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Excitatory motor innervation in the canine rectoanal region: Role of changing receptor populations

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1 Motor innervation in the canine rectoanal region was examined in isolated strips of the circular muscle layer. Contractile responses to electrical field stimulation began at lower frequencies and were more persistent in the internal anal sphincter (IAS) than in the rectum.

2 Motor innervation to the IAS was almost exclusively sympathetic, since it was blocked by guanethidine (Guan 3 μM) while the response in the proximal rectum was approximately 50% muscarinic, and sensitive to the M_3 selective antagonist 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, 0.1 μM) and 50% tachykinergic, and sensitive to the neurokinin 2 (NK_2) receptor antagonist GR 94800 (1 μM). From IAS to rectum there was a gradual shift in the relative contribution of intrinsic and extrinsic neural innervation.

3 Responses to exogenously applied transmitters exhibited a similar pattern to that observed with motor innervation. Norepinephrine (NE) was most potent in the IAS and acetylcholine (ACh) and NK-A were most potent in the proximal rectum. The responses were inhibited by prazosin, 4-DAMP and GR 94800 respectively.

4 A gradient in the density of adrenergic α_1, muscarinic and NK_2 receptors also existed from IAS to rectum as determined by measuring the binding of [^{3}H]-prazosin, [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB and [^{3}H]-SR-48968 to smooth muscle membranes.

5 In summary, these data suggest that the shift in motor innervation in the rectoanal region is achieved in part by changes in receptor populations available for activation by sympathetic and enteric motor neurons.

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Abbreviations: 4-DAMP, 4-diphenylacetone-N-methylpiperidine; HEPES, 4-[2-hydroxyethyl]piperazine-[2-N′-ethanesulfonic acid]; L-NNA, N^{ω}-nitro-L-arginine; QNB, quinuclidinyl benzilate; Tris-HCl, [hydroxymethyl]aminomethane hydrochloride

Introduction

The rectoanal region is the final site for controlling the storage, transport and evacuation of gastrointestinal contents. The internal anal sphincter (IAS) is usually closed and aids in maintaining continence, while during the defecation reflex it briefly relaxes to allow the passage of faecal matter. In contrast, the rectum is involved in storage prior to defecation as well as participating in the defecation reflex. These functional differences are associated with significant differences in the motility patterns in the two regions as well as the mechanisms controlling this activity. While the predominant inhibitory neurotransmitterin the dog colon is nitric oxide (Keef *et al*., 1994), the predominant excitatory motor control of the rectum is due to enteric cholinergic nerves, while extrinsic sympathetic nerves play an important role in the sphincter (Krier, 1989). This dichotomy of motorinnervation therefore provides a means to differentially control the motility patterns of the IAS and rectum. The goal of the present study was to examine in detail the transition in excitatory motor innervation occurring from IAS to rectum, and to determine the extent to which these changes are associated with differences in the receptor populations available for activation. The notion that postjunctional receptor populations differ throughout the rectoanal region for sympathetic, cholinergic, and tachykinergic nerves is established here for the first time and suggests that receptor distribution, rather than receptor sensitivity or amplification of signal transduction, may go a long ways towards explaining the physiological consequence of nerve stimulation.

Methods

Tissue preparation

Mongrel dogs of either sex were killed with an overdose of sodium pentobarbital (100 mg kg^{-1}) under an approved institutional animal use protocol. The pelvic rectoanal region was exposed by sawing through the midline of the pelvic

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bone. Incisions were made through the skeletal muscle on either side of the rectum and through the skin adjacent to the anus to allow removal of a 10–12 cm length of the anus and rectum. The dissected segment was cut open from anus to proximal rectum and washed repeatedly. All adhering skeletal muscle and glands were removed after pinning the segment in a dissecting dish containing oxygenated Krebs Ringer bicarbonate solution (KRB) of the following composition (in mM): NaCl 118.5, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, KH_2PO_4 1.2, dextrose, 11.0. This solution had a pH of 7.4 at 37°C when bubbled to equilibrium with 95% O_2-5% CO_2. Unless otherwise specified, all experiments were performed in the presence of 100 μM N^{ω}-nitro-L-arginie (L-NNA).

Contractile studies

Two mm wide strips of the tunica muscularis were cut parallel to the circular muscle fibres 1, 2, 4 and 8 cm from the anal verge. The mucosa was removed from strips by sharp dissection. Muscle strips were attached with sutures to a stable mount and to a Gould strain gauge and immersed in tissue baths containing 10 ml KRB solution, maintained at 37°C. A basal tension of 1 g was maintained for all muscle strips. Tissues were initially equilibrated by exposure to either 10 μM NE (strips obtained 1 or 2 cm from the anal verge) or 10 μM ACh (strips obtained 4 or 8 cm from the anal verge) for 3 min every 15 min. Experiments were undertaken after the tissue had reached a steady response to exogenous drug addition, which typically required around 2 h.

To determine the effects of various antagonists of neurotransmitter function, muscle strips were stimulated three times with electrical field stimulation (EFS; 15 Hz at 15 V and 0.3 ms pulse duration for 1 min) at 10 min intervals to ensure reproducible neural responses. Tissues without reproducible responses were discarded. Next, an antagonist of either adrenergic, cholinergic or tachykinergic responses was added to the bath and EFS repeated. The response obtained in the presence of antagonist was then normalized to the control response. Maximum contraction was defined as the response elicited with either 1 mM NE (for muscle strips 1 and 2 cm from the anal verge) or 3 mM ACh (for muscle strips 4 and 8 cm from the anal verge). In preliminary studies we tested combined drug addition (i.e., NE, ACh, NK-A, histamine) along with high KCl (100 mM) and found that single additions of either ACh (4 and 8 cm) or NE (1 and 2 cm) produced as much contraction as these combined additions.

Radioligand binding studies, membrane preparation

Circular smooth muscle preparations from eight dogs were obtained by dissecting off all mucosa and longitudinal muscle from three muscle sections, namely, 0–1.5, 4–5 and 7–8 cm from the anal verge. Muscles were then frozen in liquid nitrogen and powdered with a mortar and pestle cooled to the same temperature. Powdered samples were homogenized (1 : 1 wt/vol) for 30 s using a stainless steel tissue grinder at high speed in ice-cold sucrose buffer (HEPES 6 mM, sucrose 25 mM, pH 7.4) and brought to a final volume of 30 ml.

