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3-Aminopropylphosphinic acid—a potent, selective $GABA_B$ receptor agonist in the guinea-pig ileum and rat anococcygeus muscle

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1 3-Aminopropylphosphinic acid, a γ-aminobutyric acid (GABA) analogue, was tested for activity on guinea-pig isolated ileum and rat isolated anococcygeus muscle preparations. The effects of 3-aminopropylphosphinic acid were compared with those of GABA and baclofen.

2 In the electrically stimulated ileum, 3-aminopropylphosphinic acid, like GABA and baclofen, caused a concentration-dependent inhibition of the cholinergic twitch contraction, the IC_{50} value being $1.84 \pm 0.23\,\mu M$ ($n = 12$). Unlike GABA, but like baclofen, 3-aminopropylphosphinic acid did not produce an initial contraction.

3 The inhibitory effects of 3-aminopropylphosphinic acid and baclofen in the guinea-pig ileum were not significantly antagonized by bicuculline ($10\,\mu M$), phentolamine plus propranolol (both $1\,\mu M$), yohimbine ($1\,\mu M$), naloxone ($1\,\mu M$), impromidine ($1\,\mu M$) or 8-phenyltheophylline ($10\,\mu M$). The inhibitory effects of 3-aminopropylphosphinic acid, but not of baclofen, were however antagonized by phaclofen ($500\,\mu M$). In addition the effects of 3-aminopropylphosphinic acid were abolished by baclofen desensitization in the guinea-pig ileum.

4 3-Aminopropylphosphinic acid, GABA and baclofen reduced the twitch contraction evoked by electrical field stimulation in the rat anococcygeus muscle. The IC_{50} for 3-aminopropylphosphinic acid inhibition of the anococcygeus contraction was $0.89 \pm 0.15\,\mu M$ ($n = 8$).

5 It is concluded that 3-aminopropylphosphinic acid is a potent, selective $GABA_B$ agonist, being seven times more potent than baclofen in the guinea-pig ileum and five times more potent than baclofen in the rat anococcygues muscle preparations.

Introduction

In the guinea-pig ileum, γ-aminobutyric acid (GABA) causes both contraction and inhibition of the electrically-induced cholinergic contractions (Krantis *et al.*, 1980). In the presence of bicuculline, a $GABA_A$ receptor antagonist, GABA causes inhibition of the ileal twitch contraction by an action on presynaptic $GABA_B$ receptors (Bowery *et al.*, 1981; Kaplita *et al.*, 1982; Ong & Kerr, 1983). The β-*p*-chlorophenyl analogue of GABA, baclofen, a selective $GABA_B$ agonist, mimics this effect but is no more potent than GABA itself (Bowery *et al.*, 1981; Giotti *et al.*, 1983). Several compounds, have been reported to show $GABA_B$ receptor agonist activity (Bowery, 1982), but to date, none have shown an increase in potency over baclofen.

In binding studies on rat brain membranes, a new compound, 3-aminopropylphosphinic acid (Figure 1) has been claimed to have an affinity for the $GABA_B$ receptor some 20 times greater than the affinity of baclofen (Figure 1) (Dingwall *et al.*, 1987a, b). We have investigated the pharmacological activity of 3-aminopropylphosphinic acid in the guinea-pig ileum and rat anococcygeus muscle *in vitro*.

Methods

Guinea-pig ileum

Male guinea-pigs (300–450 g) were killed by a blow to the head and bled out. Segments of ileum of approximately 3 cm in length were removed from an

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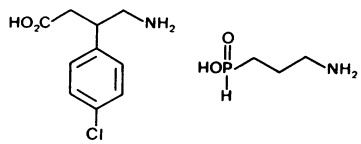

Figure 1 Chemical structures of (left) baclofen (±)-4-amino-3-(4-chlorophenyl) butanoic acid and (right) 3-aminopropylphosphinic acid. In this study racemic baclofen was used.

area 10–15 cm proximal to the ileo-caecal junction. Preparations were immediately placed in a modified Krebs solution (Bülbring, 1953) which was continually bubbled with 95% O_2 and 5% CO_2. Segments were freed of their mesenteric attachment and suspended in 10 ml organ baths containing Krebs solution under an isometric tension of 1 g. Isometric contractions to transmural stimulation (Paton, 1954) were recorded with strain gauge transducers (Biomed Dynamometer UF1) and displayed on an Ormed Multitrace pen recorder. Electrical stimulation of preparations was achieved by passing rectangular pulses (duration 0.5 ms; frequency 0.1 Hz; supramaximal voltage (25–35 V)), from a Grass SD11 stimulator via platinum electrodes. Preparations were allowed to equilibrate for 1 h prior to addition of compounds to the organ bath.

