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THE EFFECTS OF VASOACTIVE INTESTINAL POLYPEPTIDE AND OF ADENOSINE 5′-TRIPHOSPHATE ON THE ISOLATED ANOCOCCYGEUS MUSCLE OF THE MOUSE

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1 Vasoactive intestinal polypeptide (VIP, 0.01–5 μM) produced dose-related relaxations of the mouse anococcygeus muscle.

2 Following incubation with indomethacin (2.8 μM 1 h) adenosine 5′-triphosphate (ATP, 0.5–10 mM) produced dose-related relaxations of the mouse anococcygeus.

3 Haemolysed blood reduced inhibitory responses of the mouse anococcygeus to field stimulation but had no effect on relaxations to VIP or ATP.

4 Apamin (0.5 μM) had no effect on the relaxation of mouse anococcygeus to field stimulation, VIP, or ATP.

5 2-2′-Pyridylisatogen tosylate (PIT, 50 μM) itself reduced muscle tone but it did not abolish inhibitory responses to field stimulation, VIP, or ATP.

6 During prolonged inhibitory nerve stimulation the relaxation of the mouse anococcygeus in response to VIP was reduced greatly while that to ATP was unaffected.

7 Bundles of VIP-immunoreactive sites were detected in sections of the mouse anococcygeus treated by the peroxidase-antiperoxidase (PAP) immunocytochemical technique.

8 The results suggest that the mechanisms underlying non-adrenergic, non-cholinergic inhibitory transmission in the mouse anococcygeus are similar to those in the bovine retractor penis and unlike those in the guinea-pig taenia caeci.

9 The possibility that VIP or ATP might be involved in inhibitory neurotransmission in the mouse anococcygeus is discussed.

Introduction

The anococcygeus muscle of the mouse receives an inhibitory non-adrenergic, non-cholinergic innervation, the transmitter of which is as yet unidentified (Gibson & Wedmore, 1981). Substances proposed as putative transmitters of such nerves in other tissues include adenosine 5′-triphosphate (ATP, Burnstock, 1972; 1975; Satchell, 1981) and vasoactive intestinal polypeptide (VIP, Bryant, Bloom, Polak, Albuquerque, Modlin & Pearse, 1976; Bloom, Polak, Bishop & Buchan, 1978; Daniel, 1978; Goyal, Rattan & Said, 1980; Willis, Ottesen, Wagner, Sundler & Fahrenkrug, 1981). Since the isolated anococcygeus of the mouse relaxes in response to VIP and, after incubation with indomethacin, to ATP, both substances must be considered as candidates for the role of inhibitory transmitter in this tissue (Gibson & Wedmore, 1981). Indeed, Burnstock, Cocks & Crowe (1978) have already proposed ATP as the inhibitory transmitter of the rat anococcygeus.

The mechanisms underlying non-adrenergic, non-cholinergic autonomic transmission may vary among tissues. For example, the bee venom toxin, apamin, reduces the inhibitory response to field stimulation and to ATP in the guinea-pig taenia coli (MacKenzie & Burnstock, 1980) but not in the bovine retractor penis (Bowman & Gillespie, 1981). Conversely, haemolysed blood inhibits relaxations of the bovine retractor penis but not those of the guinea-pig taenia caeci (Bowman & Gillespie, 1981), in response to non-adrenergic, non-cholinergic stimulation. We have now examined the characteristics of the relaxations induced by VIP and by ATP in the mouse isolated anococcygeus, and conducted an immunocytochemical study designed to detect possible sites of VIP-immunoreactivity in this tissue.

Methods

Male mice (25–35 g; LACA strain) were stunned and bled. The paired anococcygeus muscles were dissected and set up in series, joined at the ventral bar

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(Gibson & Wedmore, 1981), in a 1 ml organ bath 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) which was 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 were measured by a Grass FTO3 force-displacement transducer and displayed on a Devices MX2 pen recorder. Field stimulation was applied by two parallel platinum electrodes running down either side of the tissue. These were attached to a square wave pulse generator (1 ms pulse width; supramaximal voltage).

