Neuropeptide-induced contraction and relaxation of the mouse anococcygeus muscle

(neurohypophysial peptides/neurotensin/thyrotropin-releasing hormone/urotensin II/vasoactive intestinal polypeptide)

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ABSTRACT  Isometric tension responses to neuropeptides were recorded from anococcygeus muscles isolated from male mice. This smooth muscle tissue is innervated by inhibitory nonadrenergic, noncholinergic nerves that resemble, ultrastructurally, the peptidergic neurons of the gastrointestinal tract; the physiological function of the anococcygeus is not known. Slow sustained contractions were produced by oxytocin (0.2–20 nM), [Arg8]vasopressin (0.4–200 nM), and [Arg8]vasotocin (0.4–100 nM); the mouse anococcygeus is, therefore, one of the few examples of nonvascular smooth muscle from male mammals to respond to low concentrations of oxytocin and related peptides. Substance P (0.5–8 μM) caused distinctive, biphasic increases in muscle tone of some, but not all, preparations. Other neuropeptides producing contractions were neurotensin (2–100 μM) and thyrotropin-releasing hormone (2–100 μM); the responses were of similar time course and displayed selective cross-desensitization, suggesting that these two peptides act through a common distinct mechanism. Tetradecapeptide somatostatin (10–80 μM) and its analog urotensin II (0.1–5 μM), a dodecapeptide from the urophysis of the teleost fish Gillichthys mirabilis, produced similar slowly developing relaxations of carbachol-induced tone. Piscine urotensin II, of which there are no reported effects on nonvascular mammalian systems, was 20–50 times more potent than somatostatin, a well-established mammalian hormone. Of the peptides studied, only vasoactive intestinal polypeptide (0.05–1 μM) caused rapid powerful relaxations in low concentrations; this is consistent with its proposed involvement in nonadrenergic, noncholinergic neurotransmission in the mouse anococcygeus.

The anococcygeus muscles are two thin sheets of smooth muscle that arise, separately, from tendinous origins on the posterior sacral vertebrae and run caudal around both sides of the rectum to unite on its ventral aspect; a physiological function of the tissue has yet to be elucidated (1). The muscles of all species studied to date (rat, cat, rabbit, dog, mouse, and ox) are innervated by motor sympathetic fibers, of which the transmitter is norepinephrine, and by inhibitory nonadrenergic, noncholinergic (NANC) fibers, of which the transmitter is unidentified (1–6). Ultrastructurally, the NANC nerves of the anococcygeus resemble the p-type fibers of the gastrointestinal tract, suggesting that the transmitter may be a peptide (7). It has been proposed recently that vasoactive intestinal polypeptide (VIP) might be involved in NANC transmission in the mouse anococcygeus (8). However, NANC relaxations of the mouse anococcygeus in response to field stimulation are complex, and it is possible that there is more than one NANC transmitter in this tissue (9). Consequently, we have investigated the effects of a wide range of neuropeptides on tone of the mouse anococcygeus in vitro.

MATERIALS AND METHODS

Male mice (LACA strain from A. Tuck & Son, Battlesbridge, Essex, U.K.; 25–35 g) were stunned and bled. Both anococcygeus muscles were dissected from the animal and set up in series, joined at the point of unification on the ventral rectum, in 1-ml glass organ baths that contained Krebs bicarbonate solution (mM: NaCl, 118; KCl, 4.7; MgSO4, 1.0; KH2PO4, 1.2; CaCl2, 2.5; NaHCO3, 25.0; glucose, 11.1). Organ bath temperature was maintained at 37°C and the Krebs solution was gassed continuously with 95% O2/5% CO2. A resting tension of 200–400 mg (1 mg tension = 9.8 μN) was placed on the tissue, and changes in tension were recorded by a Grass FT03 force-displacement transducer attached to a Lectromed pen-recorder. Each preparation was allowed to equilibrate for 30 min before the effects of neuropeptides were tested.

The anococcygeus muscle is unusually sensitive to substances that act by releasing norepinephrine from sympathetic nerve endings (1). To prevent such effects, each preparation was incubated with the adrenergic neuron-blocking drug guanethidine (30 μM) for 15 min before the experiment was begun (during the 30-min equilibration period) and, in addition, the Krebs solution contained the α-adrenoceptor antagonist phentolamine (1 μM) throughout.

Peptides were added to the organ bath in volumes not exceeding 50 μl. Since the mouse anococcygeus, in vitro, has no spontaneous tone, it was necessary to raise tone pharmacologically in order to determine, initially, whether each peptide would contract or relax the tissue. Tone was raised by the muscarinic receptor agonist carbachol, which was added at submaximal concentrations (2–4 μM) in order to produce a background tone of 250–350 mg. The maximal response to carbachol in this preparation is about 500 mg (6). If, in the initial experiment, the peptide produced further contraction of the tissue, then the experiment was continued in the absence of carbachol. If, however, the peptide relaxed the tissue, then carbachol was used routinely to raise tone. The peptide was not added until carbachol had produced a steady, sustained rise in tone (usually within 2–3 min). When the response to the peptide had been obtained, both it and the carbachol were washed out of the bath and muscle tone was allowed to return to base line for 15 min before it was raised again with carbachol. A peptide was considered inactive if it caused no significant change in carbachol-induced tone within 5 min. Each peptide was studied on a minimum of four separate muscle preparations.