Rat anococcygeus

Male Wistar rats (200–300 g) were killed by a blow to the head and bled out and their anococcygeus muscles removed as previously described (Gillespie, 1972). The muscles were mounted in organ baths (10 ml) containing modified Krebs solution which was continually gassed with 95% O_2 and 5% CO_2. A resting tension of 0.5 g was applied and the preparations were field stimulated from Grass SD11 stimulators via platinum ring electrodes with the following stimulus parameters: pulse duration 1 ms; frequency 10 Hz; for 1 s. Isometric muscle responses were measured with a strain gauge transducer (Biomed UF1 dynamometer) and displayed on an Ormed Multitrace pen recorder.

Typical tension responses generated in either preparation as a result of electrical stimulation were between 2 and 4 g force. Preparations generating less tension were rejected.

In both preparations, sequential agonist concentration-response curves were constructed, allowing 30 min between additions of agonist to minimize tachyphylaxis. When antagonists were used, concentration-response curves to 3-aminopropylphosphinic acid in the presence of the antagonist were constructed after an initial equilibration period of 10 min. $GABA_B$ receptor desensitization was achieved by 3 additions of baclofen (100 μM) without washout from the tissue.

Drugs used

The following drugs were used: γ-amino-n-butyric acid (Sigma), (±)-baclofen (Research Biochemicals Inc.), (±)-propranolol (ICI), phentolamine mesylate (Ciba-Geigy), yohimbine hydrochloride (Sigma), naloxone hydrochloride (Sigma), 8-phenyltheophylline (Aldrich Chem. Co), impromidine oxylate (SK&F), bicuculline methiodide (Sigma), phaclofen (Tocris Neuramin) and 3-aminopropylphosphinic acid (prepared by the method of Dingwall *et al.*, 1987a, b). With the exception of 8-phenyltheophylline, all compounds were dissolved in distilled water, subsequent dilutions being made in distilled water and compounds were added to the organ bath in volumes no greater than 1% total volume. 8-Phenyltheophylline was made up in 80% methanol/2 M NaOH, subsequent dilutions being made in distilled water and all vehicle controls were negative. Propranolol and phentolamine were added directly to the Krebs solution.

Statistical analysis

EC_{50} values for concentration-response curves were derived by use of a pharmacological data handling package (PDH, SK&F software) on a VAX computer. Statistically significant differences between concentration-response curves were estimated using "Allfit' (De Lean *et al.*, 1977) and P values of less than 0.05 were taken to be significant. Where n numbers are given in the text, they refer to number of animals.

Results

Guinea-pig ileum

GABA and baclofen produced a concentration-related inhibition of the cholinergic twitch response in the guinea-pig ileum (Figures 2, 3). Concentrations inducing 50% inhibition (IC_{50}) for the inhibition of contraction were 13 ± 1.63 μM ($n = 8$) and 12.6 ± 1.8 μM ($n = 8$) for GABA and baclofen respectively. 3-Aminopropylphosphinic acid produced an inhibition of the electrically stimulated guinea-pig ileal twitch response which had the same characteristics as the GABA and baclofen responses (Figure 2). However, like baclofen but unlike GABA, the inhibitory response to 3-aminopropylphosphinic acid (up to 100 μM) was not preceded by a contraction. The IC_{50} for 3-aminopropylphosphinic acid

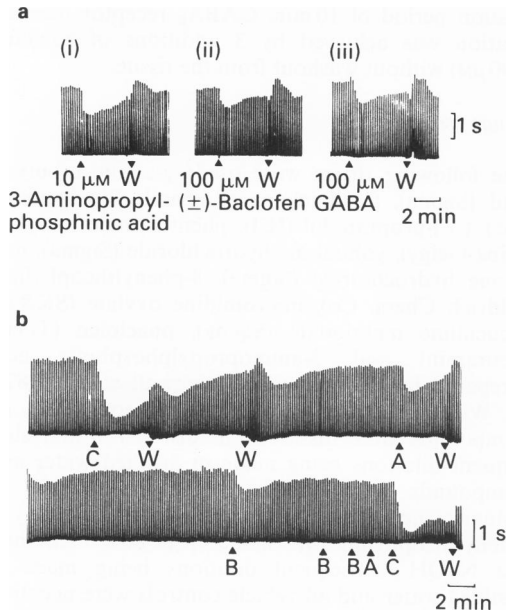

Figure 2 (a) Effect of 3-aminopropylphosphinic acid, (10 μM) (i), baclofen (100 μM) (ii), and GABA (100 μM) (iii), on the contractions of the guinea-pig ileum induced by field stimulation. (b) The effect of $GABA_B$ receptor desensitization on the responses to 3-aminopropylphosphinic acid (A) (10 μM) and clonidine (C) (0.1 μM). Control 3-aminopropylphosphinic acid (10 μM) and clonidine (0.1 μM) responses are shown prior to baclofen (B) (100 μM). Repeated applications of baclofen (100 μM) were without effect, thus indicating desensitization of $GABA_B$ receptors. Although under these circumstances 3-aminopropylphosphinic acid (10 μM) was without effect, clonidine, which reduces the twitch through an α_2-receptor mechanism, was as effective as it was prior to $GABA_B$ receptor densitization. The trace is continuous. Stimulus parameters were 0.5 ms duration, 0.1 Hz at supramaximal voltage.