Drugs were added to the organ bath in volumes not exceeding 40 μl. To obtain consistent inhibitory responses to field stimulation the motor effects of sympathetic nerve stimulation were removed. Usually, this was achieved by incubation with guanethidine (30 μM) for 15 min before beginning the experiment. During this period, muscle tone was raised (Gibson & Wedmore, 1981) but it quickly returned to baseline following washout. However, the motor effects of sympathetic nerve stimulation remained blocked for the rest of the experiment. This reflects the persistence of the adrenergic neurone blocking activity of guanethidine compared with its rapidly reversible sympathomimetic effect (Maxwell, 1982). Subsequently, muscle tone was raised by carbachol (10–50 μM) in order to observe relaxations to field stimulation or drugs. Inhibitory stimuli were not given until carbachol had produced a steady, maintained rise in tone, usually some 2 min after addition of the agonist. The contact time for VIP and ATP was 2 min and following washout muscle tone was allowed to return to baseline for 15 min before raising it again with carbachol. There were two exceptions to this general procedure. When testing the effect of apamin which itself produced no inhibition of inhibitory field stimulation it was not necessary to produce repeated increases in muscle tone and therefore guanethidine-induced tone was sufficient (Figure 5a). Second, when the effects of haemolysed blood on inhibitory responses were studied (Figure 3) phentolamine (1 μM) was included in the Krebs solution in order to prevent any motor effects of blood which might be mediated through α-adrenoceptors. Phentolamine (1 μM) did not reduce tone. When responses to ATP were to be studied the muscle was incubated with indomethacin (2.8 μM) 1 h before beginning the experiment (Gibson & Wedmore, 1981).

For immunocytochemistry, the anococcygeus was dissected rapidly and frozen in isopentane cooled to the temperature of liquid nitrogen. The tissues were then freeze-dried overnight, vapour-fixed in diethylpyrocarbonate, embedded in paraffin, and sectioned (6 μM). Deparaffinized sections were then subjected to the peroxidase-antiperoxidase (PAP) immunocytochemical procedure (Sternberger, 1974) for the demonstration of VIP-immunoreactive sites. The VIP antiserum No 89N (kindly supplied by Prof. S. Said) was applied in a dilution of 1:5,000 to the tissue sections for 48 h. The affinity and specificity of this antiserum has been detailed elsewhere (Goyal *et al.*, 1980).

The following drugs were used: adenosine 5'-triphosphate disodium salt (Sigma); apamin (Sigma); carbachol (Koch Light); guanethidine sulphate (Ciba); indomethacin (Sigma); phentolamine mesylate (Ciba); 2-2'-pyridylisatogen tosylate (donated by Dr J.H. Botting); tetrodotoxin (Sigma); vasoactive intestinal polypeptide (Sigma, and donated by Prof V. Mutt).

The doses in the text refer to final bath concentrations.

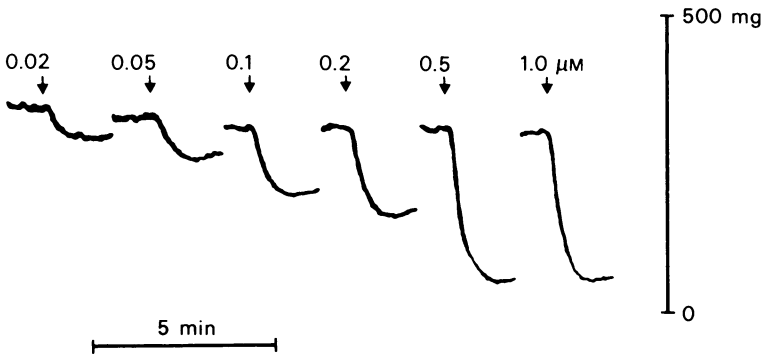

Figure 1 Vasoactive intestinal polypeptide (VIP)-induced relaxations of the mouse isolated anococcygeus. Muscle tone was raised by carbachol (10 μM).