Samples were filtered using nylon strainers (100 μm) to remove connective tissue and then centrifuged for 5 min (4°C) at $500 \times g$ to remove connective tissue and to enrich the supernatant for smooth muscle plasma membrane (Schiemann *et al*., 1990). The supernatant from this low-speed spin was then centrifuged at high speed ($88{,}000 \times g \times 90$ min, 4°C) to form a pellet enriched in membrane suitable for radioligand binding. Pellets were again frozen and powdered in liquid nitrogen and stored at −80°C prior to use.

Powdered membrane protein (150–250 mg) was re-suspended in binding buffer (mM) TRIS-HCl 20, $MgCl_2$ 5 , and EDTA 1) and protein content determined using standard methods (Butcher & Lowry, 1976). For quantification of adrenergic and muscarinic receptors, [^{3}H]-radioligands were diluted in binding buffer such that equilibrium binding could be determined above and below the expected K_D for binding of the antagonist radioligand. For muscarinic receptors, the non-selective antagonist [^{3}H]-quinuclidinyl benzilate (QNB: sp. act. = 38 Ci mmol^{-1}) was employed at 0.008–2.0 nM, and non-specific binding determined in the presence of 1 μM atropine (Atr). For α-adrenergic receptors, the α_1-selective antagonist radioligand [^{3}H]-prazosin (sp. act. = 19.5 Ci mmol^{-1}) was employed from 0.032–8.0 nM and non-specific binding determined in the presence of non-radioactive prazosin (10 μM). For α_2 adrenergic receptors, the α_2 selective radioligand [^{3}H]-rauwolscine (sp. act. = 76 Ci mmol^{-1}) was employed from 0.2–20 nMM and non-specific binding measured in the presence of its racemate yohimbine (10 μM). For tachykinin receptors, the NK_2 specific radioligand [^{3}H]-SR-48968 (sp. act. = 27 Ci mmol^{-1}) was employed from 0.01–2.2 nM, while non-specific binding was measured in the presence of 1 μM SR-4896. The presence of NK_3 receptors was determined using the antagonist radioligand [^{3}H]-SR-20000 (sp. act. = 37 Ci mmol^{-1}) employed from 0.20–80 nM with non-specific binding measured in the presence of 10 μM non-radioactive SR-20000.

Assays (200 μg of membrane protein) were carried out at 30°C for 90 min in a reciprocating water bath. Total binding, performed in triplicate at each radioligand concentration, was defined as binding of the radioligand in the absence of non-radioactive competitor while non-specific binding was determined in duplicate in the presence of excess non-radioactive competitor. For studies of tachykinin receptors, equilibrium binding was sampled at 75 min incubation at 25°C. Bound and free radioligand were separated by filtration of reactions over Whatman glass fibre filters (α adrenergic and muscarinic receptors, G/F-D; tachykinin receptors, G/F-A) and analysed for radioactivity using a scintillation counter (Beckman LS6000Ic).

NK_3 receptor binding was examined in an effort to quantify the extent to which our smooth muscle membrane preparation may be contaminated with neuronal membranes. NK_3 receptors are prominent on neurons including those of the GI tract but not on GI smooth muscle (Holzer & Holzer-Petsche, 2001). As a positive control for NK_3 binding, we employed canine diencephalon membrane prepared in a fashion identical to that of smooth muscle membrane. Binding studies using the NK_3 specific radioligand [3H]-SR-20000, non-radioactive SR-20000 to define non-specific binding and brain membranes revealed a K_D of 19.5 nM and a density of 5600 fmols mg^{-1}. In rectoanal smooth muscle, total and non-specific binding of [^{3}H]-SR-20000 increased linearly and were indistinguishable, whereas detectable levels for receptor density is possible to levels as

low as 5 fmols mg^{-1} protein. We conclude that our preparation contained no quantifiable NK_3 receptors and thus was minimally contaminated with nerve membrane.

Data analysis

Radioligand binding data were analysed by computer assisted nonlinear least-squares regression using software particularly well suited to this purpose (GraphPAD Prism v. 3, GraphPAD Software, San Diego, CA, U.S.A.). For concentration-effect curves (Figures 1b, 6a, 7a and 8a) Prism® compares the best-fit values of two curves plotted as mean data ($n=5$) from a standard four-parameter logistic equation (dose-effect, variable slope) using a two-tailed paired t-test to compare a difference with the standard error of that difference reported by nonlinear regression.

$$t = \frac{M_1 - M_2}{\sqrt{SE_1{}^2 + SE_2{}^2}} \quad (1)$$

The numerator is the difference between best-fit values. The denominator is an estimate of the standard error of that difference, computed as the square root of the sum of the squares of the two standard error values.

Significant differences between means were calculated by a two-tailed Student t-test and values were considered significantly different when $P<0.05$. The levels of significance are indicated in graphs by the number of symbols. A single symbol indicates $P<0.05$, two symbols $P<0.005$ and three symbols $P<0.0005$.

Only one muscle strip per rectoanal region was used from any one animal thus 'n' values represent both the number of animals and the number of muscle strips used.

Materials

Drugs used in this study included the NK ligands [^{3}H]-SR-20000, SR-20000, [^{3}H]-SR-48968, SR-48968 purchased from Tocris Cookson (Ellisville, MO, U.S.A.): The adrenergic ligands [^{3}H]-prazosin and [^{3}H]-rauwolscine as well as the muscarinic ligand [^{3}H]-QNB were purchased from NEN (Perkin Elmer NEN Life Sciences, Wellsley, MA, U.S.A.): prazosin, yohimbine, Atr, tetrodotoxin, NE, NK-A, 4-diphenylacetoxy-N-methylpiperidie, ACh, N^{ω}-nitro-L-arginine, Guan, GR 94800, and GR 82334 were all purchased fromSigma (St Louis, MO, U.S.A.).