inhibition of twitch contractions was 1.84 ± 0.23 μM ($n = 12$). As with the GABA and baclofen responses, the inhibition reached a maximum within approximately 1 min, and began to return to control levels within approximately 2 min even though the compound was still in contact with the tissue. The total duration of inhibition of twitch contractions in the presence of 3-aminopropylphosphinic acid without washout was 4–5 min.

When the guinea-pig ileum was desensitized to baclofen by three additions of baclofen (100 μM) at 2–6 min intervals, 3-aminopropylphosphinic acid (10 μM), was completely without effect (Figure 2b), whereas relaxations to clonidine (0.1 μM) persisted ($n = 8$). Similarly, the guinea-pig ileum was desensitized to 3-aminopropylphosphinic acid (10 μM) by three repeated additions of the compound and found to be unresponsive to baclofen 100 μM ($n = 4$).

The selectivity of the 3-aminopropylphosphinic acid inhibition was evaluated by use of a number of pharmacological antagonists at concentrations previously shown to cause significant antagonism of the relevant agonist responses. The control concentration-response curves to either 3-aminopropylphosphinic acid or baclofen were not significantly altered by incubation of tissue with either bicuculline (10 μM), propranolol plus phentolamine (both 1 μM), yohimbine (1 μM), 8-phenyltheophylline (10 μM), impromidine (1 μM), or naloxone (1 μM).

This suggests that 3-aminopropylphosphinic acid is not inhibiting the cholinergic twitch contraction in the guinea-pig ileum by interacting with $GABA_A$, β- or α-adrenoceptors, α_2-adrenoceptors, adenosine, histamine H_3 or opioid receptors, respectively.

Phaclofen, a new compound reported to have $GABA_B$ antagonist activity (Kerr *et al.*, 1987), significantly antagonized the 3-aminopropylphosphinic acid concentration-response curve in the guinea-pig ileum at a concentration of 500 μM ($P = 0.03$) ($n = 4$) but not at a concentration of 200 μM ($n = 8$). However, no significant antagonism of the baclofen concentration-response curve was obtained at concentrations of phaclofen up to 500 μM ($n = 7$).

Rat anococcygues muscle

As previously reported, GABA and baclofen inhibited the nerve-mediated contractions of the rat anococcygeus muscle produced by electrical field stimulation. The IC_{50} values obtained in the present experiments were 3.08 ± 0.45 μM ($n = 4$) for GABA and 5.47 ± 0.68 μM ($n = 4$) for baclofen. The inhibition produced by GABA and baclofen was much more long-lasting than that seen in the guinea-pig ileum. The agonists could be left in contact with the tissue for 15 min with no diminution in the response.

3-Aminopropylphosphinic acid inhibited the contractions of the rat anococcygeus in a concentration-dependent manner ($IC_{50} = 0.89 \pm 0.15$ μM ($n = 8$)) (Figure 3.) As with GABA and baclofen, the inhibition was more long-lasting than the inhibition seen in the ileum with 3-aminopropylphosphinic acid, and could be sustained for periods in excess of 15 min.

When baclofen (100 μM) was repeatedly added to the rat anococcygeus until no further inhibitory response could be obtained, 3-aminopropylphosphinic acid had no further inhibitory effect. However, since the effects of baclofen are long lasting in this preparation, the contractions did not return to normal during repeated application, and therefore no firm conclusions could be drawn from these data.