Abbreviations: NANC, nonadrenergic noncholinergic; TRH, thyrotropin-releasing hormone; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide.

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The following drugs were used: carbachol (Koch-Light Laboratories, Bucks, England), glaucagon (Sigma), luteinizing hormone-releasing hormone (LHRH; Sigma), [Met]enkephalin (Cambridge Research Biochemicals), neurotensin (Sigma), oxytocin (preservative-free; Sandoz Pharmaceutical), somatostatin (tetradecapeptide; Sigma), substance P (Sigma), tetrodotoxin (TTX; Sigma), thyrotropin-releasing hormone (TRH; Sigma), urotensin II (synthetic Gillichthys; Peninsula Laboratories, San Carlos, CA), VIP (Sigma), [Arg]vasopressin (Sigma), [Arg]vasotocin (Sigma).

**RESULTS**

**Peptides Having no Effect.** The following peptides neither increased nor decreased carbachol-induced tone (all in concentrations up to 10 μM): luteinizing hormone-releasing hormone, [Met]enkephalin, and glucagon.

**Peptides Producing Contraction.** Oxytocin (0.2-20 nM), [Arg]vasopressin (0.4-200 nM), and [Arg]vasotocin (0.4-100 nM) produced slow, sustained contractions (Fig. 1). The characteristics of the responses to all three peptides were similar, with the exception that the contractions to oxytocin required a longer time (3-4 min) to reach equilibrium than those to the other two peptides (2-3 min).

Substance P (0.5-8 μM) produced a variable response, causing three preparations to contract but having no effect on two others. When present, the contractions produced by lower concentrations of substance P were biphasic (Fig. 2); at higher concentrations the two phases merged.

Neurotensin (2-100 μM) and TRH (2-100 μM) caused contractions that peaked within 2 min but were poorly sustained (Fig. 3). If either peptide was left in contact with the tissue, tone returned to baseline within 5 min and, at this time, the responses of the tissue to further doses of the peptide were reduced greatly, although carbachol (4 μM) gave its normal 250- to 350-mg rise in tone (Fig. 3 a and b). Peptide responses were restored 20 min after washout.Because of the similar-

ity of the responses to neurotensin and TRH, it seemed possible that these peptides were acting via a common mechanism. This possibility was confirmed by the type of experimental shown in Fig. 3c. When the muscle was exposed to TRH it became desensitized to neurotensin and vice versa, but in neither case was the response to carbachol reduced. In other experiments it was found that contractions in response to [Arg]vasotocin were normal in muscles desensitized to TRH and neurotensin. These experiments show that the responses of the mouse anococcygeus to TRH and neurotensin display selective cross-desensitization.

**Peptides Producing Relaxation.** Tetradecapeptide somatostatin (10-80 μM) produced slowly developing relaxations of carbachol-induced tone (Fig. 4 a and b), which required 5 min to reach equilibrium. At higher concentrations these relaxations were preceded by small transient contractions. The somatostatin analog urotensin II (0.1-6 μM), a 12-amino-acid neuropeptide from the urophysis of the teleost fish* Gillichthys mirabilis* (10), produced changes in tone similar to those of somatostatin but was 20-50 times more potent (Fig. 4 c and d). The relaxations produced by both peptides were well sustained and showed no sign of desensitization. The effects of somatostatin and urotensin II were not blocked by TTX (1 μM), which abolishes nerve-mediated relaxation of the mouse anococcygeus (9).

VIP (0.05-1 μM) produced a different pattern of relaxation (Fig. 5); the response was much more rapid, equilibrium occurring within 2 min. These relaxations were also unaffected by TTX (1 μM).

**DISCUSSION**

This study has shown that a variety of neuropeptides produce distinctive changes in tone of the mouse anococcygeus. Peptide-induced contractions were obtained in muscles that were both pretreated with guanethidine and maintained in phentolamine-containing Krebs solution, indicating that nor-

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**Fig. 1.** Contractions of mouse anococcygeus muscle preparations exposed to oxytocin (Oxy; a), [Arg]vasopressin (AVP; b), and [Arg]vasotocin (AVT; c). After each response the organ bath was washed out and muscle tone was allowed to return to baseline. The time interval between each break in the trace was 20 min. Time calibration, 1 min; tension calibration, 0-400 mg.
epinephrine, released from sympathetic nerve endings, did not contribute to the effect. Relaxations were not blocked by TTX and, therefore, did not result from generation of action potentials in NANC nerves, with subsequent release of inhibitory transmitter. Thus, it is likely that the contractions and relaxations observed were due to a direct effect of the peptides on smooth muscle.