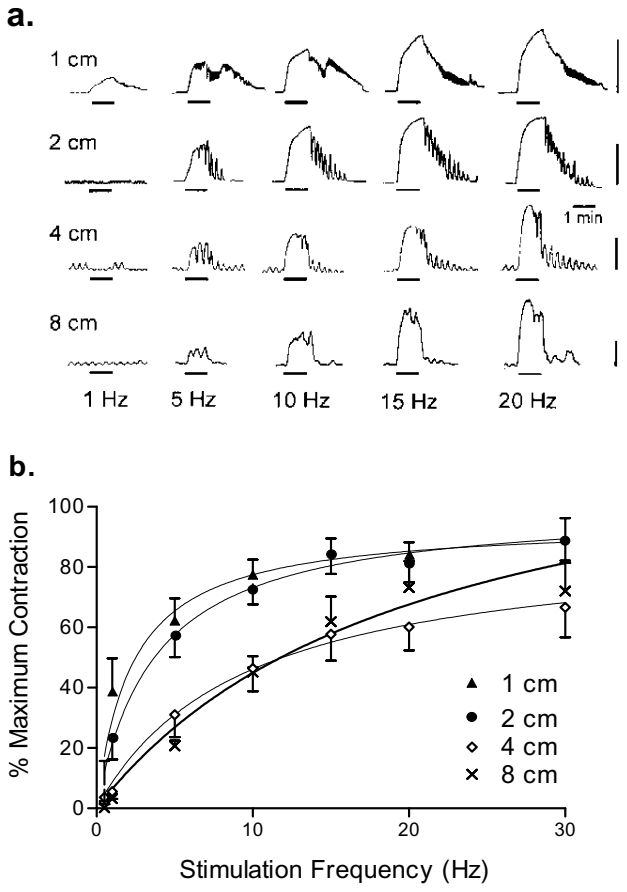

Figure 1 Frequency response relationships for stimulation of motor neurons in various segments isolated from the rectoanal region. (A) Sample traces of contractile responses recorded with increasing frequency EFS from muscle strips isolated 1–8 cm from the anal verge. Bar in each case represents 5 g tension. (B) Graph of frequency response relationship for nerve stimulation in muscle strips from the various regions. The relationships at 1 and 2 cm are significantly to the left of those at 4 and 8 cm (2 cm *versus* 4 cm, $P=0.0008$). Values are mean ± s.e.mean, $n=4-7$.

Results

Characteristics of excitatory neural responses in the rectoanal region

Contractile responses to electrical field stimulation (EFS, 1-30 Hz, 1 min, 15 V, 0.3 ms pulse duration) were recorded in strips of muscle isolated at various distances from the anal verge. Each region exhibited frequency dependent contractions (Figure 1a). Responses in the IAS (i.e., 1 and 2 cm from the anal verge) were not different from one another, nor were responses in the rectum (i.e., 4 and 8 cm from the rectum). However responses in the IAS were significantly to the left of those in the rectum: (2 cm *versus* 4 cm, $P=0.0008$) (Figure 1b). Neural response in the IAS also returned more slowly to baseline at the end of the stimulus train than those of the rectum. All responses to EFS were abolished by 1.0 μM tetrodotoxin (TTX, $n=3$).

Neural responses were normalized to the maximum contraction produced with either ACh, (4 and 8 cm) or NE (1 and 2 cm). The contraction recorded from segments of IAS was approximately half as large as that recorded in the rectum even though all muscle strips were cut to the same dimensions (i.e., 8.3 ± 0.6, 8.7 ± 1.2, 13.4 ± 1.3, 18.8 ± 1.8 g for 1, 2, 4 and 8 cm respectively, $n=8-9$). This difference likely reflects the greater amount of connective tissue septa present in sphincteric muscle *versus* rectum.

To investigate the neurotransmitter(s) responsible for EFS-induced contraction across the rectoanal region, experiments were first undertaken with Guan to block sympathetic responses and Atr to block cholinergic responses. Guan treatment led to almost complete blockade of the EFS-induced response (15 Hz) in the IAS (1 cm) whereas at the proximal rectum (8 cm) there was no significant reduction of the response to EFS in the presence of 3 μM Guan. The response in the presence of Guan at 8 cm was significantly

greater than responses at 1, 2 or 4 cm (8 cm *versus* 4 cm; 103±4% *versus* 81±8%, $P=0.032$) (Figure 2a). In 5 of 10 rectal muscle strips (8 cm) the response elicited in the presence of Guan was significantly enhanced (119±7% of control) whereas in the other five tissues, no significant difference was seen (92±2.3). Between 1 and 8 cm there was a stepwise increase in the 'Guan resistant' component of the neural response. The opposite pattern was observed for Atr (1 μM) blockade, that is, Atr (1 μM) produced significantly greater inhibition of the 8 cm strip than the 1 cm strip (Figure 2a). Some direct sympathetic motor innervation appears to extend at least 4 cm proximal to the anal verge since neural responses were blocked to a greater extent by combined Guan and Atr than with Atr alone at 4 but not 8 cm (Figure 2b). These data suggest that the predominant motor innervation of the IAS is sympathetic whereas in the rectum the neural response is due to ACh plus one or more other transmitters.

Excitatory neural response in the rectum It is known that tachykinins are often colocalized with ACh in enteric motor neurons in the gastrointestinal tract (Steele *et al*., 1991; Sang & Young, 1998). Thus, tachykinins are likely candidates for the noncholinergic, non-sympathetic component of the neural response in the rectum. To further investigate this possibility antagonists of tachykinin receptors were tested. Control responses to EFS (15 Hz) were initially obtained in the absence of antagonists. Thereafter Guan (3 μM) was added to the bathing medium and all subsequent responses were elicited in the presence of Guan. The role of NK receptors was then investigated by adding at 1 μM concentration, an NK_1 (GR 82334) and an NK_2 receptor antagonist (GR 94800). Neural responses were reduced by roughly half when either Atr (1 μM) or NK receptor antagonists were added to the bath whereas they were largely eliminated in the presence of combined Atr and NK receptor blockade (Figure 3a, $n=4-10$). These data suggest that the motor innervation of the rectum is comprised of a cholinergic and tachykinergic component. The same results were obtained when the M_3 muscarinic receptor antagonist 4-diphenylacetoxy-N-methyl-piperidine (4-DAMP, 100 nM) was used instead of Atr (Figure 3b).

