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PRESYNAPTIC γ-AMINOBUTYRIC ACID RECEPTORS IN THE RAT ANOCOCCYGEUS MUSCLE AND THEIR ANTAGONISM BY 5-AMINOVALERIC ACID

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1 The effects of γ-aminobutyric acid (GABA) and related drugs on the isolated anococcygeus muscle of the rat were determined.

2 GABA caused a dose-related inhibition of the electrically-evoked twitch response.

3 The maximum response to GABA was a 56.8% depression of twitch response, with an EC_{50} of 0.68 μM.

4 (±)-Baclofen mimicked the effect of GABA (EC_{50} 0.9 μM). (+)-Baclofen was more than 100 times less active than (−)-baclofen.

5 The response to GABA was unaffected by picrotoxin or bicuculline but was antagonized by 5-aminovaleric acid (0.5 mM).

6 Our results suggest that $GABA_B$ receptors are present on motor nerve terminals in the rat anococcygeus muscle and that 5-aminovaleric acid is an antagonist of these receptors.

Introduction

The role of γ-aminobutyric acid (GABA) as an inhibitory transmitter in the CNS is now well-established. Although in mammals there is no known functional role for GABA outside the central nervous system, GABA receptors occur in the periphery. For example, Bowery & Brown (1974) showed that GABA and some related compounds had a depolarizing effect on the rat superior cervical ganglion. The receptors mediating this response to GABA appeared to resemble conventional GABA receptors previously investigated in the central nervous system, since the effect of GABA was mimicked by 3-aminopropanesulphonic acid and blocked by the convulsant alkaloid, bicuculline (Curtis, Phillis & Watkins, 1961; De Groat, 1970; 1972; Bowery & Brown, 1974; Johnston, 1978).

Recently, Bowery, Doble, Hill, Hudson, Shaw, Turnbull & Warrington (1981) reported the existence of a novel type of GABA receptor, termed $GABA_B$ receptor, on sympathetic nerve terminals in the mouse vas deferens and rat atria. These receptors were characterized by studying the effects of a range of agonists on the stimulus-evoked release of noradrenaline and on mechanical responses to nerve stimulation. $GABA_B$ receptors appear to differ from conventional GABA receptors in that they are not blocked by bicuculline. Furthermore, baclofen is a potent agonist on $GABA_B$ receptors whereas 3-aminopropanesulphonic acid, which is at least equipotent with GABA at bicuculline-sensitive GABA sites, is inactive on these receptors (Bowery, Doble, Hill, Hudson, Shaw & Turnbull, 1979; Bowery *et al.*, 1981).

In the present investigation we obtained evidence for the existence of $GABA_B$ receptors on sympathetic nerve terminals in the rat anococcygeus muscle. We have also shown that 5-aminovaleric acid is an antagonist of the GABA responses in this preparation.

Methods

Male Wistar rats (200–300 g) were killed by cervical dislocation. The two anococcygeus muscles were dissected out as described by Gillespie (1972) and mounted in a 10 ml organ bath containing Krebs-Henseleit solution of the following composition: (mM): NaCl 118.4, KCl 4.74, $CaCl_2$ 2, KH_2PO_4 1.18, $MgSO_4.7H_20$ 1.19, $NaHCO_3$ 25 and glucose 11.1, at 37°C. The solution was gassed continuously with a mixture of 95% O_2 and 5% CO_2. A resting tension of about 0.5 g was applied to the muscle. Field stimulation was applied to the muscle by platinum ring electrodes. Muscle responses were measured with a UFI dynamometer, strain gauge and recorded on a pen recorder. In most experiments, trains of supramaximal pulses (10 Hz) were applied every 10 s for

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0.5 s; however, the effect of varying the frequency of stimulation (in the range of 2–15 Hz) was also investigated. The individual pulse duration was 0.5 ms.

