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INHIBITORY ACTION OF γ-AMINOBUTYRIC ACID ON THE EXCITATORY BUT NOT INHIBITORY INNERVATION OF THE RAT ANOCOCCYGEUS MUSCLE

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1 The effects of γ-aminobutyric acid (GABA), ethylenediamine, 3-aminopropane sulphonic acid and (±)-baclofen have been examined on the responses to stimulation of the adrenergic excitatory and non-adrenergic non-cholinergic inhibitory innervation of the rat anococcygeus muscle *in vitro*.

2 GABA produced a dose-related depression of the contractile responses to field stimulation. Ethylenediamine and baclofen also depressed the contractile responses, though they were less potent than GABA. 3-Aminopropane sulphonic acid was almost inactive. The inhibitory action of GABA was not modified by phentolamine, propranolol or bicuculline methylbromide.

3 GABA did not affect the contractile responses of the anococcygeus muscle to noradrenaline, phenylephrine or carbachol in untreated muscles or those treated with 6-hydroxydopamine *in vitro*.

4 In preparations in which tone was raised by continuous perfusion with carbachol in the presence of phentolamine, field stimulation relaxed the muscle. GABA had no effect on this inhibitory response, and did not itself produce any relaxation.

5 It is concluded that GABA exerts a presynaptic inhibitory action on the excitatory adrenergic but not on the inhibitory innervation of the anococcygeus muscle, and that the GABA receptor involved exhibits properties of the previously described $GABA_B$ site.

Introduction

γ-Aminobutyric acid (GABA) is widely recognized as an important inhibitory neurotransmitter in the mammalian central nervous system (Krnjević, 1974; Curtis & Johnston, 1974). GABA receptors also exist on components of the peripheral nervous system, including sympathetic ganglion cells (Bowery & Brown, 1974) and dorsal root ganglion cells (Desarmenien, Santangelo, Linck, Headley & Feltz, 1981) axons (Brown & Marsh, 1978) and adrenergic nerve terminals of the mouse vas deferens and rat atria (Bowery, Doble, Hill, Hudson, Shaw, Turnbull & Warrington, 1981). The latter receptors appear to inhibit neurotransmitter release. GABA receptors in the central nervous system are pharmacologically different from those causing inhibition of transmitter release in that the latter cannot be blocked by bicuculline. This distinction has given rise to the nomenclature of $GABA_A$ and $GABA_B$ sites respectively for these two species of receptor (Hill & Bowery, 1981).

The present study was undertaken to determine whether GABA and ethylenediamine, which has been found to have GABA-mimetic properties in other systems (Perkins, Bowery, Hill & Stone, 1981; Bokisch, Bold, Perkins, Roberts, Stone & Walker, 1982; Lloyd, Perkins & Stone, 1982; Blaxter & Cottrell, 1982; Morgan & Stone, 1982) had any action on the rat anococcygeus muscle, a preparation that possesses both a dense adrenergic excitatory innervation as well as a non-adrenergic inhibitory innervation (Gillespie, 1972).

Methods

Male Wistar rats, 200–350 g were killed and the anococcygeus muscles removed according to the method of Gillespie (1972). Preparations were suspended in a 10 ml organ bath under approximately 1–1.5 g tension and perfused at a rate of 3 ml/min with Krebs-Henseleit solution (composition (mM) NaCl 118, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1), gassed with 95% O_2–5% CO_2, at a temperature of 37 C. Preparations were left for 45–60 min before experiments were begun. Induced responses are displayed superimposed upon the 1–1.5 g resting tension.

Field stimulation was carried out via a pair of parallel silver or platinum wire electrodes connected to a Digitimer and DS2 stimulus isolator. Trains of pulses at a frequency of 10 Hz, duration 1 ms and of supramaximal voltage (50 V) were delivered for three s, every 30 s. Contractions were measured using an isometric transducer and displayed on a Devices

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four channel penrecorder. Drugs were normally administered into the bath in a volume of 0.1 ml, 15 s before the arrival of a train of stimuli. Where this volume was exceeded an equivalent volume of Krebs solution was also given as a control. All drug concentrations quoted are final bath concentrations.

When an interaction between two drugs was being studied, these were administered either 30 s apart or perfused for several minutes before the administration of GABA. Dose-response curves for noradrenaline and carbachol were constructed in the absence of field stimulation.

