Br. J. Pharmac. (1983), **79**, 611–615

Biphasic non-adrenergic, non-cholinergic relaxations of the mouse anococcygeus muscle

A. Gibson & O. Yu

Department of Pharmacology, Chelsea College, University of London, Manresa Road, London SW3 6LX

1 Trains of field stimulation of 60 s duration caused a biphasic relaxation of carbachol (50 μM)-induced tone in the mouse anococcygeus. The optimal pulse frequency and width were 10 Hz and 1 ms respectively.

2 Tetrodotoxin (31, 124, and 310 nM) caused a dose-dependent reduction in the magnitude of both phases. Neither phase was affected by (±)-propranolol (1 μM), neostigmine (1 μM), (+)-tubocurarine (100 μM), or apamin (500 nM). Biphasic relaxations were observed in muscles from 6-hydroxydopamine pretreated mice.

3 Haemolysed blood (10, 40, and 100 μl/ml) reduced the magnitude of the first phase of nerve-induced relaxation to a greater extent than the second. This effect was reversible.

4 Following a prolonged train of inhibitory nerve stimulation (10 Hz; 10 min) the magnitude of the first phase was reduced only slightly, but the second markedly.

5 The possible relationships between the biphasic relaxation to field stimulation and putative non-adrenergic, non-cholinergic transmitters in the mouse anococcygeus are discussed.

Introduction

The mouse anococcygeus muscle receives an inhibitory non-adrenergic, non-cholinergic (NANC) innervation (Gibson & Wedmore, 1981). During an investigation of the actions of putative inhibitory transmitters, excitation of these nerves by means of prolonged trains of field stimulation, caused a biphasic relaxation of the muscle (Gibson & Tucker, 1982). In this paper the results of some experiments carried out to characterize this phenomenon are detailed.

Methods

Male mice (LACA strain; 20–35 g) were stunned and bled. The paired anococcygeus muscles were dissected out and set up in series, joined at the ventral bar, in 1 ml glass organ baths containing Krebs bicarbonate solution (composition mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1) maintained at 37°C and gassed continuously with 95%O_2:5%CO_2. A resting tension of 200–400 mg was placed on the muscle and changes in tension measured with a Grass FTO3 force-displacement transducer attached to a Lectromed pen-recorder. Field stimulation, using supramaximal voltage, was applied with two parallel platinum electrodes running down either side of the tissue. These were attached to a square wave pulse generator. To prevent the effects of sympathetic nerve stimulation, the Krebs solution contained phentolamine (1 μM) throughout. In addition, each muscle was preincubated with guanethidine (30 μM) for 15 min before beginning the experiment. This single exposure to guanethidine produces a long-lasting inhibition of sympathetic responses in the mouse anococcygeus (Gibson & Tucker, 1982), reflecting the persistent nature of the adrenergic neurone blocking activity of the drug. During the preincubation, motor responses due to the indirect sympathomimetic activity of guanethidine were not observed, presumably due to the presence of phentolamine (1 μM) in the Krebs solution. In all cases muscle tone was raised with carbachol (50 μM).

Haemolysed blood was prepared by making a 1:1 dilution of heparinized mouse blood with distilled water.

Some mice were pretreated with 6-hydroxydopamine (6-OHDA, 2 × 50 mg/kg on day 1; 2 × 100 mg/kg on day 4; experiment on day 5). This dose schedule has been shown to produce an effective sympathectomy of the mouse anococcygeus (Gibson & Wedmore, 1981).

Drugs were added to the organ bath in volumes not exceeding 50 μl. The following drugs were used: apamin (Sigma); carbachol (Koch Light); guanethidine sulphate (Ciba); 6-hydroxydopamine hydrobromide (Sigma); neostigmine methylsulphate (Roche); phentolamine mesylate (Ciba); (±)-propranolol hydrochloride (ICI); tetrodotoxin (Sigma); (+)-tubocurarine chloride (Wellcome). The doses in the text refer to final bath concentrations.

Statistical analysis was by Student's *t* test.

Results

The effect of varying pulse frequency and pulse width

A train length of 60 s was used; longer train lengths might have caused a marked desensitization of nerve-induced relaxations (see later).

The effects of varying frequency were investigated using a pulse width of 1 ms (Figure 1). Below 5 Hz the relaxation consisted of only one component. However, stimulation at 5, 10, and 25 Hz produced a clear biphasic relaxation of carbachol-induced tone. There was an initial rapid relaxation during the first 10 s followed by a slower relaxation over the succeeding 50 s. The magnitude of each phase was calculated as the peak percentage reduction in tone compared with the immediate prestimulation level. In the majority of muscles the second phase of relaxation reached a plateau before 60 s, but in a few cases relaxation was still in progress at 60 s. In either case, the degree of relaxation at 60 s was used to calculate the results. The magnitude of both phases was maximal at 10 Hz, the second phase usually being about 10% greater than the first (Figure 1). Muscle tone returned to prestimulation levels between each period of field stimulation suggesting that neither phase was due to breakdown of carbachol.