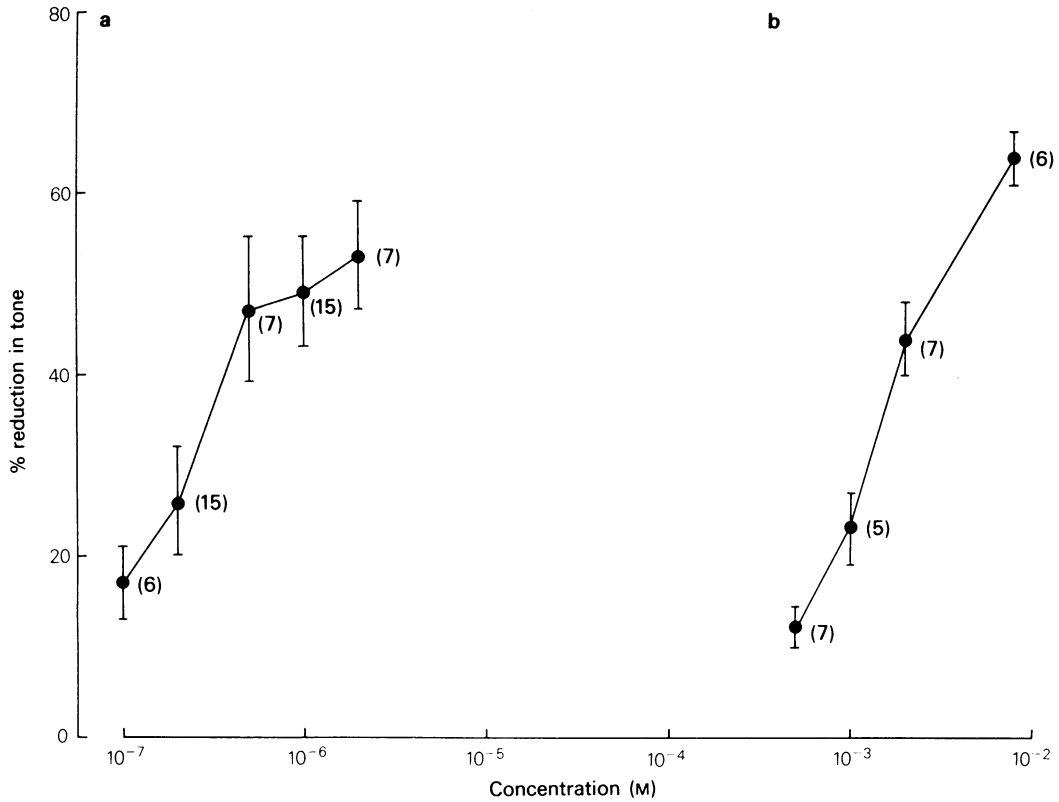

Figure 2 Dose-response curves for the relaxations of the mouse anococcygeus induced by vasoactive intestinal polypeptide (a) (VIP) or ATP (b). Relaxations were calculated as percentage reductions of carbachol (50 μM)-induced tone. Responses to ATP were obtained from tissues preincubated with indomethacin (2.8 μM; 1 h). Since indomethacin had no effect on the responses of the muscle to VIP the values shown in the figure include results from untreated and indomethacin-treated tissues. The values shown are means ± s.e. Figures in parentheses represent the number of observations.

Results

Dose-response relationships

When tone was raised by carbachol (10–50 μM), VIP produced dose-related relaxations (Figures 1 and 2); the threshold was approximately 20 nM. A comparison of the dose-response relationships for VIP and ATP showed the former to be approximately 10,000 times the more potent (Figure 2). Tetrodotoxin (0.5 μM), which blocks the responses of the mouse anococcygeus to inhibitory nerve stimulation (Gibson & Wedmore, 1981), had no effect on the relaxations produced by VIP or ATP.

The effect of haemolysed blood

Mouse blood was collected, in heparinized tubes, and haemolysed by making a 1:1 dilution with distilled water. Addition of 40 μl of haemolysed blood reduced greatly the inhibitory responses to field stimulation but had no effect on those produced by VIP or ATP (Figure 3). Although not shown in Figure 3, the effect of haemolysed blood on the responses to inhibitory nerve stimulation was reversed rapidly by washout. This inhibitory effect of haemolysed blood was not due to generalized blockade of neuronal activity; 40 μl of haemolysed blood did not reduce, but rather potentiated, the responses of the muscle to motor nerve stimulation, and by itself caused a small increase in tone.