In a number of experiments the tone of the anococcygeus muscle, normally very low, was raised by perfusion with carbachol in the presence of phentolamine. Under these circumstances the same parameters of stimulation used above induced a non-adrenergic inhibition of the muscle, as described previously (Gillespie, 1972). The carbachol-induced tone tended to decline over a period of 60–90 min with a corresponding decrease in the size of evoked inhibition. Experiments were therefore performed during the first 30–60 min of carbachol perfusion. Both the contractile and inhibitory responses to field stimulation could be prevented by tetrodotoxin (5×10^{-7} M).

The following drugs were used: 3-aminopropanesulphonic acid; atropine sulphate; carbamylcholine chloride (carbachol); ethylenediamine dihydrochloride; γ-aminobutyric acid (GABA); 6-hydroxydopamine hydrobromide; (−)-noradrenaline hydrochloride; (−)-phenylephrine hydrochloride; (±)-propranolol hydrochloride; tetrodotoxin (all from Sigma Chemical Co); (±)-baclofen hydrochloride (Dr N.G. Bowery); bicuculline methylbromide (Dr J.F. Collins); phentolamine methanesulphonate (a gift from Ciba Labs).

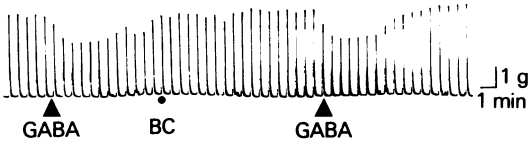

Figure 1 Contractile responses of the rat anococcygeus muscle to field stimulation. At each arrow γ-aminobutyric acid (GABA) was added to the bath to a concentration of 10^{-5} M. At the dot bicuculline methylbromide (BC, 20 μM final concentration) was added to the perfusing solution and remained present thereafter. Bicuculline did not influence the inhibitory action of GABA.

Results

Effect of γ-aminobutyric acid and analogues

GABA (Figure 1) caused a dose-related inhibition of the contractions caused by field stimulation, reaching a peak effect of abour 44% inhibition at approximately 3×10^{-5} M (Figure 2a). The putative GABA-mimetics ethylenediamine (Perkins *et al.*, 1981) 3-aminopropane sulphonic acid and baclofen were all less active than GABA (Figures 2b–d).

Effect of potential antagonists on γ-aminobutyric acid inhibition

Since phentolamine (10^{-6} M) completely abolished the excitatory responses to field stimulation, its effect on the GABA-induced inhibition of these responses could not be examined. Propranolol (10^{-6} M) or atropine (10^{-6} M) did not alter the effect of GABA.

Bicuculline, an antagonist of the actions of GABA at the $GABA_A$ site did not block the GABA-induced reduction of developed tension when perfused at a concentration of 20 μM for 10 min before the injection of GABA (Figure 1).

Effect of γ-aminobutyric acid on the response to phenylephrine, noradrenaline and carbachol

Phenylephrine (about 3×10^{-7} M) exerts relatively specific postsynaptic effects on the anococcygeus muscle (Gillespie, 1980) but to exclude any presynaptic actions, pretreatment with 6-hydroxydopamine (10^{-3} M for 3 h) in the organ bath was carried out. This procedure abolished all contractile responses to field stimulation. Phenylephrine (3×10^{-7} M) then led to well sustained contractions that were unaltered by GABA (up to 10^{-4} M) given 30 s previously. Likewise, in untreated preparations, dose-response curves for noradrenaline and carbachol were unaffected by the presence of GABA (10^{-5} M) (Figure 3).

Effect of γ-aminobutyric acid on the inhibitory response to field stimulation

When tone was increased by carbachol (5×10^{-5} M) in the presence of phentolamine (5×10^{-5} M) field stimulation relaxed the muscle (Figure 4). GABA did not alter the size of this relaxation, nor did it produce any change in tone (Figure 4).

