti 1978). As already discussed, the application of 4-AP resulted mainly in the spontaneous appearance of postsynaptic spikes. Since (1) the frequency of these spikes was clearly increased during the application of GABA, and (2) this effect was blocked by hexamethonium it would seem that this reflects a presynaptic action of GABA.

The precise presynaptic action of GABA in 4-AP is, as yet, unknown. However, assuming that 4-AP induces axonal membrane potential oscillations and/or action potentials (see above), a further GABA-induced axonal depolarization (Brown and Marsh 1978) would probably increase the likelihood of such oscillations exceeding threshold. This interaction would most probably occur on unmyelinated axons, since GABA-receptors are restricted to these fibres (Brown and Marsh 1978).

Regardless of the mechanism of the 4-AP/GABA interaction, the question remains as to whether GABA acts on the nerve terminals, the axonal membrane or both sites. The following points would seem to suggest an axonal site of action. a) A depolarizing action of GABA on sympathetic axons has already been described (Brown and Marsh 1978). b) The excitation of a single nerve terminal is probably not sufficient to release enough acetylcholine to induce a suprathreshold postsynaptic potential (cf Wallis and North 1978). c) GABA does not increase the frequency of miniature EPSPs, as would be expected of a terminal depolarization (Kato et al. 1980). Furthermore, the subthreshold potentials observed in the presence of 4-AP and GABA were also not miniature EPSPs, since they were blocked by TTX.

On the other hand, an axonal interaction might be expected to result in antidromic (hexamethonium-insensitive) action potentials, which we have not yet observed.

The effect of GABA in 4-AP was replicated by 3-APS and muscimol but not by glycine, taurine or glutamate. In addition, since bicuculline methochloride blocked the presynaptic actions of GABA, the receptors responsible for these effects appear similar to those occurring on rat unmyelinated axons (Brown and Marsh 1978) and at postsynaptic sites in the mammalian peripheral and central nervous systems (Nistri and Constanti 1979). Baclofen, suggested to be an agonist at a second type of bicuculline-insensitive, peripheral, presynaptic GABA-receptor (Bowery et al. 1979), was ineffective in our experiments.

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