The effect of apamin

In the presence of guanethidine to induce tone apamin (0.1–0.5 μM) had no effect on the inhibitory responses to field stimulation (Figure 4); higher doses (1 μM) reduced guanethidine-induced tone

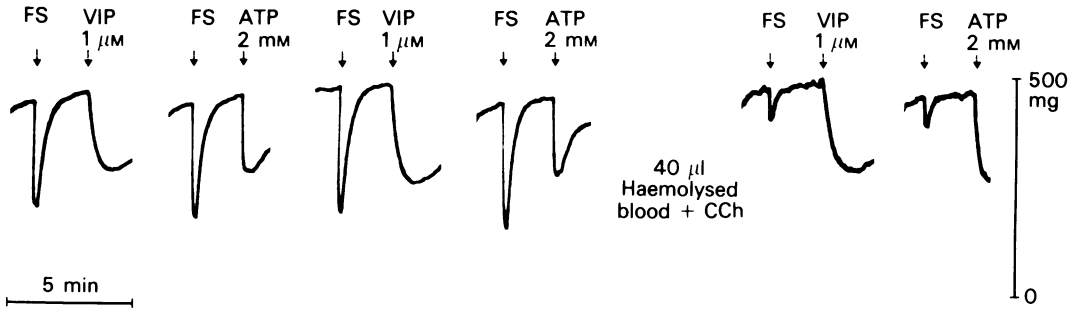

Figure 3 The effect of haemolysed blood (40 μl added to the bath 2 min before raising muscle tone with 50 μM carbachol (CCh)) on the responses of the mouse anococcygeus to 10 s trains of field stimulation (FS; 10 Hz; 1 ms pulse width; supramaximal voltage), vasoactive intestinal polypeptide (VIP) and ATP. The Krebs solution bathing the tissue contained phentolamine (1 μM) to block sympathetic responses and indomethacin (2.8 μM). Haemolysed blood reduced the effect of field stimulation but had no effect on VIP or ATP.

making it difficult to determine its effects on nerve-induced responses. Relaxations of carbachol-induced tone by field stimulation, VIP, or ATP were unaffected by 0.5 μM apamin (Figure 4).

The effect of 2-2'-pyridylisatogen tosylate (PIT)

Although incubation of the mouse anococcygeus muscle with PIT (50 μM; 30 min) greatly reduced the

