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Evidence against VIP-involvement in neurogenic relaxations of the mouse anococcygeus muscle

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Vasoactive intestinal polypeptide (VIP) antiserum, α-chymotrypsin, and repeated exposure to VIP markedly reduced relaxations of the mouse anococcygeus muscle to VIP but not to field stimulation. This evidence suggests that VIP does not mediate non-adrenergic, non-cholinergic relaxations in the mouse anococcygeus.

The transmitter(s) mediating non-adrenergic, non-cholinergic (NANC) relaxations of the anococcygeus muscle remains unidentified. In recent years, three substances have emerged as candidates; adenosine 5′-triphosphate (Burnstock et al 1978), an extract of rat anococcygeus and bovine retractor penis (Gillespie & Martin 1980), and vasoactive intestinal polypeptide (VIP). The evidence supporting VIP has been threefold; (1) VIP has been detected by immunocytochemistry and by radioimmunoassay in the anococcygeus (Gibson & Tucker 1982; Hunter et al 1984; Larson et al 1985), (2) VIP is a potent relaxant of anococcygeus muscles from several species (Gibson & Tucker 1982; Hunter et al 1984), and (3) VIP desensitization occurs following a prolonged period of NANC stimulation (Gibson & Tucker 1982), which also reduces the second phase of nerve-induced relaxations. The first two pieces of evidence support the presence of VIP and its receptors in the anococcygeus, while the third suggests a link between VIP and neurogenic relaxations. In this paper, we report the results of some experiments designed to test more directly the proposition that VIP mediates NANC relaxations of the mouse anococcygeus.

Methods

Male mice (LACA strain; 25–35 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 1·0, $CaCl_2$ 2·5, $NaHCO_3$ 25·0, 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 tissue and changes in tension measured with a Grass FTO3 force-displacement transducer attached to a Lectromed pen-recorder. Field stimulation (10 Hz; 60 s train; 1 ms pulse width; 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 (Gibson & Tucker 1982). Drugs used during the study were; carbachol (BDH), α-chymotrypsin (Sigma), (Ac-Tyr[1], D-Phe[2])-GRF(1-29)NH_2 (Peninsula), guanethidine sulphate (Ciba), phentolamine mesylate (Ciba), vasoactive intestinal polypeptide (Sigma), vasoactive intestinal polypeptide antiserum (Peninsula).

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Results

VIP (0·05–2 μM) produced dose-related relaxations of carbachol (50 μM)-induced tone, as has been reported previously (Gibson & Tucker 1982). VIP, 1 μM reduced tone by about 50% and was used in all subsequent experiments.

The first procedure to be studied was VIP desensitization (Fig. 1). In these experiments, consecutive doses of VIP were added every 15 min without washout. The first dose produced a normal relaxation, which peaked within 2 min, and this was followed by a gradual recovery in muscle tone. Relaxations to the second and third dose of VIP were reduced greatly (Fig. 1); normal sensitivity to VIP returned 15 min after washout. In parallel experiments, the effect of VIP desensitization on NANC relaxations was studied (Fig. 1); at a time when VIP responses were reduced, NANC relaxations to field stimulation were potentiated (by 12 ± 3%, n = 8).

The proteolytic enzyme α-chymotrypsin (2 u ml^{-1}; 5 min incubation) slightly reduced the tone induced by 50 μM carbachol (by 15 ± 3%, n = 12). In the presence of the enzyme relaxations to VIP were abolished (Fig. 2a), although relaxations to field stimulation were unaltered.

The effect of VIP antiserum (1 : 50 dilution in the organ bath) is shown in Fig. 2b. Incubation with the antiserum for 30 min resulted in reduced responses to VIP (by 50 ± 2%, n = 3), with no reduction in relaxations to field stimulation.

It has been suggested that (Ac-Tyr[1], D-Phe[2])-GRF-(1-29)NH_2 acts as a VIP antagonist (Waelbroeck et al 1985). However, in the mouse anococcygeus it had no effect on relaxations induced byVIP or nerve stimulation, when used in concentrations up to 8 μM.