Drugs

The following drugs were dissolved in 0.1 mM ascorbic acid solution: GABA, taurine, (±)-allylglycine, (±)-2,6-diamino-heptanedioic acid, apomorphine, (±)-2-aminoadipic acid (Sigma); (±)-baclofen, (−)-baclofen, (+)-baclofen, guanethidine (Ciba); β-*m*-chlorophenyl-γ-aminobutyric acid (β-m-CPG), β-*p*-fluorophenyl-γ-aminobutyric acid (β-*p*-FPG), β-*p*-isopropylphenyl-γ-aminobutyric acid (β-p-IPG), β-2-naphthyl-γ-aminobutyric acid (β-NG); β-alanine, glycine, 2,4,-diaminobutyric acid diHCl (BDH); sodium-3-hydroxybutyrate, 3-aminopropylphosphonic acid (Calbiochem); DL-4-amino-3-hydroxybutyric acid (Research Chemicals); 3-amino-isobutyric acid, imidazole-4-acetic acid (Koch Light), 5-amino-valeric acid, DL-3-amino-phenylpropionic acid, D-(-)-2-aminobutyric acid (Aldrich); N-methyl GABA (ICN); (±)-2-amino-5-phosphonovaleric acid, 2-amino-3-phosphonopropionic acid, 2-amino-6-phosphonohexanoic acid; bicuculline methiodide, picrotoxin, muscimol, 2-phenyl-3-aminobutyric acid, 3-aminopropyl sulphonic acid; clonidine hydrochloride (Boehringer). Chlordiazepoxide and diazepam (Roche) were dissolved in a minimum of dimethylformamide and made up to volume with distilled water. Doses refer to salts.

Results

Field stimulation of the rat isolated anococcygeus muscle, using standard stimulus parameters (see Methods) produced rapid contractions. Twitches of constant magnitude were usually obtained after 10–20 min of stimulation, with a peak tension of between 0.8 and 1.8 g.

GABA caused a concentration-dependent depression of muscle contractions (Figure 1). Cumulative, concentration-effect curves to GABA (0.1–100 μM) and GABA analogues were obtained; no desensitization was observed under these conditions. At maximally effective concentrations, GABA caused a 56.8 ± 3.4 (s.e.mean, $n = 13$)% depression of electrically-evoked contraction. The EC_{50} value (concentration causing 50% of maximum response) for GABA was 0.62 μM. GABA in concentrations of up to 0.1 mM did not affect muscle tone when this was raised by guanethidine (0.03 mM) or potassium chloride (40 mM).

Concentration-effect curves to GABA were obtained at different frequencies of stimulation (not shown). When the muscle was stimulated continuously at 2 Hz (pulse duration 0.5 ms, supramaximal voltage), the EC_{50} for GABA was reduced to 0.3 μM. The inhibition of muscle twitches increased from 38% to 62% as the frequency of stimulation was reduced from 15 to 5 Hz.

Effects of agonists

A series of GABA analogues was examined for their ability to inhibit electrically-evoked contractions of the anococcygeus muscle. The results obtained with some of the compounds are summarized in Table 1. The effects of GABA on this preparation were mimicked by (−)-baclofen (Figure 2), which had an EC_{50} of 0.6 μM. (+)-Baclofen was more than 100 times less active than (−)-baclofen (Table 1). The GABA receptor agonist, muscimol, was also active, although it had low activity compared to that of GABA (Table 1). All of the compounds listed in Table 1 produced a maximum response which was similar to that produced by GABA. A number of other GABA analogues were tested. Thus 3-aminopropanesulphonic acid, β-m-CPG, β-p-IPG, β-alanine, glycine, 3-hydroxybutyric acid, 2-amino-3-phenylbutyric acid, 3-aminopropylphosphonic acid, 2-aminobutyric acid, 3-aminobutyric acid, 5-aminovaleric acid, 3-amino-3-phenylpropionic acid, N-methyl GABA, 2-amino-4-phosphonobutyric acid, 4-amino-3-hydroxybutyric acid, imidazole-4-acetic acid, allylglycine, 2,6-diamino-heptanedioic acid, 2-aminoadipic acid, 5-aminopentanoic acid, 2,4-diaminobutyric acid, 2-amino-5-phosphonovaleric acid, 2-amino-3-phosphonopropionic acid, 2-amino-phosphonohexanoic acid, were all inactive at concentrations up to 10 μM.