The mouse anococcygeus appears to be one of the few examples of nonvascular smooth muscle from male mammals to respond to low concentrations of neurohypophysial hormones and to have greater sensitivity to oxytocin than to vasopressin. The anococcygeus is associated with both the lower alimentary tract and male urogenital tract, since some of its smooth muscle cells merge with the longitudinal muscle of the rectum while others, in some species, merge with the retractor penis (1). This may be of significance because vasopressin is known to cause certain gastrointestinal smooth muscles to contract (11), albeit in some cases by an indirect action (12), while there are reports that oxytocin may activate smooth muscle of the male reproductive system (13). The literature on the effects of neurohypophysial peptides on the anococcygeus muscles of species other than the mouse is confusing. Vasopressin has been reported to cause the rat anococcygeus to contract (3), although other workers found that vasopressin had no effect on this species but did cause the dog anococcygeus to contract (5). Further, vasopressin is relaxant in the cat anococcygeus (3). It may be that the effects of these peptides are modified by certain conditions, such as hormonal status, as has been shown for oxytocin acting on the uterus (11) and, possibly, on the seminal vesicle (14). Certainly, the effects of neurohypophysial hormones on the anococcygeus merit further attention, since they may represent an as-yet-unknown physiological function for this family of peptides and for the muscle itself.

TRH and neurotensin have been localized, by immunocytochemistry and radioimmunoassay, in the gastrointestinal tract and have been shown to modify gastrointestinal motility (15-18). In the mouse anococcygeus they produce a distinctive pattern of contraction and display a selective cross-desensitization suggesting that, in this tissue, they may act through a common mechanism, possibly on the same receptor. They share a common NH2-terminal amino acid (pyroglutamic acid), and there are recent reports of interaction between these peptides in the brain (19, 20).

Somatostatin and urotensin II also produced similar responses in the mouse anococcygeus, in this case slow relaxations, and this is consistent with their belonging to the same family of peptides (10). Somatostatin has been found within

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**Fig. 2.** Contractions of a mouse anococcygeus muscle preparation exposed to substance P (SP). After each response the organ bath was washed out and muscle tone was allowed to return to base line. The time interval between each break in the trace was 20 min. Time calibration, 1 min; tension calibration, 0-250 mg.

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**Fig. 3.** Contractions of mouse anococcygeus muscle preparations exposed to neurotensin (NT; a, c) and TRH (b, c). After each response the organ bath was washed out and muscle tone was allowed to return to base line. The time interval between each break in the trace was 20 min. In a and b, the second panel from the right shows that if either peptide was left in contact with the tissue, tone returned to base line and responses to further doses of the peptide were reduced greatly, although carbachol (CARB; 4 μM) produced a normal 250- to 350-mg rise in tension. Peptide responsiveness was restored 20 min after washout. In c, the middle trace shows that if TRH was left in contact with the tissue, the responses to NT, but not to CARB, were reduced greatly. Thus, TRH and NT showed selective cross-desensitization. Time calibration, 1 min; tension calibration, 0-300 mg.
nerves in the gut and may modify gastrointestinal motility (15).
Urotensin II has hypertensive, smooth muscle-contracting, and osmoregulatory effects (21) in fishes and birds but was thought to be devoid of significant activity in mammalian systems (22). There is one report of urotensin II causing relaxation of rabbit aorta in vitro (23); however, this effect was believed to be due to oxidation of the catecholamines used to induce tone in the preparation, since the effect was absent when tone was raised by noncatecholamines. In the present study, tone was raised by carbachol, a noncatecholamine, and so the mouse anococcygeus appears to be a mammalian smooth muscle tissue directly sensitive to urotensin II. The mammalian muscle proved to be 20–50 times more sensitive to the piscine hormone than to somatostatin. The muscle also contracted in response to [Argivaso-tocin, which is found in some teleost fish urophyses in small amounts (24). The second major neuropeptide of the fish caudal neurosecretory system, urotensin I, was not studied; however, its analogues, sauvenile and corticotropin-releasing factor, had minimal or no effects (25).

Field stimulation of the mouse anococcygeus, using train lengths of 60 s, produces a biphasic relaxation due to activation of NANC nerves (9); there is an initial rapid reduction in tone during the first 10 s, followed by a slower relaxation over the succeeding 50 s. On the basis of immunocytochemistry, time course of relaxation, and sensitivity to block by various procedures, it has been suggested that VIP might mediate the second-phase relaxation (8, 9), similar to its proposed role as mediator of delayed vasodilatation in cat salivary glands and tongue (26, 27). The results of the present study are consistent with this hypothesis since, of the peptides studied, only VIP produced relaxations with a time course and intensity likely to reflect a neurotransmitter role. A high concentration of VIP has recently been reported in the stimulated penis of several mammalian species (28), into which anococcygeal muscle fibers are known to pass (1). Substance P has been reported to relax the rat anococcygeus (29), but was motor in the mouse. If VIP is responsible for the second-phase relaxation of the mouse anococcygeus, then the mediator of the first phase remains a mystery. It could be an as-yet-untested peptide, ATP (8, 30), or a substance, extracted from rat anococcygeus and bovine retractor penis, which has still to be characterized chemically (31).

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