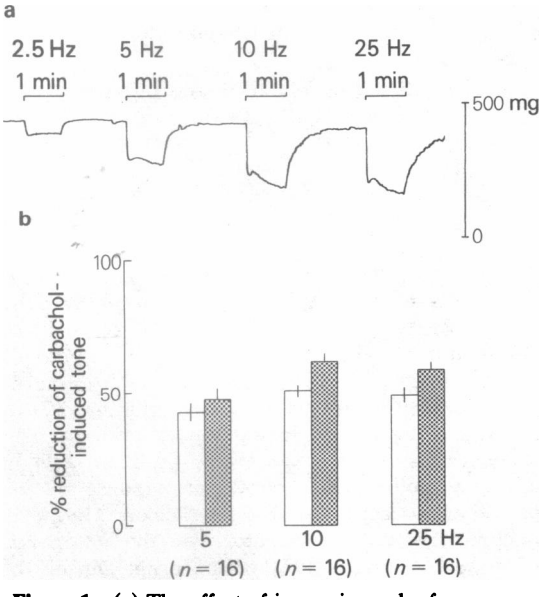

Figure 1 (a) The effect of increasing pulse frequency on relaxations of the mouse anococcygeus in response to 60 s trains of field stimulation (1 ms pulse width; supramaximal voltage). The muscle had been preincubated with guanethidine (30 μM; 15 min) and the Krebs solution contained phentolamine (1 μM). Muscle tone was raised with carbachol (50 μM). (b) The effect of increasing pulse frequency on the relative magnitude of the first (open columns) and second phases (stippled columns) of the relaxation of the mouse anococcygeus in response to 60 s trains of field stimulation (1 ms pulse width; supramaximal voltage). Values represent means (s.e. are shown by vertical lines), and were calculated as the peak percentage reduction of prestimulation tone induced by carbachol (50 μM).

The effects of pulse width were investigated using a standard frequency of 10 Hz. The threshold pulse width was about 50 μs and the magnitude of both phases increased in parallel with increasing pulse width to reach a maximum at 1 ms. At 5 ms the magnitude of both phases was reduced.

From these results it was decided to investigate the effects of drugs and of other procedures on the biphasic responses using a frequency of 10 Hz and a pulse width of 1 ms.

The effects of drugs

In these experiments, drug action was terminated by washing out the organ bath and therefore repeated additions of carbachol (50 μM) were necessary to raise tone. Consequently, a different experimental protocol was adopted. After addition of carbachol, field stimulation was applied when a steady, maintained rise in muscle tone had been produced (usually after 2 min); 1 min after field stimulation the organ bath was washed out and muscle tone allowed to return to baseline for 15 min before it was raised again with carbachol. The first train of stimuli after setting up the muscle in the organ bath did not give a clearly defined biphasic relaxation. This developed with the second and third periods of stimulation and remained constant thereafter. Therefore, in each muscle preparation, the effects of drugs and other procedures on the responses to field stimulation were not studied until constant biphasic relaxations were observed.

Tetrodotoxin (TTX; 31, 124, and 310 nM) produced a dose-dependent reduction in the magnitude of both phases (Figure 2). Higher doses of TTX (10 μM) completely abolished relaxations to 60 s trains of field stimulation. The biphasic response was unaffected by (±)-propranolol (1 μM), neostigmine