Discussion

It has been proposed that the ability of GABA to hyperpolarize or depolarize nerve cells and its ability

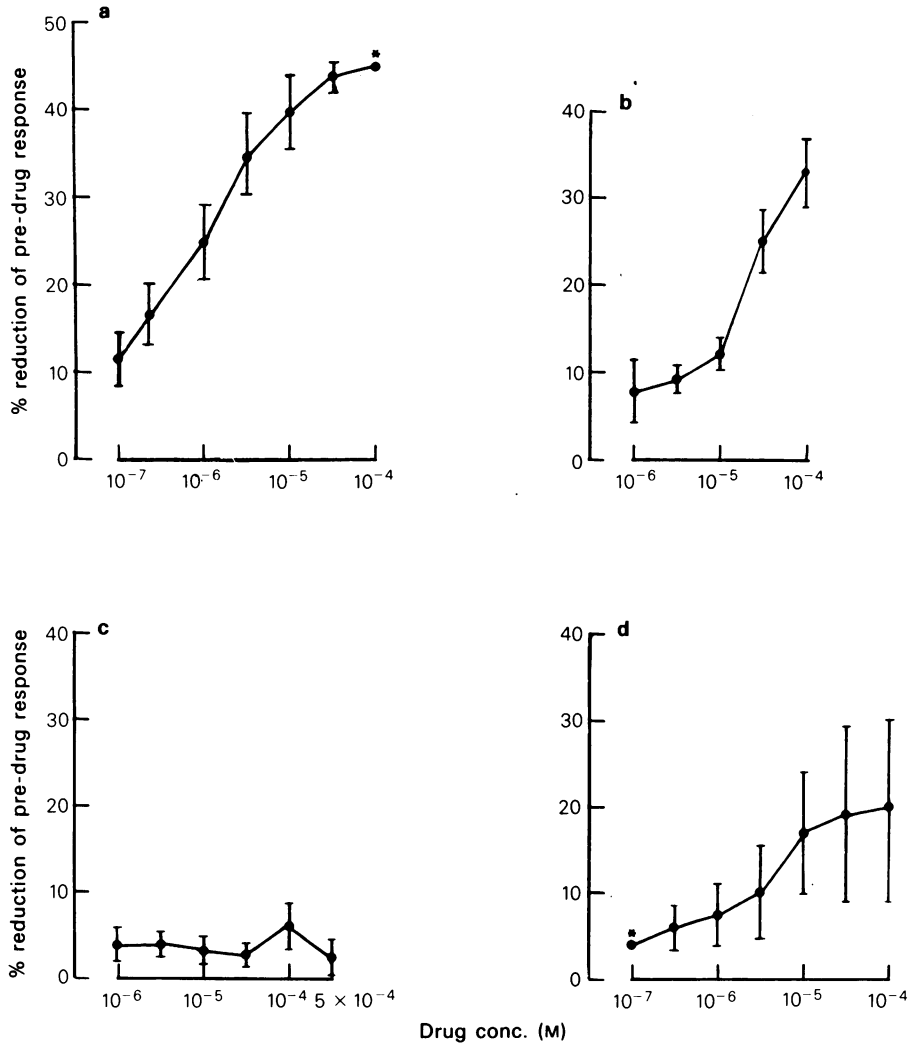

Figure 2 The effects of (a) γ-aminobutyric acid (GABA), (b) ethylenediamine, (c) 3-aminopropane-sulphonic acid and (d) baclofen on responses of the rat anococcygeus muscle to field stimulation (1 ms pulses, 10 Hz, 50 V). In (a)–(c) each point is the mean of at least four observations with vertical lines showing s.e.mean except at the asterisk (*) where $n = 1$. In (d) each point is the mean of three observations except at the asterisk (*) where $n = 2$. Ordinate scales: percentage reduction of control contraction size. Abscissa scales: drug concentration (M).

to reduce transmitter release at nerve terminals are mediated by different receptors since only the former changes can be inhibited by bicuculline (Brown & Higgins, 1979; Bowery, Hill, Hudson, Doble, Middlemiss & Turnbull, 1980; Bowery *et al.*, 1981; Kato & Kuba, 1980). The former site has been referred to as $GABA_A$ and the latter as $GABA_B$ (Hill & Bowery, 1981).