The benzodiazepines, diazepam and chlordiazepoxide, each at 10 μM, had no effect on the electrically-evoked contractions of the anococcygeus muscle, nor did they enhance or reduce the response to GABA.

Effects of antagonists

The convulsant alkaloids, bicuculline methiodide and picrotoxin which block inhibitory electrophysiological responses to GABA in the brain were completely inactive in concentrations of up to 100 μM, as GABA antagonists on the anococcygeus.

The GABA homologue, 5-aminovaleric acid, had only slight agonist activity in this preparation. At maximally effective concentrations, 5-aminovaleric acid caused only a 7.9 ± 1.6% ($n = 14$) inhibition of electrically-induced contractions. However, this compound blocked responses to GABA, although it was rather weak in this respect. Figure 3 shows that in the presence of 5-aminovaleric acid (0.5 mM) the dose-response curve to GABA was shifted to the

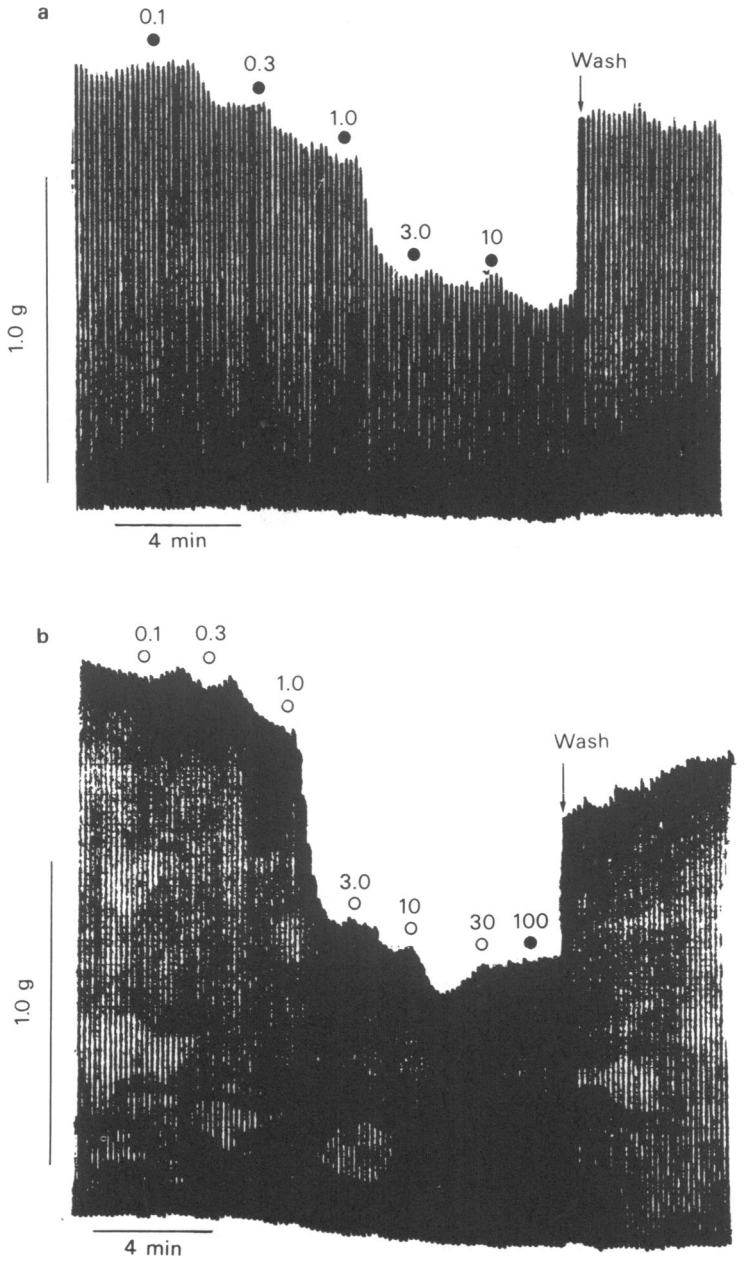

Figure 1 Inhibition by (a) GABA (●) and (b) (−)-baclofen (○) of the twitch response to electrical stimulation (10 Hz every 10 s, 0.5 ms pulse duration and with supramaximal voltage). The final drug concentrations shown (dots) are micromolar. Note the inability of GABA (100 μM) to increase further the maximal twitch inhibition produced by 10 μM (−)-baclofen.