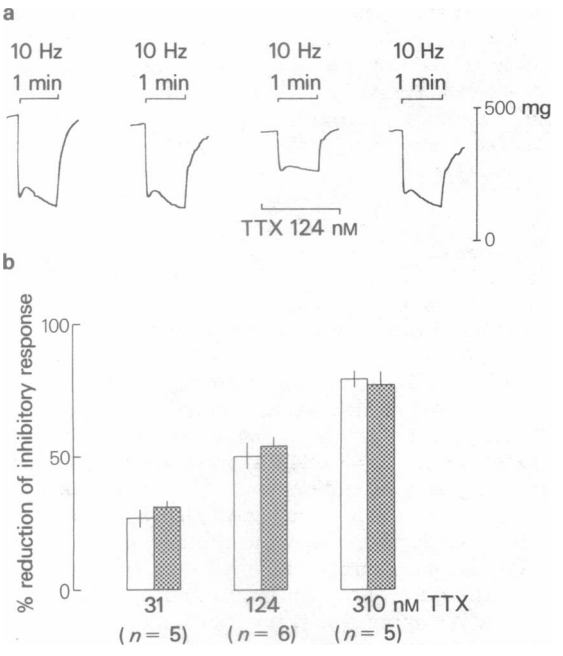

Figure 2 (a) The effect of tetrodotoxin (TTX; 124 nM; added 4 min before raising tone with 50 μM carbachol) on the biphasic relaxation of the mouse anococcygeus in response to 60 s trains of field stimulation (10 Hz; 1 ms pulse width; supramaximal voltage). The muscle had been preincubated with guanethidine (30 μM; 15 min) and the Krebs solution contained phentolamine (1 μM). The time between each period of stimulation was 15 min. TTX produced a reversible reduction of both phases. (b) The effect of increasing concentrations of TTX on the relative magnitude of the first (open columns) and second phases (stippled columns) of the relaxation of the mouse anococcygeus in response to 60 s trains of field stimulation (10 Hz; 1 ms pulse width; supramaximal voltage). Values represent means (s.e. are shown by vertical lines) and were calculated as the percentage reduction of each phase compared with the response obtained immediately before TTX.

(1 μM), (+)-tubocurarine (100 μM), or apamin (500 nM). Muscles from animals pretreated with 6-OHDA displayed biphasic relaxations similar to those of muscles from control animals.

The effect of haemolysed blood

Haemolysed blood (10, 40, and 100 μl/ml) reduced the magnitude of the first phase of nerve-induced relaxations to a greater extent than the second phase (Figure 3). Higher concentrations of haemolysed blood were not studied since frothing of the bathing medium became excessive. The effects of haemolysed blood were easily reversed by washout.

The effect of a 10 min train of field stimulation

The effect of continuing the train of field stimulation beyond 60 s is shown in Figure 4. Following the peak of the second phase relaxation, muscle tone slowly returned towards the prestimulation level and usually reached a plateau, the level of which varied from muscle to muscle. In the example shown in Figure 4a (third panel from the left) muscle tone recovered to a plateau some 40% below the prestimulation level within 4 min and remained there for the rest of the 10 min stimulation period. After 10 min the Krebs

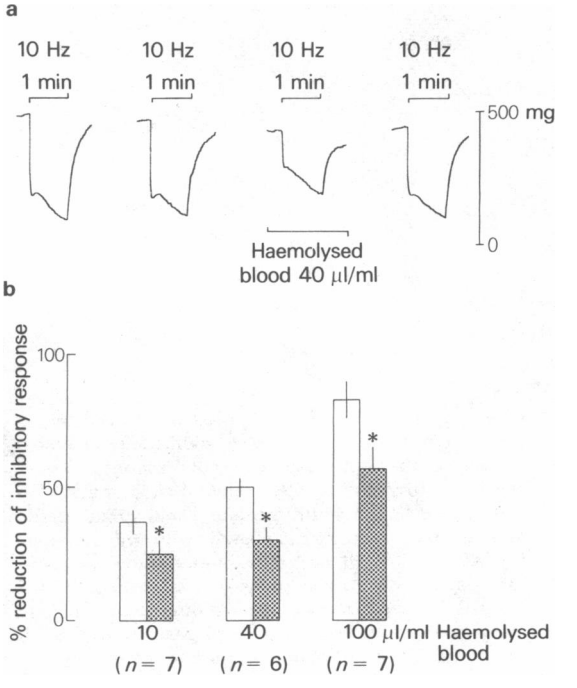

Figure 3 (a) The effect of haemolysed blood (40 μl/ml; added 4 min before raising tone with 50 μM carbachol) on the biphasic relaxation of the mouse anococcygeus in response to 60 s trains of field stimulation (10 Hz; 1 ms pulse width; supramaximal voltage). The muscle had been preincubated with guanethidine (30 μM; 15 min) and the Krebs solution contained phentolamine (1 μM). The time between each period of stimulation was 15 min. Haemolysed blood reduced the first phase to a greater extent than the second. (b) The effect of increasing concentrations of haemolysed blood on the relative magnitude of the first (open columns) and second phases (stippled columns) of the relaxation of the mouse anococcygeus in response to 60 s trains of field stimulation (10 Hz; 1 ms pulse width; supramaximal voltage). Values represent means (s.e. shown by vertical lines) and were calculated as the percentage reduction of each phase compared with the response obtained immediately before haemolysed blood. $^*P < 0.05$ (t test).