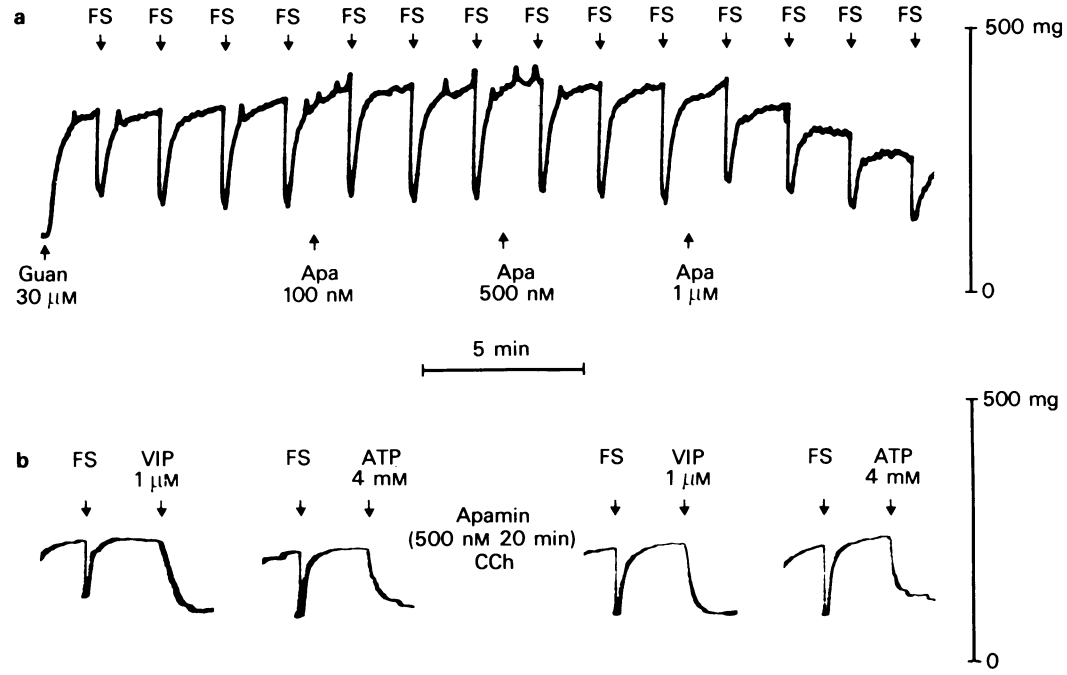

Figure 4 (a) The effect of increasing concentrations of apamin (Apa) on relaxations of the mouse anococcygeus in response to 10 s trains of field stimulation (FS; 10 Hz; 1 ms pulse width; supramaximal voltage), tone having been raised by guanethidine (Guan); 30 μM. (b) The lack of effect of apamin (0.5 μM added 20 min before raising tone with 50 μM carbachol (CCh)) on relaxations to vasoactive intestinal polypeptide (VIP), ATP, and to 10 s trains of field stimulation (FS; 10 Hz; 1 ms; supramaximal voltage). The muscle had been preincubated with guanethidine (30 μM; 15 min) in order to block sympathetic responses and the Krebs solution contained indomethacin (2.8 μM).

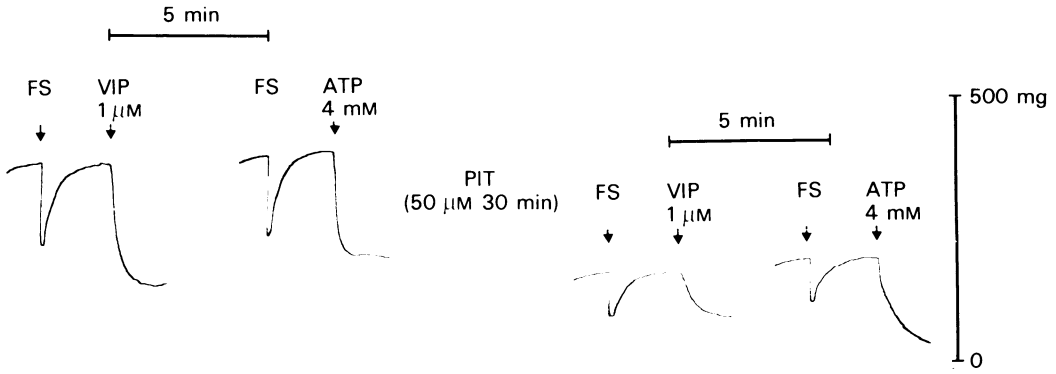

Figure 5 The effect of 2,2'-pyridylisatogen tosylate (PIT, 50 μM added 30 min before raising tone with 50 μM carbachol) on relaxations of the mouse anococcygeus to 10 s trains of field stimulation (FS; 10 Hz; 1 ms pulse width; supramaximal voltage) vasoactive intestinal polypeptide (VIP), and ATP. The muscle had been preincubated with guanethidine (30 μM; 15 min) in order to block sympathetic responses and the Krebs solution contained indomethacin (2.8 μM). PIT reduced muscle tone but did not block responses to field stimulation, VIP, or ATP.

ability of carbachol (50 μM) to raise tone (Figure 5), field stimulation, VIP, and ATP continued to relax the muscle.

The effect of prolonged inhibitory nerve stimulation

The duration of the trains of nerve stimulation commonly employed was 10 s. However, longer (10–20 min) periods of stimulation were used occasionally. As can be seen in Figure 6 the relaxation seemed to consist of two components; an initial rapid reduction in tone during the first 30 s of stimulation followed by a slower relaxation over 2 min. This biphasic relaxation has been observed in all muscles in which prolonged inhibitory nerve stimulation has been studied.

During prolonged inhibitory nerve stimulation muscle tone often returned towards prestimulation levels, although, in some cases (as in Figure 6) it was necessary to replace the carbachol in order to regain sufficient tone to permit examination of the inhibitory effects of drugs. During prolonged stimulation the inhibitory effects of VIP, but not of ATP, were reduced greatly (Figure 6). Exposure of the tissue to carbachol for 20 min, without field stimulation, did not affect the responses to VIP.

Immunocytochemistry

Treatment of sections of the mouse anococcygeus muscle with the PAP immunocytochemical procedure revealed bundles of VIP-immunoreactive sites, indicated by dark staining, running through the muscle layers (Figure 7).