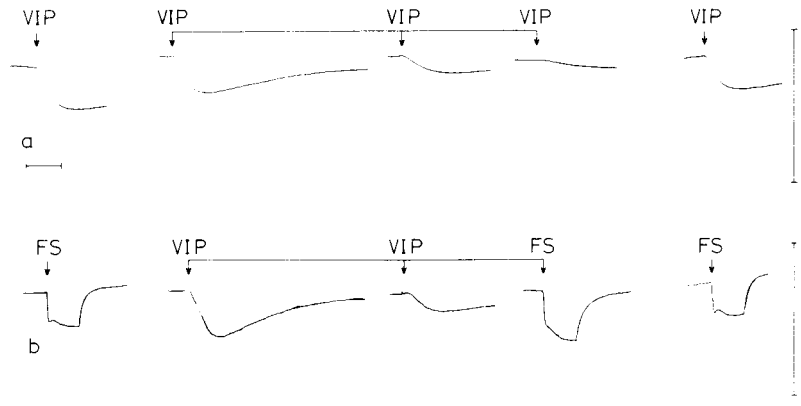

FIG. 1. Relaxations of mouse anococcygeus muscles in response to vasoactive intestinal polypeptide (VIP; 1 μM) and field stimulation (FS; 60 s train at 10 Hz). The time interval between each response was 15 min; muscle tone was raised with carbachol (50 μM). As indicated by the solid line, the organ bath was not washed out between the second and fourth response in each trace. In the upper trace, repeated exposure to VIP, without washout, resulted in desensitization (in four such experiments the third successive response was always reduced by more than 90%). The lower trace shows that repeated exposure to VIP, without washout, did not similarly reduce, but rather potentiated, relaxations to FS. In both cases, responses to VIP and FS returned to original values 15 min after washout (right hand response in each trace). Time calibration, 1 min; tension calibration, 0–500 mg.

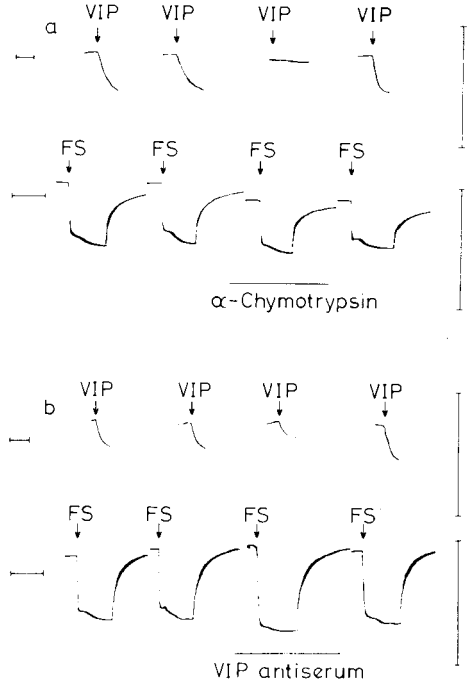

FIG. 2. Relaxations of mouse anococcygeus muscles in response to vasoactive intestinal polypeptide (VIP; 1 μM) and field stimulation (FS; 60 s train at 10 Hz). Tone was rasied with carbachol (50 μM). The time interval between each response was 15 min in (a), and 35 min in (b). The traces in (a) show the effect of α-chymotrypsin (2 u ml^{-1}; 5 min incubation), while those in (b) show the effect of VIP antiserum (1 : 50 dilution; 30 min incubation). Both procedures reduced relaxations to VIP but not to field stimulation. Time calibration, 1 min; tension calibration, 0–400 mg.

Discussion

The evidence from the experiments reported in this paper suggests that VIP is not involved in neurogenic relaxations of the mouse anococcygeus muscle, since three procedures (repeated exposure to VIP, α-chymotrypsin, and VIP antiserum) markedly reduced relaxations induced by VIP but not those induced by NANC stimulation. It could be argued that α-chymotrypsin or VIP antiserum might not reach sufficiently high concentrations within the synapse to block the actions of neurally-released VIP. However, α-chymotrypsin has been shown to block NANC transmission in dog muscularis mucosa (Angel et al 1983), and VIP antiserum inhibits tracheal NANC activity (Matsuzaki et al 1980).

Probably the most convincing evidence for or against a neurotransmitter role for VIP would come from experiments using selective VIP antagonists. It was for this reason that the experiments with (Ac-Tyr^1, D-Phe^2)-GRF(1-29)NH_2 were carried out. However (Ac-Tyr^1, D-Phe^2)-GRF(1-29)NH_2 did not inhibit relaxations to VIP, even in concentrations some 10 times higher than the reported K_i value (Waelbroeck et al 1985). The original experiments with this putative antagonist were carried out on rat pancreatic plasma membranes (Waelbroeck et al 1985); the lack of effect in the anococcygeus may suggest some difference in the VIP receptors of the two tissues.

