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Inhibition of sympathetic neurotransmission in the rat anococcygeus muscle by diazepam

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In the rat anococcygeus muscle, diazepam ($1{\cdot}8 \times 10^{-6} - 2{\cdot}8 \times 10^{-5}$ M), dipyridamole ($2 \times 10^{-6} - 3{\cdot}2 \times 10^{-5}$ M) and ATP ($2 \times 10^{-4} - 6{\cdot}4 \times 10^{-3}$ M) concentration-dependently inhibited the motor response to field stimulation. Glycine (up to $7{\cdot}5 \times 10^{-3}$ M) and GABA (up to 10^{-4} M) had no effect on field stimulation. Concentrations of diazepam and dipyridamole which reduced motor response to field stimulation did not relax the tonically contracted rat anococcygeus muscle. However, at these concentrations, diazepam and dipyridamole potentiated the inhibitory responses of the tonically contracted rat anococcygeus muscle to ATP and field stimulation. It is suggested that diazepam and dipyridamole reduced the motor response to field stimulation by potentiating the inhibitory transmitter released during nerve stimulation. Also, since the time-course of the effect of diazepam and dipyridamole on the inhibitory responses to ATP and field stimulation are closely similar, the results would tend to provide additional support for the concept of purinergic transmission in the rat anococcygeus muscle.

Intravenous administration of diazepam is accompanied by a marked but transient decrease in arterial pressure (Bradshaw 1976). In-vitro experiments have shown that diazepam may have a direct vasodilator effect on blood vessels (Chai & Wang 1966; Abel et al 1970). This is supported by the demonstration of a non-selective depression of the myogenic activity of the rat isolated portal vein as well as its responses to electrical stimulation and exogenous noradrenaline (Bradshaw 1976). Recently it has been observed that concentrations of diazepam having no direct action themselves could modulate the effects of other active compounds. Thus Savage & Adetunji (personal communication) observed that diazepam exerts a dual action (potentiation at low concentrations and inhibition at higher concentrations) on acetylcholine-induced contraction of the guinea-pig ileum. Also Clanachan & Marshall (1980) demonstrated that therapeutic concentrations of diazepam significantly potentiated the inhibitory effect of adenosine on isolated cardiac muscle and the rat vas deferens. This paper reports an inhibitory effect of diazepam on sympathetic neurotransmission in the rat anococcygeus muscle in-vitro. Its interaction with adenosine triphosphate (ATP) on the tonically contracted rat anococcygeus muscle was also studied and compared with dipyridamole, a known purine uptake inhibitor (Stafford 1966; Satchell et al 1972; Christie & Satchell 1980).

METHODS

Adult male rats (200–300 g) were killed by a sharp blow to the head and exsanguinated. The anococcygeus was dissected according to Gillespie (1972) and set up in a 20·0 ml organ bath containing Tyrode solution (37 °C) gassed with air. The composition of the Tyrode solution was (mmol litre^{-1}): NaCl 137; KCl 2·7; NaH_2PO_4, 0·31; $MgCl_2$ 0·9; $CaCl_2$ 1·8; $NaHCO_3$ 11·9 and glucose 5·6. The initial tension on the tissue was approximately 1·0 g and isotonic contractions (magnification ×7) were recorded on smoked paper. For electrical stimulation, the anococcygeus was passed through a platinum ring electrode of the type described by Burn & Rand (1960). Field stimulation of intramural nerves was carried out with pulses of 1 ms duration at supramaximal voltage. Frequencies of stimulation are as indicated in the text and stimulation was for 30 s at 3 min intervals. In all cases the preparation was allowed to equilibrate for at least 60 min (during which the bath fluid was changed every 15 min) before field stimulation or commencement of drug administration.

Experiments on the 'high tone' preparation

Effect of ATP. The relaxant effect of ATP was demonstrated after the tissue had been contracted with carbachol ($2{\cdot}2 \times 10^{-5}$ M). The doses of ATP were added cumulatively, each dose being allowed to act for 3 min. EC50 values (concn reducing the height of contraction by 50%) were obtained from dose-response curves.

Field stimulation. The muscle was contracted with carbachol ($2{\cdot}2 \times 10^{-5}$ M), and phentolamine (3×10^{-6} M) was included in the Tyrode solution.

The muscle was stimulated at 5, 10 and 20 Hz with supramaximal voltage (of 1 ms duration) for a period of 30 s at 3 min intervals.

Interaction with diazepam and dipyridamole
Following control responses to ATP and field stimulation, the dose/frequency-response curves were re-established in the presence of diazepam ($2 \times 10^{-6} - 2{\cdot}8 \times 10^{-5}$ M) and dipyridamole ($2 \times 10^{-6} - 5 \times 10^{-5}$ M) after allowing 15 min equilibration. The degree of potentiation (ATP) was expressed as the 'potentiation factor' (PF) which was calculated using the equation: PF = EC50 Control/ EC50 treated.