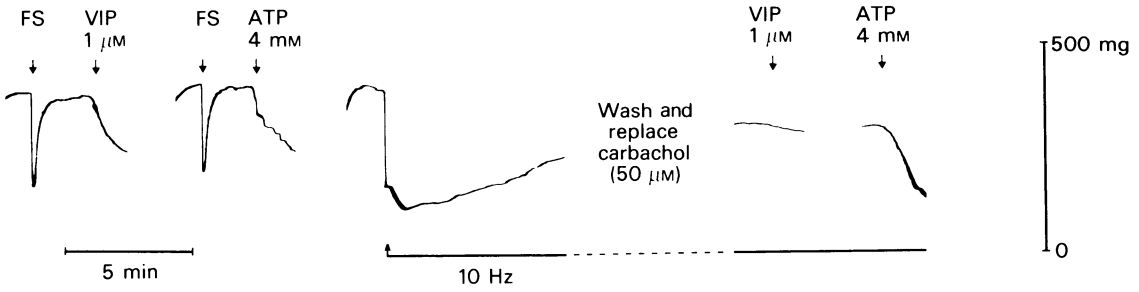

Figure 6 The effect of prolonged inhibitory field stimulation (20 min train; 10 Hz; 1 ms pulse width; supramaximal voltage; indicated by the arrow and line) on relaxations of the mouse anococcygeus in response to vasoactive intestinal polypeptide (VIP) and ATP. The muscle had been preincubated with guanethidine (30 μM; 15 min) in order to block sympathetic responses and the Krebs solution contained indomethacin (2.8 μM). Tone was raised by carbachol (50 μM). After 20 min of continuous field stimulation the carbachol in the organ bath was replaced. During prolonged inhibitory nerve stimulation the response to VIP, but not to ATP, was reduced. FS–field stimulation (10 s train; 10 Hz; 1 ms pulse width; supramaximal voltage).