Table 1 Potency of GABA analogues as inhibitors of electrically-evoked contractions of rat anococcygeus muscle

GABA analogues	EC_{50} (μM)	*Relative potency*	n
GABA	0.68 ± 0.05	1	5
(±)-Baclofen	0.91 ± 0.08	0.747	8
(−)-Baclofen	0.57 ± 0.04	1.19	6
β-p-FPG	7.6 ± 1.2	0.089	7
β-o-CPG	18.7 ± 3.2	0.036	6
Muscimol	55.1 ± 8	0.012	6
β-OH-GABA	33.6 ± 10.2	0.020	5
β-phenyl GABA	58 ± 7.9	0.012	6
(+)-Baclofen	> 50	< 0.010	4

Cumulative log dose-response curves were constructed for each of the agonist drugs (0.1–100 μM) and the EC_{50} values determined.
β-p-FPG = β-*p*-fluorophenyl-γ-aminobutyric acid; β-o-CPG = β-*o*-chlorophenyl-γ-aminobutyric acid.

right, indicating a competitive antagonism. In concentrations from 0.1 to 1 mM, 5-aminovaleric acid also blocked inhibitory responses to (±)-baclofen. The selectivity of the compound for prejunctional GABA receptors was indicated by the finding that 5-aminovaleric acid did not antagonize the depressant effects of the presynaptic α-agonist, clonidine (0.001–0.01 μM) or of the presynaptic dopamine agonist, apomorphine (0.01–1 μM).

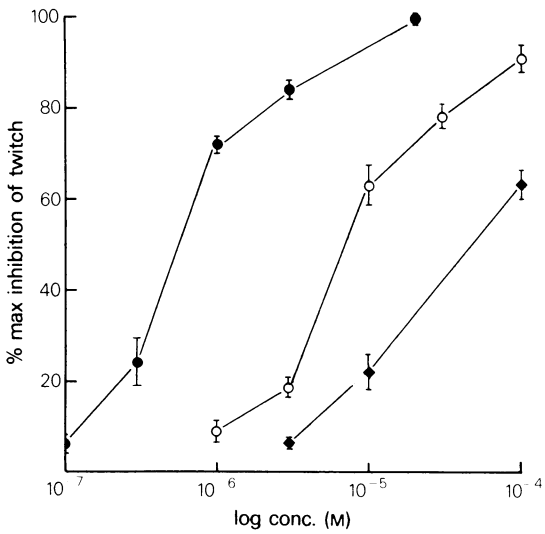

Figure 2 Log dose-response curves for (−)-baclofen (●), β-*p*-fluorophenyl-γ-aminobutyric acid (○) and muscimol (◆) on twitch responses of the rat anococcygeus muscle. Inhibitory effect of agonists is expressed as a percentage of the maximal inhibition (100%) produced by 0.1 mM GABA. Each point is mean of 6–7 determinations; vertical bars show s.e.mean.

Discussion

The rat isolated anococcygeous muscle, first described by Gillespie (1972) is highly suitable for studying various aspects of noradrenergic transmission. Using the stimulus parameters described in the present paper, field stimulation of the muscle produces contractions that seem to be due entirely to the release of noradrenaline from sympathetic nerves (Gillespie, 1972; Leighton, Butz & Parmeter, 1979). A range of presynaptic receptors, activation of which modulates the release of noradrenaline, is known to occur on sympathetic nerve terminals (Langer, 1977). Recently, Bowery & Hudson (1979) have demonstrated the presence of presynaptic GABA receptors on sympathetic nerve terminals in the mouse vas deferens and rat atria. These receptors appear to differ from the GABA receptors in the