Drugs used. These included carbamyl choline chloride (carbachol, BDH); diazepam (commercial ampoules, Roche) dipyridamole (Boehringer Ingelheim); adenosine-5 triphosphate ATP, Aldrich), Yohimbine hydrochloride (sigma): Glycine (BDH) phentolamine mesylate (CIBA); γ-aminobutyric acid (Aldrich chemical); (−)-noradrenaline base (BDH). Dipyridamole was dissolved in 0·1 M hydrochloric acid. Other drugs were made up in distilled water.

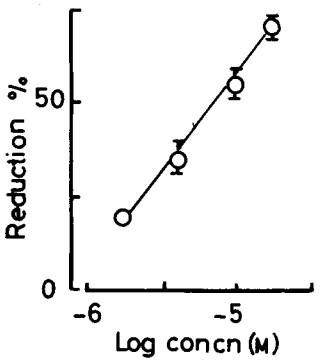

FIG. 1. The inhibitory effect of diazepam on the transmurally stimulated ($20H_z$; 70V) rat anococcygeus muscle. Each point on the graph represents the mean ± s.e. of 7 determinations.

RESULTS

Experiments on 'low tone preparation'
The rat anococcoygeus lacks tone and inherent rhythmic activity. In all experiments diazepam ($1{\cdot}8 \times 10^{-6}$ to $2{\cdot}8 \times 10^{-5}$ M) had no effect on the 'low tone' muscle preparation.

The anococcygeus contracts in response to field stimulation (2–20 Hz). Diazepam ($1{\cdot}8 \times 10^{-6}$ to $2{\cdot}8 \times 10^{-5}$ M) inhibited the motor response to field stimulation in a concentration-dependent manner. All frequencies of stimulation were affected. The inhibitory effect was long-lasting and about 60–80% recovery was obtained within 60 min after washing out of the bath. The inhibitory effect was not due to the solvent since the concentration of ethanol which contain the highest concentration of diazepam was 0·08% and this concentration had no effect on nerve transmission. In order to quantify the degree of inhibition, various concentrations of diazepam were tested on the motor response to field stimulation at a fixed frequency of stimulation. In this particular series of experiments, stimulation at 20 Hz produced a significant contraction and was used throughout this study. The maximum response to any given concentration of the drug was assumed to have occurred when two consecutive contractions were of the same amplitude. Fig. 1 shows the inhibitory effect of diazepam. The EC50 value (diazepam) obtained from concentration response curves was $6{\cdot}9 \pm 0{\cdot}6 \times 10^{-6}$ M. Noradrenaline ($7{\cdot}4 \times 10^{-7}$ to $1{\cdot}2 \times 10^{-5}$ M) produced concentration-dependent contraction of the anococcygeus muscle. These contractions were not significantly ($P > 0{\cdot}05$) reduced by diazepam ($7{\cdot}2 \times 10^{-6}$ M). Since diazepam, however, reproducibly inhibited the motor response to field stimulation at this concentration ($7{\cdot}2 \times 10^{-6}$ M), it seems very likely that diazepam does not act postjunctionally. In two experiments, yohimbine (3×10^{-8} M) did not antagonize the inhibitory effect of diazepam. Higher concentrations of yohimbine ($\geq 7{\cdot}5 \times 10^{-8}$ M) reduced the motor response to field stimulation probably by an action at post-junctional noradrenergic receptors since these concentrations have been shown to reduce the contractile responses to exogenous noradrenaline (Adenekan 1981). Since benzodiazepines have been shown to facilitate glycinergic (Young et al 1974) or GABAergic (see Costa 1980) transmission and also to potentiate the effects of purine nucleotides (for example see Clanachan & Marshall 1980); glycine, GABA, adenosine triphosphate (ATP) and dipyridamole were studied simultaneously for comparison. The results obtained show that glycine (up to $7{\cdot}5 \times 10^{-3}$ M) and GABA (up to 10^{-4} M) had no effect on the motor response to field stimulation. However ATP (2×10^{-4} to $6{\cdot}4 \times 10^{-3}$ M) and dipyridamole (2×10^{-6} to $3{\cdot}2 \times 10^{-5}$ M) produced a concentration-dependent inhibition of the motor response to field stimulation. The time-course of the inhibitory action of ATP and dipyridamole is similar to that of diazepam suggesting a similar mechanism

of action. The EC50 values for ATP and dipyridamole are also shown in Table 1.