To further investigate the non-cholinergic, non-adrenergic neural response, additional experiments were undertaken in which NK_1 and NK_2 receptor antagonists were tested separately. Responses to EFS (15 Hz) were significantly reduced by the NK_2 receptor antagonist GR 94800 (1 μM)

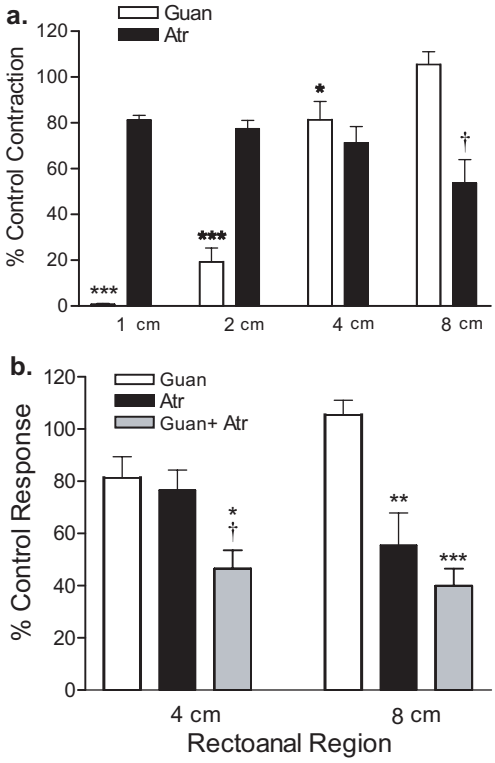

Figure 2 Comparison of the effects of Guan and Atr on responses to EFS (15 Hz) in segments isolated 1–8 cm from the anal verge. (A) Responses at 1, 2 and 4 cm in the presence of guan were significantly less (*) than at 8 cm. The response in the presence of atr at 8 cm was significantly less (†) than at 1 cm. (B) Comparison of drugs alone *versus* combined drug addition. *Indicates responses significantly less than guan alone. The response in atr plus guan at 4 cm was significantly less (†) than atr alone. Values are mean±s.e.mean, $n=5-13$.

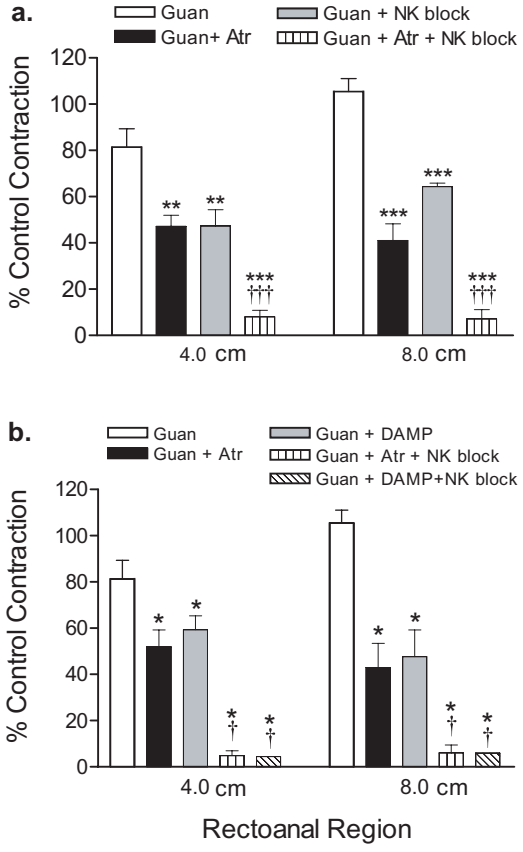

Figure 3 Effect of various antagonists on the Guan insensitive component of the neural response in the rectum (4, 8 cm). (A) Guan plus atr or neurokinin receptor blockade (NK block, GR 82334 plus GR 94800) each reduce the response to EFS (15 Hz) significantly more (*) than Guan alone. Combining all blockers produced greater inhibition (†) than guan plus atr. (B) Comparison of the effect of Atr to 4-DAMP (DAMP). There were no significant differences between Atr blockade *versus* 4-DAMP blockade. *Denotes responses significantly less than Guan alone. Responses with combined muscarinic and neurokinin blockade were significantly less (†) than muscarinic blockade alone. Values are mean±s.e.mean, $n=6-10$.

either alone or in combination with the NK1 antagonist (1 μM) but not by the NK_1 receptor antagonist GR 82334 alone suggesting a predominance of NK_2 receptors in the non-cholinergic response (Figure 4).

Excitatory neural response in the IAS In the vicinity of the IAS (1 and 2 cm) responses were either abolished or greatly reduced by application of the sympathetic neural blocker Guan. To investigate the role of α-adrenergic receptor subtypes in these responses we tested the α_1 adrenergic receptor antagonist prazosin (1 μM) and the α_2 adrenergic receptor antagonist yohimbine (1 μM). Control responses to EFS were first obtained in the absence of antagonists. Thereafter Atr (1 μM) was added to the bathing medium and all subsequent responses were elicited in the presence of Atr. Both prazosin and yohimbine significantly reduced responses to EFS but prazosin produced a significantly greater inhibition than yohimbine (Figure 5).

Responses to agonists in the rectoanal region

The differences in neural responses in the rectoanal region may be due in part to differences in the responsiveness of the muscle to the transmitters released from motorneurons. To explore this possibility we tested the concentration dependent effects of ACh, NK-A and NE in muscle strips isolated from the rectoanal region.