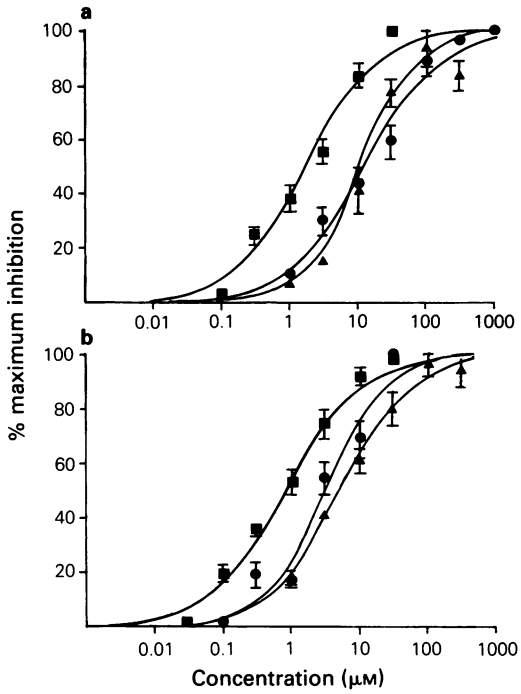

Figure 3 Concentration-response curves to agonists in the guinea-pig ileum (a) and rat anococcygeus muscle (b). (a) Concentration-response curves for the % maximum inhibition of the field stimulated guinea-pig ileum to 3-aminopropylphosphinic acid (■), baclofen (▲) and GABA (●). Stimulus parameters were 0.5 ms duration, 0.1 Hz at supramaximal voltage. The mean IC_{50} value for 3-aminopropylphosphinic acid was significantly less than the mean IC_{50} values for either baclofen or GABA ($P < 0.05$). There was no statistically significant difference between the mean IC_{50} values for GABA and baclofen. (b) Concentration-response curves for the % maximum inhibition of the field stimulated rat anococcygeus to 3-aminopropylphosphinic acid (■), baclofen (▲) and GABA (●). Stimulus parameters were 1 ms duration, 10 Hz for 1 s every 10 s, at supramaximal voltage. The mean IC_{50} value for 3-aminopropylphosphinic acid was significantly less than mean IC_{50} value for either baclofen or GABA, ($P < 0.05$). In addition, the mean IC_{50} value for GABA was significantly different from the mean IC_{50} value for baclofen ($P < 0.05$). The points represent mean for at least four preparations from separate animals; s.e. mean shown by vertical bars.

Discussion

There have been several studies on the structural requirements for $GABA_B$ receptor activation from which it would appear that even minor manipulations of the baclofen molecule result in total loss of activity (Olpe *et al.*, 1980; Krogsgard-Larsen, 1988). To date, no $GABA_B$ agonist has been shown to be more potent than baclofen on the *in vitro* systems described here. 3-Aminopropylphosphinic acid was found to be seven times more potent than racemic baclofen in the guinea-pig ileum and five times more potent than racemic baclofen in the rat anococcygeus muscle. In studies reported by Dingwall *et al.* (1987a, b), 3-aminopropylphosphinic acid has been reported to have an affinity for the $GABA_B$ receptor that is twenty times more than the affinity of baclofen.

It is interesting to compare the structures and activities of the phosphinic analogue of GABA, described here as a potent agonist, to the phosphonic analogue which has been described as a weak partial agonist/antagonist (Luzzi *et al.*, 1986), although the only difference in structure between the two is the acidic moiety. 3-Aminopropylphosphinic acid has a distorted tetrahedral arrangement of atoms around the phosphorus, has only one acidic proton and has the negative charge distributed over two oxygen atoms, thus it is similar in many respects to GABA. While 3-aminopropylphosphoninic acid has a near tetrahedral arrangement of atoms around the phosphorus, has two acidic protons (depending on pH) and has the negative charge distributed over three oxygen atoms.

In the guinea-pig ileum, it would appear that 3-aminopropylphosphinic acid is interacting with $GABA_B$ receptors located prejunctionally on cholinergic terminals. Since 3-aminopropylphosphinic acid did not cause an initial contraction, it is likely to be devoid of $GABA_A$ agonist activity. Although final validation of this assumption awaits a more specific and potent $GABA_B$ antagonist, the results with a number of known specific receptor antagonists suggest that 3-aminopropylphosphinic acid is not interacting with any other class of receptor. Further, tissues desensitised to baclofen, no longer responded to 3-aminopropylphosphinic acid. Clonidine, which was employed to test the specificity of the $GABA_B$ receptor desensitization, was equieffective both before and during $GABA_B$ receptor desensitization. Phaclofen has been claimed to be a weak but selective $GABA_B$ antagonist (Kerr *et al.*, 1987; Dutar & Nicholl, 1988). We were able to demonstrate inhibition of the 3-aminopropylphosphinic acid responses in the guinea-pig ileum but not those of baclofen. It is unclear why this should be so, although since phaclofen has more recently been claimed to show $GABA_B$ antagonist activity in a variety of test systems (Karlsson *et al.*, 1988; Soltesz *et al.*, 1988), it does support our hypothesis that 3-aminopropylphosphinic acid, is interacting with $GABA_B$ receptors.

The time course of the 3-aminopropylphosphinic acid response in both the guinea-pig ileum and the rat anococcygeus muscle was similar to that of GABA and baclofen. The response to $GABA_B$ agonists in the guinea-pig ileum is commonly observed to be transient in nature while in the rat anococcygeus muscle it is more long-lasting (Bowery *et al.*, 1981; Muhyaddin *et al.*, 1982).

Our pharmacological investigations support the claims of Dingwall *et al.* (1987a, b) that 3-aminopropylphosphinic acid is a highly potent, selective $GABA_B$ agonist. Further experiments with this compound may shed light on the physiological role of $GABA_B$ receptors in the mammalian intestine.

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