Table 1. Effect of drugs on the motor response of the rat anococcygeus muscle stimulated at a frequency of 20 Hz (70 V; 1 ms) for 30 s and at 3 min intervals. Values represent the mean ± s.e. of at least 4 experiments (n = 7 for diazepam.

Agonist	EC50 (M)*
Diazepam	$6{\cdot}9 \pm 0{\cdot}6 \times 10^{-6}$
ATP	$3{\cdot}2 \pm 0{\cdot}4 \times 10^{-3}$
Dipyridamole	$1{\cdot}1 \pm 0{\cdot}2 \times 10^{-5}$
Glycine	$>7{\cdot}5 \times 10^{-3}$ (if any)
GABA	$>10^{-4}$ (if any)

* EC50 values were obtained from concentration - response curves. Only one agonist was tested on any particular preparation and the different concentrations were added cumulatively.

Effect of diazepam ATP and dipyridamole on the tonically contracted ('high tone') anococcygeus muscle

Concentrations of diazepam ($1{\cdot}8 \times 10^{-6}$ to $2{\cdot}8 \times 10^{-5}$ M) and dipyridamole (2×10^{-6} to 5×10^{-5} M) that inhibited motor response to field stimulation did not relax the tonically contracted rat anococcygeus muscle. However ATP (2×10^{-6} to $6{\cdot}4 \times 10^{-3}$ M) produced a concentration-dependent relaxant effect which was preceded by an initial contraction. Usually between 85–95% inhibition could be obtained in the presence of the highest concentration of ATP used.

Potentiation of the effects of ATP by diazepam and dipyridamole

At concentrations lower than 10^{-5} M, diazepam did not modify the relaxant effect of ATP. But at higher concentrations (10^{-5} to $2{\cdot}8 \times 10^{-5}$ M), diazepam produced a concentration-dependent potentiation of the inhibitory effects of exogenously applied ATP (Fig. 2). Higher concentrations of diazepam ($>2{\cdot}8 \times 10^{-5}$ M) were not tested so as to avoid possible interference from the vehicle. The EC50 values before and after the various concentrations of diazepam as well as the 'potentiation factors' are shown in Table 2. Dipyridamole (2×10^{-6} to 5×10^{-5} M) also produced a graded potentiation of the inhibitory effect ATP on the rat anococcygeus muscle (Fig. 2). The highest concentration of dipyridamole used in this study (5×10^{-5} M) produced a slight reduction in the response to carbachol. However, in two preparations, this concentration of dipyridamole produced more than 50% reduction in the contractile response to carbachol; such tissues were discarded. Table 2 shows the EC50 values for ATP before and after dipyridamole. The corresponding potentiation factors are also shown.

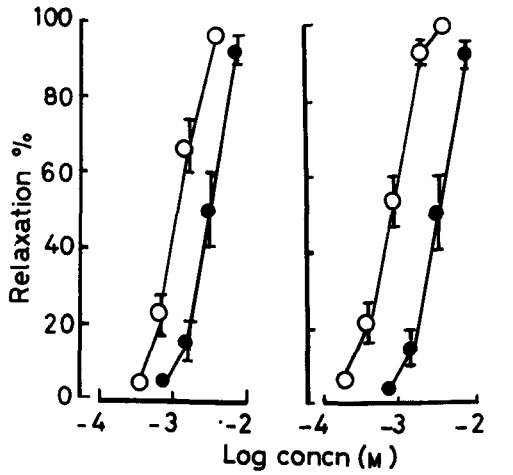

FIG. 2. Potentiation of the inhibitory effect of ATP on the tonically contracted rat anococcygeus muscle by (a) diazepam (2.8×10^{-5}M) and (b) dipyridamole (5×10^{-5}M). On the graph, (○) represents response before and (●) response in the presence of diazepam and dipyridamole. Each point is the mean ± s.e. of 4 experiments.

Table 2. Potentiation of the inhibitory effects of exogenously applied ATP by diazepam and dipyridamole (means of 4–6 experiments).

Treatment	Concn (M)	ATP (EC50 × 10^{-3} M	*Potentiation factor
Control	—	$3{\cdot}10 \pm 0{\cdot}20$	—
Diazepam	10^{-5}	$1{\cdot}95 \pm 0{\cdot}05$	1·65 (1·50–1·80)
	$2{\cdot}8 \times 10^{-5}$	$1{\cdot}20 \pm 0{\cdot}10$	(2·6–2·90)
Dipyridamole	2×10^{-6}	$2{\cdot}04 \pm 0{\cdot}20$	1·50 (1·30–1·60)
	10^{-5}	$1{\cdot}30 \pm 0{\cdot}12$	2·2 (2·0–2·40)
	5×10^{-5}	$0{\cdot}77 \pm 0{\cdot}02$	3·8 (3·0–4·8)

* EC50 control/EC50 treated the range for each concentration is given in parentheses.