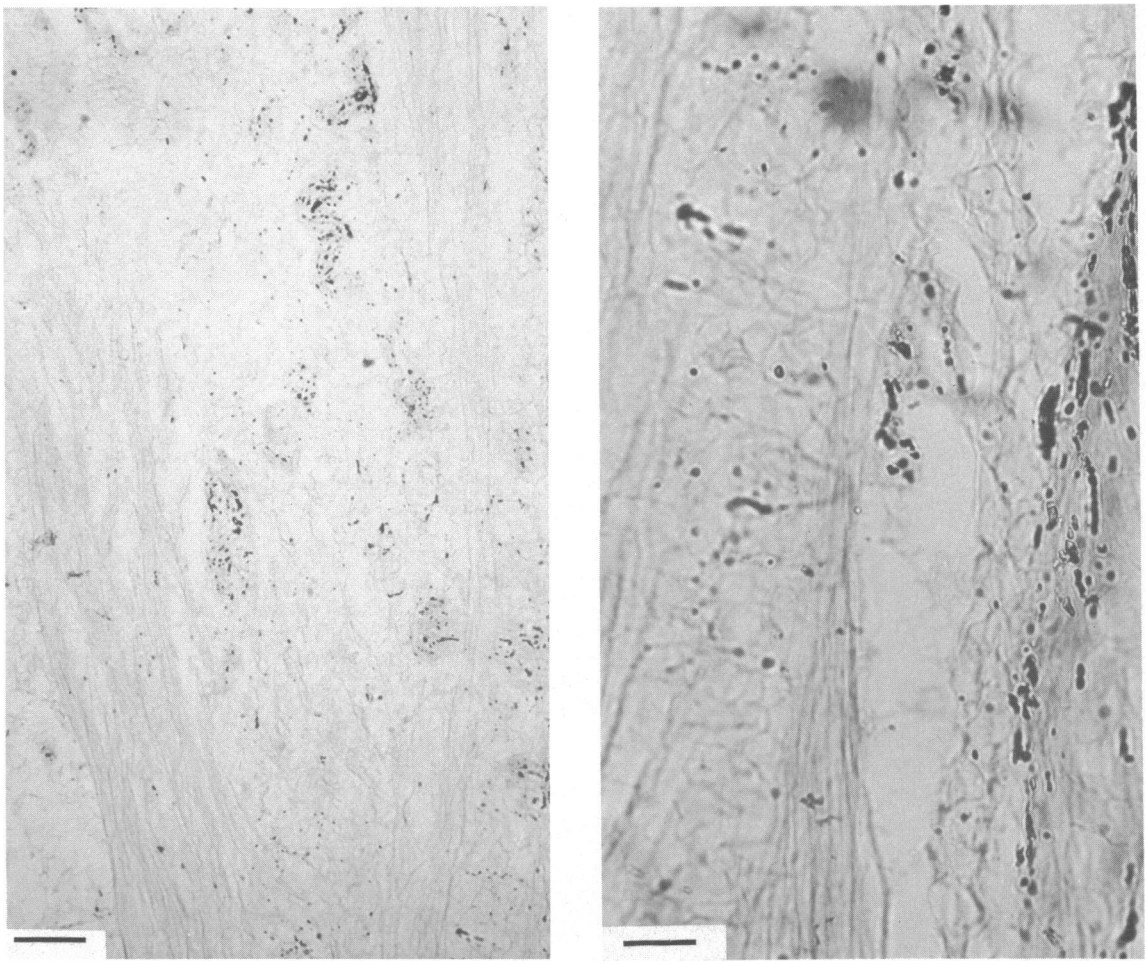

Figure 7 Photomicrographs showing dark-stained sites of vasoactive intestinal polypeptide ((VIP)-immunoreactivity in sections of the mouse anococcygeus subjected to the PAP immunocytochemical technique: (a) bundles of VIP-immunoreactive sites running through the muscle (bar = 50 μm); (b) higher magnification of one such bundle (bar = 20 μm).

Discussion

The present results confirm and extend the previous observation that VIP and ATP produce dose-related relaxations of the isolated anococcygeus muscle of the mouse (Gibson & Wedmore, 1981), with VIP some 10,000 times the more potent.

The mechanisms underlying non-adrenergic, non-cholinergic transmission in the mouse anococcygeus resemble those in the bovine retractor penis, but differ from those in the guinea-pig taenia caeci. Thus haemolysed blood reduced the inhibitory responses of the mouse anococcygeus and the bovine retractor penis to field stimulation, but not those of the guinea-pig taenia caeci (Bowman & Gillespie, 1981). Further, apamin, in low concentrations, failed to reduce the inhibitory responses of the mouse anococcygeus and the bovine retractor penis to field stimulation, but was effective in the guinea-pig taenia (MacKenzie & Burnstock, 1980; Bowman & Gillespie, 1981).

The identity of the mediator, or mediators, of the inhibitory responses to field stimulation in the anococcygeus remains unknown. Burnstock *et al.* (1978) have proposed ATP in the rat anococcygeus, based on evidence which has included the demonstration of quinacrine-staining nerve fibres and an increased overflow of ATP following field stimulation. Quinacrine-staining nerve fibres have also been detected in the mouse anococcygeus (Olson & Alund, 1979). The present study has shown that ATP produces relaxations of the mouse anococcygeus, although the doses required are high and the effect is

apparent only after preincubation with indomethacin as previously shown by Gibson & Wedmore (1981). PIT (Spedding & Weetman, 1976) was found to be unsuitable for use as an ATP antagonist in the mouse anococcygeus. The results present no contradictory evidence for a transmitter role for ATP in this tissue.

Some evidence that VIP should be considered as a possible mediator of inhibitory nerve responses in the mouse anococcygeus was obtained. First, VIP produced rapid relaxations in low concentrations. Second, the presence of VIP-immunoreactive sites in the muscle was demonstrated by immunocytochemistry. However, clarification of the exact location of these sites, whether neuronal or muscular, awaits more detailed histological examination involving selective denervation as suggested previously by Gibson & James (1977) and Olson & Alund (1979). Third, during prolonged inhibitory nerve stimulation the tone of the muscle gradually returned towards prestimulation levels, and at this time the responses to VIP, but not to ATP, were reduced. The attenuation of the responses to both inhibitory nerve stimulation and VIP suggests that there might be a common mechanism involved in the relaxation produced by these stimuli. It is likely that this mechanism is located postsynaptically, although the possibility that VIP might act by releasing the inhibitory transmitter cannot be excluded (Williams & North, 1979).