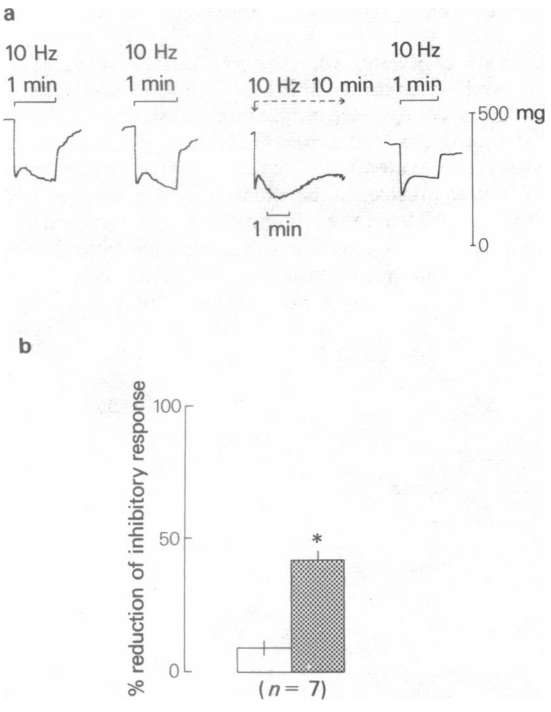

Figure 4 (a) The effect of a 10 min train of field stimulation (10 Hz; 1 ms pulse width; supramaximal voltage) on the biphasic relaxation of the mouse anococcygeus in response to 60 s trains of field stimulation (10 Hz; 1 ms pulse width; supramaximal voltage). The muscle had been preincubated with guanethidine (30 μM; 15 min) and the Krebs solution contained phentolamine (1 μM). Muscle tone was raised with carbachol (50 μM). The time between the first three periods of field stimulation was 15 min. The third panel from the left shows the initial part of the response to the 10 min train (note that chart speed has been reduced to save space); following the peak of the second phase of the relaxation muscle tone recovered to some 40% of the prestimulation level, where it remained for the rest of the 10 min stimulation period. After 10 min the Krebs solution and carbachol (50 μM) were replaced, causing a further rise in tension, and 2 min later a 60 s train of field stimulation applied (right hand panel). At this time the first phase was reduced only slightly, but there was a marked reduction in the second phase. (b) The effect of a 10 min train of field stimulation (10 Hz; 1 ms pulse width; supramaximal voltage) on the relative magnitude of the first (open column) and second phases (stippled column) of the relaxation of the mouse anococcygeus in response to 60 s trains of field stimulation (10 Hz; 1 ms pulse width; supramaximal voltage; protocol as in (a)). Values represent means (s.e. shown by vertical lines) and were calculated as the percentage reduction of each phase compared with the response obtained immediately before the 10 min train. $^*P < 0.05$ (t test).

solution and carbachol (50 μM) were replaced, causing a further rise in tension, and 2 min later a 60 s train of field stimulation applied (Figure 4a; right hand panel). At this time the first phase of the relaxation was reduced slightly, but there was a marked reduction in the magnitude of the second phase (Figure 4).

Discussion

The results of the present study confirm and extend the previous observation of a biphasic relaxation of the mouse anococcygeus during field stimulation (Gibson & Tucker, 1982). Both phases appear to be non-adrenergic and non-cholinergic since they were unaffected by (±)-propranolol, neostigmine, (+)-tubocurarine, or 6-OHDA pretreatment. In addition, biphasic relaxations could be elicited in muscles which were both preincubated with guanethidine and maintained in phentolamine-containing Krebs solution. Further, since muscarinic receptor activation causes contraction of the mouse anococcygeus (Gibson & Wedmore, 1981) involvement of these receptors is unlikely.

Two procedures were found to differentiate between the phases. First, haemolysed blood reduced the first phase more than the second. Bowman & Gillespie (1982) have shown that haemolysed blood blocks NANC transmission in the bovine retractor penis and the rat anococcygeus. Although the exact mechanism of this action is unknown it has recently been shown to be associated with oxyhaemoglobin (Bowman, Gillespie & Pollock, 1982). In contrast, Bowman & Gillespie (1982) found that NANC inhibition of the guinea-pig taenia caeci was not blocked by haemolysed blood. The bee venom toxin, apamin, blocked NANC inhibition of the taenia but not of the bovine retractor penis or rat anococcygeus (Bowman & Gillespie, 1982). Since in the present study the first phase of nerve-induced relaxation of the mouse anococcygeus was blocked by haemolysed blood but not by apamin, this phase would appear to be similar to NANC relaxations in the bovine retractor penis and rat anococcygeus. The second phase of the relaxation of the mouse anococcygeus was not blocked by apamin and was relatively resistant to block by haemolysed blood suggesting that it is unlike NANC transmission in the guinea-pig taenia, bovine retractor penis, and rat anococcygeus. However, it should be noted that the effects of haemolysed blood and of apamin in these latter three tissues were studied only on short trains of nerve stimulation (up to 10 s). In addition, although haemolysed blood reduced the second phase, the time course of each phase, independent of the other, is not known. Some overlap is likely, and therefore any change in the

magnitude of one phase will probably result in a similar, though smaller, change in the magnitude of the other.