Functional response to ACh in the rectoanal region The concentration-dependent effects of ACh were tested by cumulative addition of drug (0.01 μM – 3 mM). All muscle strips (1 – 8 cm) contracted in response to ACh but differences were apparent from rectum to IAS. ACh was most potent and produced maximum contraction of the tissue in the proximal rectal segment (8 cm), whereas in the distal direction there was a successive decrease in the ACh response such that even 3 mM ACh produced less than a 50% maximum contraction in the IAS (Figure 6a). In these experiments we were struck by the relatively low potency of ACh in all muscle strips. To clarify this observation, additional experiments were undertaken using circular muscle strips isolated from the proximal colon, where responses to ACh have been well characterized (Huizinga *et al*., 1984; Keef *et al*., 1992). The effective concentration 50% for ACh incolonic strips ($EC_{50} \sim 1$ μM) was the same as that previously reported (Keef *et al*., 1992) and was significantly to the left of

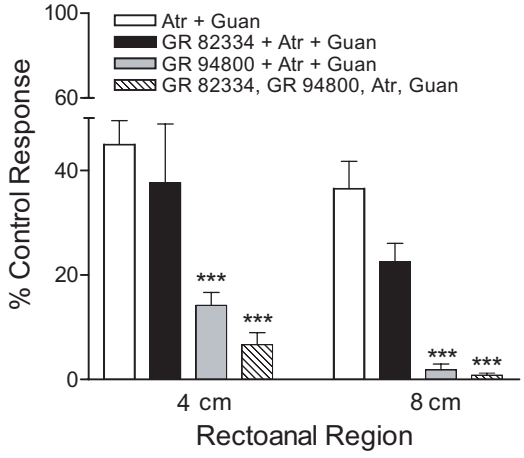

Figure 4 Comparison of the effect of NK_1 and NK_2 receptor antagonists on the Guan and Atr resistant component of the neural response in rectum (4.8 cm). Responses to EFS (15 Hz) were significantly (*) reduced by the NK_2 receptor antagonist GR 94800 (1 μM) either alone or in combination with the NK1 antagonist (1 μM) but not by the NK_1 receptor antagonist GR 82334 alone. Values are mean ± s.e.mean, $n = 4-9$.

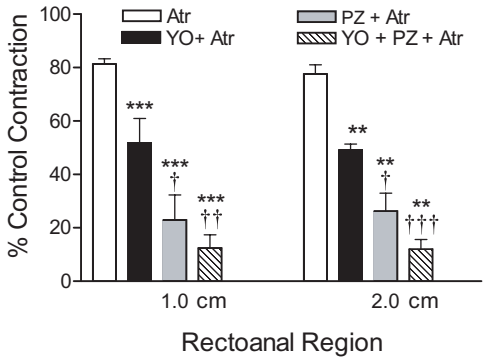

Figure 5 Comparison of the effect of prazosin (PZ) and yohimbine (YO) on the atr resistant component of the neural responses in the IAS. Responses to EFS (15 Hz) were significantly reduced (*) by PZ (1 μM), YO (1 μM) or PZ plus YO. In addition, responses in the presence of PZ or PZ plus YO were significantly (†) less than responses in the presence of YO alone. Values are mean ± s.e.mean, $n = 5-13$.

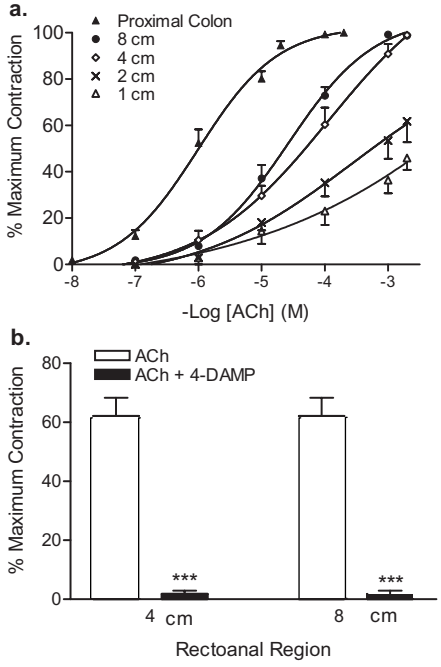

Figure 6 Effect of acetylcholine (ACh) and 4-DAMP (DAMP) in the rectoanal region and proximal colon. (A) Comparison of the concentration-response relationships for ACh ($n = 4-9$). Responses in the colon were significantly to the left of those in the rectum (8 cm, $P = 0.03$) and responses in the rectum were significantly to the left of those in the IAS (2 cm *versus* 4 cm, $P = 0.02$). (B) Effect of DAMP (100 nM) on the response to ACh (100 μM) in the rectum. The contractile response to ACh was blocked (*) by DAMP in both the 4 and 8 cm muscle strips. Values are mean ± s.e.mean, $n = 4-9$.

responses observed in the rectoanal region. These data suggest that there is a progressive decline in the potency of ACh in the large intestine from proximal colon to distal IAS. The contractile response to ACh (100 μM) in the rectal region was virtually abolished by 4-DAMP (0.1 μM) suggesting that it was due in large part to stimulation of muscarinic M_3 receptors (Figure 6b).

Functional responses to NK-A in the rectoanal region The two major tachykinins synthesized and released from enteric motor neurons are substance P (SP) and NK-A. Our experiments using neurokinin receptor antagonists suggest that the predominant postjunctional receptor involved in motor innervation is the NK_2 receptor. For this reason we examined the concentration-dependent effects of NKA which is more potent at NK_2 receptors than SP. Like ACh, there was a gradient in the responsiveness of muscle strips in the rectoanal region to NKA with the potency to NKA in the rectal segments (4 and 8 cm) being significantly greater (Figure 7a) than in the IAS (1 and 2 cm). The contractile response to NKA (1 μM) in the rectum was reduced by approximately 75% with the NK_2 antagonist GR 94800 (1 μM) compared to 10% with the NK_1 specific antagonist GR82334 (1 μM), suggesting that the response was largely due to stimulation of NK_2 receptors (Figure 7b).

Functional responses to NE in the rectoanal region The concentration-dependent effects of NE were tested by cumulative addition of drug (1 nM – 100 μM). All muscle strips (1 – 8 cm) contracted in response to NE but differences were apparent from IAS to rectum. NE was most potent and produced maximum contraction in the IAS (1 and 2 cm), whereas in the proximal direction the response to NE decreased such that even 100 μM NE produced only a 20% maximum contraction in the proximal (8 cm) rectum (Figure 8a). These data indicate that there is a progressive increase in the ability of NE to contract the tissue in the distal direction that corresponds to the progressive increase in the amount of the motor response that is Guan sensitive. The contractile response to NE (1 μM) in the IAS was reduced by yohimbine (1 μM) but virtually abolished by prazosin (1 μM) suggesting that it is due in large part to stimulation of α_1 adrenergic receptors (Figure 8b).