Finally, if VIP is not involved in neurogenic relaxations of the mouse anococcygeus the question remains as to the function of the VIP-containing nerves within the tissue (Gibson & Tucker 1982; Hunter et al 1984; Larson et al 1985). If they are associated with the blood supply to the tissue, then it would have to be assumed that the peptide has a relatively efficient inactivation

mechanism, which prevents it from leaking to the surrounding anococcygeal smooth muscle in sufficient concentration to cause relaxation.

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The effect of digoxin-specific active immunization on digoxin toxicity and distribution in the guinea-pig

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In guinea-pigs intravenously infused with digoxin, prior immunization using a digoxin-human serum albumin conjugate increased by 3- and 2·4-fold, respectively, the digoxin doses causing the first signs of cardiotoxicity and death. At death, serum digoxin concentration was four times higher in immunized than in control animals. In the immunized guinea-pigs 50% of the serum digoxin was protein bound, presumably mainly to digoxin-specific antibodies, since in the controls the bound fraction was only 1–2%. Generally, tissue digoxin concentrations were not increased to the same extent as the lethal dose, and in the heart and lungs the increase was not significant. With cardiac (ventricle) subcellular fractions, there was no difference between control and immunized animals in the digoxin concentration of the 'microsomal' pellet. This subfraction contains the plasma membrane and the associated sodium pumps which are considered to be the sites at which the pharmacologically active digoxin binds. It seems likely, therefore, that the greater digoxin resistance in the immunized animals can be explained on the basis of reduced drug access to the site of action within the heart.

Schmidt & Butler (1971) showed that rabbits actively immunized using a digoxin-human serum albumin conjugate could tolerate intravenous digoxin doses of 0·6–0·9 mg kg^{-1}, while in control animals (immunized with albumin) a dose of 0·6 mg kg^{-1} was uniformly lethal. The present study, using guinea-pigs as a further digoxin-sensitive species, investigates more fully the protective effect of digoxin-specific immunization. The study involving continuous intravenous infusion of digoxin, determines more precisely the effect of immunization on the lethal digoxin dose, as well as measuring the digoxin concentrations in the heart and other tissues at the end of the infusion.

Materials and methods

Preparation of immunogen. A human serum albumin-digoxin conjugate was prepared by the method of Smith et al (1970) in which the terminal digitoxose residue of digoxin is oxidized with sodium metaperiodate so that the resulting dialdehyde reacts with a primary amino group of the albumin to form a Schiff base linkage. The bond is then stabilized by reduction with sodium borohydride. Using the spectrophotometric method of Smith et al (1970); it was estimated that the conjugate possessed 6–7 digoxin molecules per molecule of albumin.

Digoxin-specific active immunization. Female Dunkin-Hartley guinea-pigs between 6 and 9 months old (mean weight 0·95 kg) were injected (i.m. in the hind legs) with 0·8 ml of an emulsion containing equal volumes of Freund's complete adjuvant and 2 mg ml^{-1} (in saline) of human serum albumin (control) or human serum albumin-digoxin conjugate ('immunized'). The animals were used 9 weeks after immunization, and were allowed free access to food and water up to this time.

Digoxin infusion. Seven immunized and seven control guinea-pigs were anaesthetized with urethane (25%, 1·5 g kg^{-1} i.p.) and infused via a jugular vein with [^{3}H]digoxin (500 μg ml^{-1}, 10 μCi ml^{-1} in an aqueous vehicle containing 10% ethanol, 40% propylene glycol, 0·069% citric acid and 0·45% Na_2HPO_4) at a rate of 1·9 ml h^{-1} until death (no cardiac activity for more than 60 s). Cardiac activity was monitored by lead II electrocardiographic recording.

Treatment of tissue and blood samples. After infusion, tissues were removed and weighed, and blood samples (cardiac puncture) were allowed to clot to obtain serum. Protein (antibody)-bound serum digoxin was separated by precipitation in '50% saturated' ammonium sulphate solution (Hudson & Hay 1980).

Following the removal of small samples for the determination of tissue radioactivity, the ventricles

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