The second procedure found to differentiate between the phases was a 10 min train of inhibitory nerve stimulation, after which the first phase was reduced only slightly but there was a marked reduction in the second phase. The mechanism of this effect of prolonged nerve stimulation is unknown and is at present under investigation. It seems that nerve stimulation in some way desensitizes the mechanism responsible for the second phase relaxation. This may account for the observation that a biphasic response was not apparent during the first period of nerve stimulation after mounting the tissue in the organ bath.

The initial event which triggers the NANC biphasic relaxation of the mouse anococcygeus appears to involve neuronal depolarization since both phases were reduced by TTX. However, it is not clear whether the biphasic response results from the release of a single transmitter, which then produces two effects of differing time course, or from the release of more than one transmitter. It is unlikely that either phase involves synthesis of prostaglandin-like materials since the original observation of the effect was made on indomethacin-treated tissues (Gibson & Tucker, 1982). There are three main candidates for the role of inhibitory transmitter in the anococcygeus: vasoactive intestinal polypeptide (VIP); adenosine 5'-triphosphate (ATP); and an extract of bovine retractor penis. In a previous study it was found that VIP-induced relaxations of the mouse anococcygeus were not blocked by apamin or haemolysed blood but were reduced greatly by a prolonged period of nerve stimulation (Gibson & Tucker, 1982). These results, together with those of the present study, suggest that if VIP is involved in NANC inhibitory transmission in the mouse ancoccygeus it is more likely to be associated with the second, rather than the first, phase of nerve-induced relaxation. There is evidence in other tissues that VIP might be the transmitter responsible for delayed effects of parasympathetic stimulation (Lundberg, Hokfelt, Anggard & Fahrenkrug, 1981; Fahrenkrug & Ottesen, 1982). Relaxations of the mouse anococcygeus to ATP were unaffected by apamin, haemolysed blood, or prolonged nerve stimulation (Gibson & Tucker, 1982), and therefore its possible role in NANC transmission in this tissue is less clear. However, it appears to have more in common with the first phase relaxation. The inhibitory factor from bovine retractor penis relaxes the rat anococcygeus muscle and this action is blocked by haemolysed blood but not by apamin (Bowman & Gillespie, 1982). As yet it has not been studied in the mouse anococcygeus.

The support of the Central Research Fund of London University is appreciated.

References

BOWMAN, ANNE & GILLESPIE, J.S. (1982). Block of some non-adrenergic inhibitory responses of smooth muscle by a substance from haemolysed erythrocytes. *J. Physiol.*, **328**, 11–25

BOWMAN, ANNE, GILLESPIE, J.S. & POLLOCK, D. (1982). Oxyhaemoglobin blocks non-adrenergic non-cholinergic inhibition in the bovine retractor penis muscle. *Eur. J. Pharmac.*, **85**, 221–224

FAHRENKRUG, J. & OTTESEN, B. (1982). Nervous release of vasoactive intestinal polypeptide from the feline uterus: pharmacological characteristics. *J. Physiol.*, **331**, 451–460.

GIBSON, A. & TUCKER, J.F. (1982). The effects of vasoactive intestinal polypeptide and of adenosine 5'-triphosphate on the isolated anococcygeus muscle of the mouse. *Br. J. Pharmac.*, **77**, 97–103

GIBSON, A. & WEDMORE, C.V. (1981). Responses of the isolated anococcygeus muscle of the mouse to drugs and to field stimulation. *J. auton. Pharmac.*, **1**, 225–233

LUNDBERG, J.M., HOKFELT, T., ANGGARD, A. & FAHRENKRUG, J. (1982). Functional implications of coexistence of peptides and classical transmitters: studies on exocrine glands. In Neurotransmitters-Receptors, *Adv. Pharmac. Ther.* vol. II, ed. H. Yoshida, Y. Hagihara & S. Ebashi. pp. 177–187 Oxford, New York; Toronto; Sydney; Paris; Frankfurt: Pergamon.

(*Received December 28, 1982. Revised January 28, 1983.*)