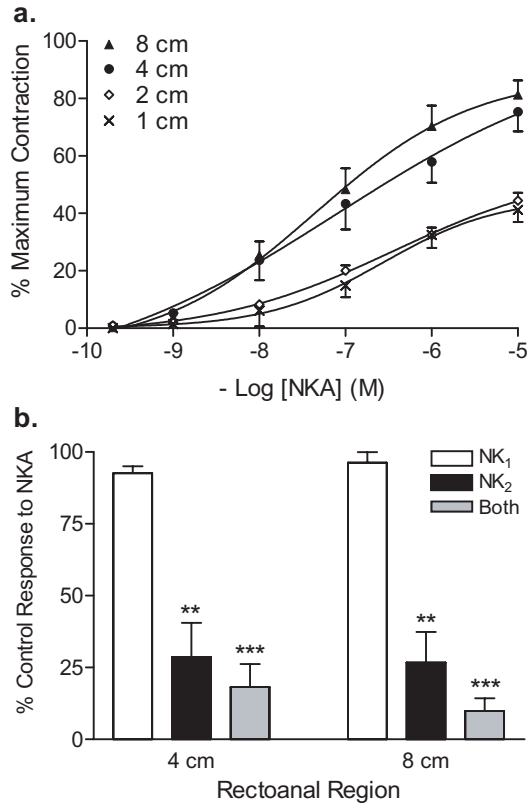

Figure 7 Effect of neurokinin A (NKA) and neurokinin receptor blockade in the rectoanal region. (A) Comparison of the concentration-response relationships for NKA in muscle strips isolated 1 – 8 cm from anal verge ($n = 5 – 6$). Responses in the rectum were significantly to the left of those in the IAS (2 cm *versus* 4 cm, $P = 0.02$). (B) Comparison of the effect of the NK_1 receptor antagonist GR 82334 (1 μM) and the NK_2 receptor antagonist GR 94800 (1 μM) on contractions elicited with NKA (1 μM). Responses were significantly (*) reduced by GR 94800 (1 μM) either alone or in combination with GR 82334 but not by GR 82334 alone. Values are mean ± s.e.mean, $n = 3 – 5$.

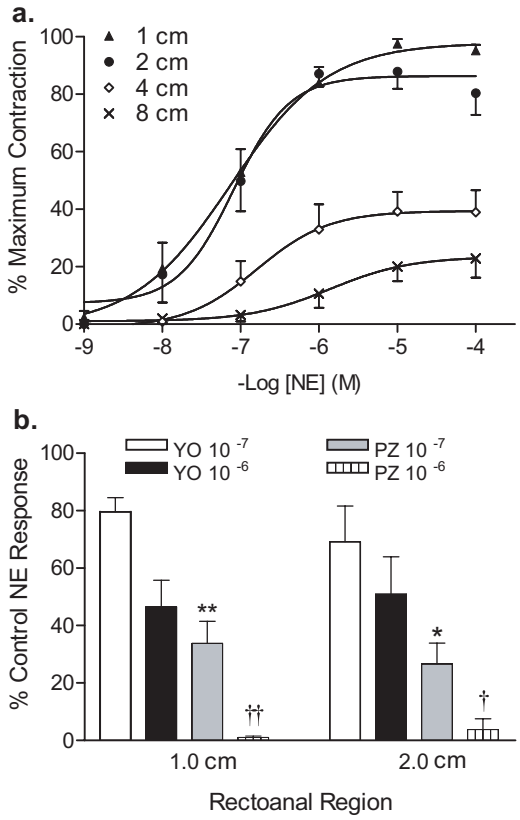

Figure 8 Effect of norepinephrine (NE) and adrenergic receptor blockade in the rectoanal region. (A) Comparison of the concentration-response relationship for NE in muscle strips isolated 1 – 8 cm from anal verge ($n = 3 – 9$). Responses in the IAS were significantly to the left of those in the rectum (2 cm *versus* 4 cm, $P = 0.03$). (B) Comparison of the effects of the α_1 adrenergic receptor antagonist PZ and the α_2 adrenergic receptor antagonist YO on contractions elicited with NE (1 μM). Responses in the presence of 0.1 μM and 1.0 μM PZ were significantly less (* and † respectively) than responses in the presence of 0.1 and 1 μM YO. Values are mean ± s.e.mean, $n = 3 – 6$.

Radioligand binding studies

Our results indicate that there is a gradient in the potency of NE, ACh and NKA, as well as their ability to produce a maximum contraction in the rectoanal region. One possible cause for these differences would be changes in the density of receptors on the smooth muscle cells throughout the rectoanal region. To explore this possibility we undertook radioligand binding studies of adrenergic, muscarinic and tachykinergic receptors in the rectoanal region. The sections evaluated were IAS (0–1.5 cm), mid-region (4–5 cm) and proximal rectum (7–8 cm).

Distribution of muscarinic receptors in the rectoanal region Muscarinic receptors were evaluated in the three rectoanal regions by measuring the binding of the non-subtype specific muscarinic antagonist [^{3}H]-QNB. The maximal binding (B_{max}) for [^{3}H]-QNB to smooth muscle partially purified from rectoanal tissues was greatest in the rectum and declined in the distal direction (Figure 9a). This gradient in receptor density corresponds well with the diminishing contractile response observed to ACh in the distal direction. The K_D for QNB was the same throughout the rectoanal region (360±33 pM). Previous studies from our laboratories have characterized [^{3}H]-QNB binding in the canine proximal colon (Zhang *et al*., 1991). The greater potency of ACh which we observed in the proximal colon in our earlier work *versus* that shown here for the rectum (Figure 6) is not the result of differences in muscarinic receptor density (Bmax) between these two regions because they are nearly identical (i.e., 120 *versus* 126 fmol mg^{-1} protein). It is likely that the difference we measure is reflected in the affinity (K_D) values for the radioligand (i.e., 90 pM for colon *versus* 360±0.03 nM across the rectoanal region).