Thus, both VIP and ATP remain candidates for the role of inhibitory transmitter in the mouse anococcygeus. Indeed, it is not inconceivable that both might contribute to nerve-induced relaxations since there is histological evidence for the existence of both in the tissue (Olson & Alund, 1979; this study). The observation of a biphasic response to prolonged inhibitory nerve stimulation might also be of significance in this respect. The possibility of VIP and ATP acting as co-transmitters has already been raised (MacKenzie & Burnstock, 1980), and there is evidence that VIP might co-exist with acetylcholine in other parasympathetic fibres (Lundberg, 1981).

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References

BLOOM, S.R., POLAK, J.M., BISHOP, A.E. & BUCHAN, A.M.J. (1978). VIPergic nerves – the missing component in the autonomic nervous system. *Fedn. Proc.*, **37,** 482.

BOWMAN, ANNE, & GILLESPIE, J.S. (1981). Differential blockade of non-adrenergic inhibitory mechanisms in bovine retractor penis and guinea-pig taenia caeci. *J. Physiol.*, **317,** 92–93P.

BRYANT, M.G., BLOOM, S.R., POLAK, J.M., ALBUQUERQUE, R.H., MODLIN, I. & PEARSE, A.G.E. (1976). Possible dual role for VIP as gastrointestinal hormone and neurotransmitter substance. *Lancet*, **i,** 991–993.

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmac. Rev.*, **24,** 509–581.

BURNSTOCK, G. (1975). Purinergic transmission. In *Handbook of Psychopharmacology*. ed. Iversen, L.L., Iversen, S.D. & Snyder, S.H., pp. 131–194. New York: Plenum.

BURNSTOCK, G., COCKS, T., & CROWE, R. (1978). Evidence for purinergic innervation of the anococcygeus muscle. *Br. J. Pharmac.*, **64,** 13–20.

DANIEL, E.E. (1978). Peptidergic nerves in the gut. *Gastroenterology*, **75,** 142–145.

GIBSON, A. & JAMES, T.A. (1977). The nature of potassium chloride-induced relaxations of the rat anococcygeus muscle. *Br. J. Pharmac.*, **60,** 141–145.

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.

GOYAL, R.K., RATTAN, S., & SAID, S.I. (1980). VIP as a possible neurotransmitter of non-cholinergic, non-adrenergic inhibitory neurones. *Nature*, **288,** 378–380.

LUNDBERG, J.M (1981). Evidence for co-existence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. Morphological, biochemical, and functional studies. *Acta. physiol. scand.*, Suppl. 496.

MACKENZIE, I. & BURNSTOCK, G. (1980). Evidence against vasoactive intestinal polypeptide being the non-adrenergic, non-cholinergic inhibitory transmitter released from nerves supplying the smooth muscle of the guinea pig taenia coli. *Eur. J. Pharmac.*, **67,** 255–264.

MAXWELL, R.A. (1982). Guanethidine after twenty years: a pharmacologists perspective. *Br. J. clin. Pharmac.*, **13,** 35–44.

OLSON, L. & ALUND, M. (1979). Quinacrine-binding nerves: Presence in the mouse ano-coccygeus muscle, disappearance after muscle transection. *Med. Biol.*, **57,** 182–186.

SATCHELL, D.G. (1981). Nucleotide pyrophosphatase antagonists responses to adenosine 5′-triphosphate and non-adrenergic, non-cholinergic inhibitory nerve stimulation in the guinea-pig isolated taenia coli. *Br. J. Pharmac.*, **74,** 319–321.

SPEDDING, M. & WEETMAN, D.F. (1976). Identification of separate receptors for adenosine and adenosine 5′-triphosphate in causing relaxations of the isolated taenia of the guinea-pig caecum. *Br. J. Pharmac.*, **57,** 305–310.

STERNBERGER, L.A. (1974). *Immunocytochemistry*. New Jersey: Prentice-Hall.

WILLIAMS, J.T. & NORTH, R.A. (1979). Vasoactive intestinal polypeptide excites neurones of the myenteric plexus. *Brain Res.*, **175,** 174–177.

WILLIS, E., OTTESEN, B., WAGNER, G., SUNDLER, F. & FAHRENKRUG, J. (1981). Vasoactive intestinal polypeptide (VIP) as a possible neurotransmitter involved in penile erection. *Acta physiol. scand.*, **113,** 545–547.

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