Effect of diazepam and dipyridamole on the inhibitory response to field stimulation

Field stimulation (5–20 Hz) of the tonically contracted rat anococcygeus muscle resulted in frequency-dependent relaxations. The maximum relaxation (usually between 20–25% reduction in the height of

contraction to carbachol) was in most cases achieved at a frequency of 10 Hz. Diazepam (10^{-5} to $2{\cdot}8 \times 10^{-5}$ M) and dipyridamole (10^{-5} to 5×10^{-5} M) produced concentration-dependent potentiation of the inhibitory response (only the effects of the higher dose of diazepam and dipyridamole are shown in Fig. 3). The potentiating effect of diazepam and dipyridamole was more marked on the lower frequency of stimulation (5 Hz). As can also be seen in Fig. 3 dipyridamole did not modify the time-course of the inhibitory responses of the rat anococcygeus muscle to field stimulation. On the contrary, diazepam significantly prolonged the recovery phase.

DISCUSSION

The above results demonstrate that diazepam can exert some direct inhibitory effect on noradrenergic transmission in the rat anococcygeus muscle. Diazepam reduced the motor response to field stimulation while having no effect on noradrenaline-induced contractions suggesting that diazepam probably does not act postjunctionally. Benzodiazepines modulate glycinergic (Young et al 1974) and GABAergic (Costa 1980) transmission. However it is unlikely that the inhibitory effect of diazepam observed in this study involves glycinergic or GABAergic receptors since neither glycine nor GABA inhibited the motor response to field stimulation. In addition to a dense adrenergic innervation, the rat anococcygeus muscle has an inhibitory innervation with a transmitter yet to be identified (Gillespie 1972; Gillespie & McGrath 1974; Creed et al 1977). Therefore field stimulation of the anococcygeus muscle activates both excitatory (noradrenergic) and inhibitory (non-adrenergic) nerves even though the net response is excitatory. The possibility therefore arises that (1) diazepam could directly inhibit the motor response to field stimulation or (2) diazepam could inhibit the motor response to field stimulation by potentiating the effect of the inhibitory transmitter released during stimulation.

The observation that (1) ATP which has been proposed as the inhibitory transmitter in the rat anococcygeus muscle (Burnstock et al 1978) concentration-dependently inhibited the motor response to field stimulation and (2) dipyridamole, a known purine uptake inhibitor (Stafford 1966; Kolassa et al 1970; Sano et al 1972; Satchell et al 1972; Kalsner 1975; Coleman 1976, 1980; Christie & Satchell 1980) also reduced the motor response, would support the suggestion that the inhibitory effect of diazepam, which has also been shown to inhibit adenosine uptake (Mah & Daly 1976; Hertz et al 1979; Phillis et al 1980), could be due to a potentiation of the inhibitory transmitter. In support of this is the observation that in the tonically contracted rat anococcygeus muscle, both diazepam and dipyridamole potentiated the inhibitory response to exogenous ATP and field stimulation.

Some of the criteria to be fulfilled before a substance could be labelled a transmitter include (i) release of the substance when the nerve is stimulated and (ii) factors which modify the proposed transmitter substance should similarly modify the response to nerve stimulation. In the rat anococcygeus, ATP is released following stimulation of the inhibitory nerve (Burnstock et al 1978).

The present observation that the purine uptake inhibitors-diazepam and dipyridamole potentiated the effect of ATP would suggest that ATP is taken up into the tissues and could possibly provide a means of terminating the action of ATP as a neurotransmitter in this tissue. Also a parallel effect of diazepam and dipyridamole on ATP, and field stimulation would tend to provide further evidence in favour of ATP or a related purine nucleotide as the inhibitory transmitter in the rat anococcygeus muscle.

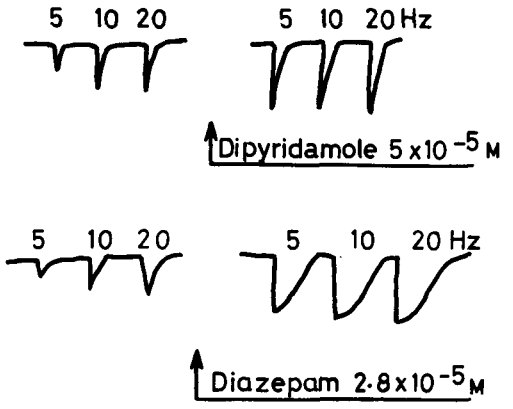

FIG. 3. Potentiation of the inhibitory response of the tonically contracted rat anococcygeus muscle to field stimulation by (a) dipyridamole and (b) diazepam. Note that diazepam but not dipyridamole prolonged the recovery phase of the response.

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