Distribution of NK_2 receptors in the rectoanal region NK_2 receptors were evaluated in the three rectoanal regions by determining the amount of specific binding of the [^{3}H]-NK2 receptor antagonist SR-4896. Like ACh, the B_{max} for [^{3}H-SR-48968 binding was greatest in the rectum and declined in the distal direction (Figure 9b). This gradient in receptor density corresponds well with the diminishing responses observed with NKA in the distal direction. The density of NK_2 receptors was greatest in the proximal rectum (B_{max}=313 fmol mg^{-1}), and decreased toward the IAS region (68 fmol mg^{-1}). The affinity of the receptor for the NK_2 receptor radioligand [^{3}H]-SR-48968 was not significantly different across the rectoanal region (K_D=0.75±0.30 nM).

Distribution of alpha adrenergic receptors in the rectoanal region α-1 adrenergic receptors were evaluated in the three rectoanal regions by determining the amount of [^{3}H]-prazosin binding. The B_{max} for prazosin binding was greatest in the IAS and declined in the proximal direction (Figure 10). This gradient in receptor density corresponds well with the diminishing responses observed with NE in the proximal direction (Figure 8). The affinity of rectoanal α_1 adrenergic receptors for [^{3}H]-prazosin was the same throughout the rectoanal region and averaged 5.1±3 nM. This is consistent with the affinity of smooth muscle α_1 adrenergic receptors for prazosin. Since a component of the NE-induced contraction in the IAS was blocked by yohimbine we also evaluated α_2 adrenergic receptors using the antagonist [^{3}H]-rauwolscine. The density of the α_2 adrenergic receptors in the IAS was approximately 1/3 that of α_1 adrenergic receptor density (B_{max}=33 *versus* 94 fmol mg^{-1} respectively) while the affinity (K_D) for [^{3}H]-rauwolscine (5.6 nM) was similar to that of prazosin (5.1 nM). These values for α_2 adrenergic receptors are very similar to those previously reported for the proximal

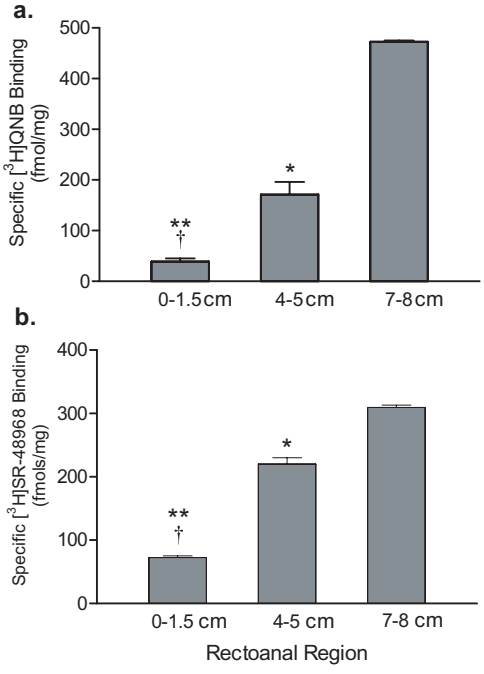

Figure 9 Density of muscarinic and NK_2 receptors in the rectoanal region. (A) Muscarinic receptors. [^{3}H]-quinuclidinyl benzilate revealed a gradient of receptors with the rectum (7–8 cm) containing significantly (*) more receptors than either the 4–5 cm region or the IAS (0–1.5). The IAS was also significantly (†) less than the 4–5 cm region (n=6–8). (B) NK_2 receptors. [^{3}H]-SR-4896 revealed a gradient of receptors with the rectum (7–8 cm) containing sigificantly (*) more receptors than either the 4–5 cm region or the IAS (0–1.5). The IAS was also significantly (†) less than the 4–5 cm region. Values are mean±s.e.mean, n=6–8.

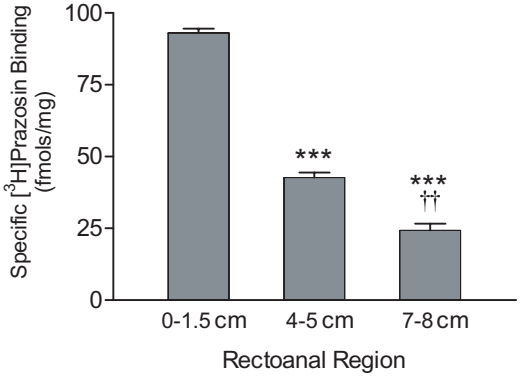

Figure 10 Density of α1 adrenergic receptors in the rectoanal region. [^{3}H]-prazosin revealed a gradient of receptors with the IAS (0–1.5 cm) containing significantly (*) more receptors than either the 4–5 or 7–8 cm regions. The 4–5 cm region was also significantly (†) greater than the 7–8 cm region. Values are mean±s.e.mean, n=6–8.

colon ($B_{max}=38$ fmol mg^{-1} protein, $K_D=5.1$ nM) while α_1 adrenergic receptors were not detected (Zhang *et al*., 1992). In contrast to the similar densities of α_2 adrenergic receptors in colon and IAS, NE produces less than a 20% maximum contraction in the proximal colon whereas NE maximally contracts the IAS. Thus, the greater response to NE in the IAS appears to be due to the presence of α_1 adrenergic receptors.

Discussion

Excitatory motor innervation undergoes marked changes from proximal rectum to distal IAS. In the IAS, motor responses are exclusively mediated by sympathetic nerves, whereas in the proximal rectum they are due to cholinergic and tachykinergic nerves. Our studies suggest that changes in functional motor innervation are associated with changes in the ability of NE, ACh and NKA to contract the tissue from rectum to IAS. Furthermore, radioligand binding studies, done for the first time to detect receptor density changes in the rectoanal region, suggest that these changes may be attributed, at least in part, to changes in the density of postjunctional receptors activated by NE, ACh and NKA.

In the dog rectoanal region nerve stimulation (EFS, 5 Hz) in the presence of antagonists of excitatory motor innervation (i.e., Atr, Guan and GR 98400) completely reverses the SP-induced contraction in both the IAS and rectum. This relaxation is abolished by either L-NA, the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-alpha]quinoxalin-1-one (ODQ) or TTX suggesting that it is due to release of NO from nerves (unpublished observations). This is not surprising since the predominant inhibitory neurotransmitter in the dog colon is also NO (Keef *et al*., 1994). NO has also been shown to play a significant role as an inhibitory neurotransmitter in the IAS of other species including man (e.g., Stebbing, 1998; Rattan & Chakder, 1992). Thus, all experiments in the present study were conducted in the presence of L-NA.