The present results show that, in the rat anococcygeus muscle GABA produced a dose-dependent inhibition of the excitatory mechanical response to field stimulation but not to exogenous noradrenaline, phenylephrine or carbachol, which act postsynaptically. This indicates that GABA is acting presynaptically. The ineffectiveness of 3-aminopropane sulphonic acid, an agonist specific for the $GABA_A$ site, and the inability of bicuculline to antagonize the effects of GABA suggest that GABA is not acting via a $GABA_A$ site. However, baclofen which is a GABA-mimetic at the bicuculline insensitive site responsible for regulating transmitter release at nerve endings (Hill & Bowery, 1981) did reduce neurally-evoked contractions in the anococcygeus though it was less active than GABA.

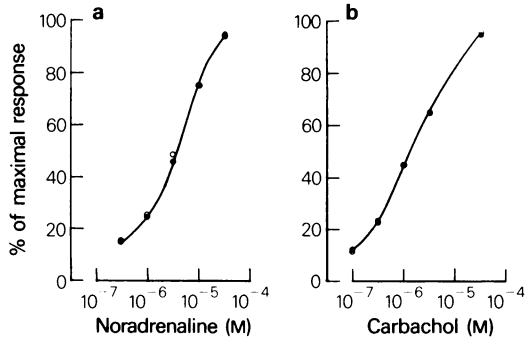

Figure 3 Contractile effects of (a) noradrenaline and (b) carbachol on the rat anococcygeus muscle in the presence (○) and absence (●) of γ-aminobutyric acid 10^{-5} M. Each point is the mean of at least 3 observations, each point having a s.e.mean of less than ±4%. Ordinate scale: percentage of maximal obtainable response. Abscissa scale: concentration (M).

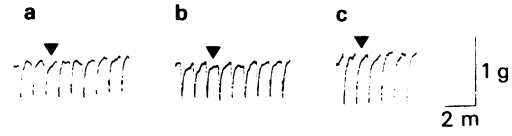

Figure 4 Inhibitory responses of the rat anococcygeus muscle to field stimulation. Tone was raised by continuous perfusion with carbachol (5×10^{-5} M) in the presence of phentolamine (5×10^{-5} M) and γ-aminobutyric acid (GABA) added to a final concentration of 10^{-5} M at the arrows. Stimulation was effected by 1 ms pulses of 50 V amplitude: (a) 5 Hz for 5 s; (b) 10 Hz for 3 s, (c) 20 Hz for 3 s. All three records are from the same preparation.

These results suggest that the GABA site on the anococcygeus muscle is analogous to the previously described $GABA_B$ receptor site on nerve terminals elsewhere though the weaker action of baclofen may also indicate tissue variations in the pharmacological profile of these receptors. This may not necessarily indicate sub-types of the $GABA_B$ receptor; differences in receptor responses may arise from changes in the membrane environment rather than from differences in molecular characteristics (Stone, 1974).

The ability of ethylenediamine to activate a receptor of the $GABA_B$ type is entirely consistent with the binding studies of Bowery, Hill, Hudson, Perkins & Stone (1982) who demonstrated the ability of ethylenediamine to displace the $GABA_B$ ligand baclofen from rat brain membranes, and clearly implies that ethylenediamine can effectively mimic the actions of GABA on both $GABA_A$ (Perkins *et al.*, 1981; Perkins & Stone, 1982) and $GABA_B$ receptors. These rssults thus support our contention that in view of the simplicity of its structure ($NH_2(CH_2)_2.NH_2$) ethylenediamine may prove a valuable tool in probing the mechanism of activation of GABA receptors at the molecular level.

As well as possessing an excitatory adrenergic innervation the rat anococcygeus muscle receives an anatomically distinct non-adrenergic, non-cholinergic inhibitory innervation (Gillespie, 1972; Gillespie & McGrath, 1973) the action of which is revealed when tone is raised. Although a wide variety of transmitter candidates have been proposed for this inhibitory system, none has thus far been proved to be the true transmitter. As GABA had no depressant action on the contracted anococcygeus it is most unlikely to function as the inhibitory transmitter (see also Gibson & Gillespie, 1973).

Furthermore, GABA does not inhibit responses of the muscle to stimulation of the inhibitory nerves. The preferential activity of GABA towards excitatory synaptic input has also been observed in electrophysiological studies (Lanthorn & Cotman, 1981; Davies, 1981) and implies that the mechanism of this action of GABA involves a receptor-mediated process on some nerve terminals (rather than a non-specific depression of transmitter release), perhaps involving a reduction of the influx of calcium that accompanies an action potential (Bowery *et al.*, 1981).

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