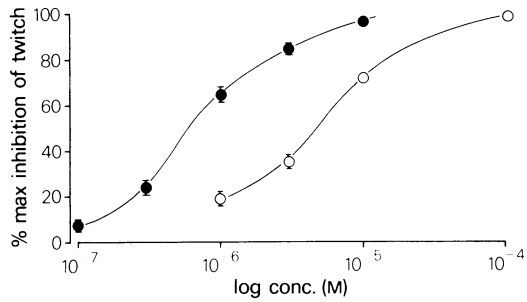

Figure 3 Inhibition by GABA alone (●) and in the presence of 0.5 mM 5-aminovaleric acid (○) (for 5 min in organ bath) on twitch responses of anococcygeus muscle to electrical stimulation (10 Hz every 10 s, 0.5 ms pulse duration and with supramaximal voltage). Each point represents mean of 6–7 determinations; vertical bars show s.e.mean.

CNS which mediated inhibition via an increase in chloride permeability. GABA receptors on sympathetic terminals have been designated $GABA_B$ receptors and conventional receptors as $GABA_A$. Bowery *et al.* (1979) have shown that the $GABA_B$ receptors on sympathetic terminals are markedly different from $GABA_A$ sites. Baclofen is a potent agonist at $GABA_B$ receptors with the (−)-enantiomer being more than 100 times more active than (+)-baclofen. 3-Aminopropanesulphonic acid and a number of other agonists which have activity at $GABA_A$ sites are inactive on $GABA_B$ receptors. Furthermore, picrotoxin and bicuculline are ineffective as antagonists at $GABA_B$ sites.

In the present investigation, we have shown that GABA and baclofen, but not 3-aminopropanesulphonic acid, are potent depressants of the electrically-evoked contractions of the rat anococcygeus muscle. Since electrically-evoked contractions of this preparation are due almost entirely to the release of noradrenaline from adrenergic motor nerves (Leighton, Butz & Parmeter, 1979), it seems likely that the responses measured are due to the agonists causing a depression of the stimulus-evoked release of noradrenaline. Support for this comes from our finding that GABA and baclofen do not reduce muscle tone in the anococcygeus muscle when this has been raised by guanethidine or potassium. The efficacy of GABA in inhibiting the electrically-evoked contractions of the muscle, was greater at lower frequencies of stimulation. A similar pattern is observed for the α_2-presynaptic adrenoceptor (Leighton *et al.*, 1979). The structure-activity profile for the depression of contraction of the anococcygeus muscle agrees closely with the studies of Bowery *et al.* (1979; 1981) on mouse vas deferens, rat atria and also on electrically-stimulated guinea-pig ileum, i.e., in contrast to classic GABA receptors (the $GABA_A$ site) these receptors were activated preferentially by GABA and baclofen, while other agonists such as 3-aminopropanesulphonic acid and muscimol were inactive or only weakly so. Picrotoxin and bicuculline were ineffective as antagonists. Thus, we conclude from our studies that $GABA_B$ receptors occur on motor nerve terminals in the anococcygeus muscle. Our results showing that baclofen depresses electrically-evoked contractions of the anococcygeus muscle are in contrast with those of Ault & Evans (1981) who failed to demonstrate an effect of baclofen in this preparation.

More recently Bowery *et al.*, have extended their studies to the central nervous system. Noradrenaline release from slices of rat brain was inhibited by (−)-baclofen (Bowery, Hill, Hudson, Doble, Middlemiss, Shaw & Turnbull, 1980) and tritiated baclofen was bound specifically to rat brain synaptic membranes (Hill & Bowery, 1981) thus providing evidence for the presence of $GABA_B$ receptors in the central nervous system. The physiological and pharmacological significance of $GABA_B$ receptors is at present unknown. Progress in this direction could be expected if a selective antagonist of these $GABA_B$ sites could be developed. Work described in the present paper shows that 5-aminovaleric acid is an antagonist of $GABA_B$ receptors. The drug is not particularly potent with a pA_2 of approximately 4.1 and it does have a slight agonist activity. Previously Bowery & Brown (1974) have shown that 5-aminovaleric acid is an agonist at $GABA_A$ receptors in sympathetic ganglia. It is hoped that the study of some structural analogues of 5-aminovaleric acid might lead to the development of more potent and more selective antagonists of GABA receptors.

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