Both the rectum and IAS receive sympathetic nerve fibres that arise predominantly from the inferior mesenteric ganglion and travel in the lumbar colonic nerves (LCN) and hypogastric nerves (HGN) (Costa & Furness, 1973; Furness & Costa, 1973; Krier, 1989; Mizutani *et al*., 1992). Sympathetic nerves exert a tonic excitatory influence on the IAS since cutting the HGN and/or the LCN as well as high spinal anaesthesia lead to a significant fall in IAS tone in a number of species, including cat, dog, opossum and human (Hedlund *et al*., 1984; Garrett *et al*., 1974; Frenckner & Ihre, 1976; Rattan & Thatikunta, 1993). In contrast, the role of sympathetic nerves in modulating rectal motility is much less clear. Cutting the sympathetic nerves or applying epidural anaesthesia to the thoraco-lumbar region has generally been reported to enhance rectal motility (Carlstedt *et al*., 1988a, b), suggesting the predominance of a tonic inhibitory influence. This action would be commensurate with the well-established presynaptic inhibitory effects of sympathetic nerves mediated *via* α_2 adrenergic receptors, described for many portions of the gastrointestinal tract (Krier, 1989).

In the IAS the neural responses were abolished by Guan suggesting that excitatory motor innervation is almost exclusively sympathetic. In addition, responses in the IAS began at lower frequencies of stimulation than in the rectum and were more persistent in nature. The functional role of the IAS is to remain in a state of contracture most of the time to aid in maintaining continence. The high degree of sensitivity of this region to nerve stimulation would therefore allow for the maintenance of contracture with low frequencies of sympathetic input. Adrenergic α_1 receptors appear to dominate the post-junctional response in this region since prazosin was a more effective antagonist of nerve evoked contractions than yohimbine.

Sympathetic motor innervation extends at least 4 cm proximal to the anal verge, since combined Atr and Guan reduced the neural response to a greater extent than Atr alone (33% *versus* 53%, Figure 2b). In contrast, in the proximal rectum (8 cm) neural responses were slightly potentiated by Guan, and there was no difference in the blockade observed with Atr *versus* Atr plus Guan. The Guan-induced potentiation likely involves blocking presynaptic sympathetic inhibition of enteric motor nerves (Krier, 1989). The pattern of response to sympathetic nerve stimulation in the rectoanal region was very similar to the pattern of responses to the sympathetic neurotransmitter NE, i.e., responses to NE were greatest in the IAS and declined by 80% at 8 cm distance from the anal verge. The decline in functional response to NE was also associated with a 74% reduction in $\alpha 1$ adrenergic receptor density, suggesting that the decline in response to NE may be due to the decline in number of available α_1 adrenergic receptors. Previous studies report no detectable α_1 adrenergic receptor binding at all in the canine proximal colon (Zhang *et al*., 1992). In contrast, α_2 adrenergic receptor density in the IAS was only 35% of the α_1 adrenergic receptor density. The same density of α_2 adrenergic receptors are also present in the proximal colon (Zhang *et al*., 1992), and here NE produces less than a 20% maximum contraction. These data suggest that the greater response to NE in the IAS is related to the high density of α_1 adrenergic receptors present in this region.

In the proximal rectum neural responses were blocked by a combination of muscarinic and neurokinin receptor antagonists, suggesting that they are due to release of ACh and tachykinins from enteric motor neurons. Cholinergic responses were due predominantly to stimulation of M_3 muscarinic receptors, since they were blocked by the selective M_3 receptor antagonist 4-DAMP. The M_3 receptor is the predominant receptor coupled to contraction with ACh in the gastrointestinal tract (for review see Ehlert *et al*., 1999). The tachykinergic response on the other hand, appears to be due largely to NK_2 receptors. This is in agreement with studies of the canine proximal colon (Shuttleworth *et al*., 1993). Previous studies have suggested that ACh and tachykinins are typically co-localized in the same enteric motor neurons (Steele *et al*., 1991; Sang & Young, 1998). Interestingly, the tachykinergic component of the neural response in the rectum was greater than in the proximal colon (40–50% *versus* 30% of the motor response; Shuttleworth *et al*., 1993). Since the contractile response to ACh declines from proximal colon to rectum (Figure 6), it is possible that this difference reflects a diminished cholinergic response rather than an elevated tachykinergic response. In assessing the density of tachykinergic receptors in our tissue we have attempted to control for the presence of nerve membrane in our preparation. The notion that nerve membrane may contaminate preparations

from dissected tissues is long-standing, and various approaches have been taken to assess this concern. Here we employ the NK_3 receptor radioligand [^{3}H]-GR-20000 in both brain, as a control, and in our circular muscle preparation, and find that we can not detect any specific binding in the rectoanal smooth muscle. The limit of detection under our conditions is approximately 1% of receptor number using the tritiated ligand. Thus, our results can clearly be assigned to NK_2 receptors, based on minimal contamination and the use of an NK_2-specific ligand.

At a distance of 2 cm from the anal verge a small neural response remains in the presence of Guan (i.e., 20%) whereas at 1 cm distance the entire neural response was abolished. Thus enteric motor innervation extends to the proximal edge of the IAS but appears to play little role within the IAS. The decline in enteric motor response from rectum to IAS was associated with a similar decline in the functional response to ACh (60% decrease in maximal effect) and NKA (50% decrease in maximal effect). These changes were also associated with a dramatic reduction in receptor density of 98% in the case of muscarinic receptors and 78% in the case of NK2 receptors. Thus, as with adrenergic innervation, changes in receptor populations along the rectoanal region appear to play an important role in the differences that are manifest in motor innervation.

In conclusion, we have mapped the pattern of functional sympathetic and enteric motor innervation in the canine rectoanal region. The results reveal that innervation shifts from exclusively sympathetic in the IAS to exclusively enteric motor neurons in the rectum. These changes are reflected in differences in the response to exogenously applied neurotransmitter substances, as well as changes in the receptor populations available for activation by neurotransmitters. The heterogeneity of motor innervation in this region likely provides a means for independently controlling the motility patterns of the functionally distinct rectum and IAS.

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