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Regional differences in signalling transduction pathways among smooth muscle cells from rabbit colon

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Abstract

Smooth muscle cells (SMC) from the circular muscle layer of rabbit colon, taken from the proximal and distal regions that are known to have different physiological and motor activities, were used to highlight distinct regional intrinsic myogenic properties and to investigate the correlations between receptor and signalling transduction pathways. Contractile agonists were shown to be more potent on proximal than on distal SMC in inducing contraction and intracellular Ca^{2+} increase. Concentration–response curves of agonists-induced Ca^{2+} increase were constantly shifted to the right, though remaining parallel, with respect to contraction curves, independently of the region analysed. Using agents activating different steps of cAMP-or cGMP-mediated intracellular cascades, main regional differences were revealed as far as relaxation was concerned. Relaxation of proximal SMC was found to be essentially cGMP mediated, while that of distal SMC was cAMP mediated. In conclusion, the motor patterns of the two regions appear to be influenced by distinct regional biochemical characteristics that are intrinsic to colonic SMC.

Keywords: Smooth muscle cells; Relaxation; Contraction; Signal transduction

1. Introduction

Increasing literature data suggest regional heterogeneity in myogenic properties of smooth muscle throughout the gastrointestinal tract. Gastrointestinal motility is controlled by a neuroendocrine regulatory system in which the nervous apparatus, namely the enteric nervous system (ENS), shows an autonomous activity. The gastrointestinal muscle is arranged in two layers, the longitudinal and the circular muscle layers, while the ENS is organised in two major plexuses, the myenteric and submucosal plexus. The former, located between the two muscle layers, controls smooth muscle activity through excitatory (contractile) and inhibitory (relaxant) motorneurons. The excitatory fibres are mainly cholinergic and tachykinergic containing, respectively, as neurotransmitters, acetylcholine (Ach) and substance P/neurokinin A. The inhibitory fibres are mainly peptidergic and nitrergic, the main inhibitory neurotransmitters being respectively vasoactive intestinal peptide (VIP) and nitric oxide (NO), and to a lesser extent sympathetic and purinergic.

The regional heterogeneity has been observed either on isolated SMC or on the responsiveness of muscle strips to neurohumoral agents and has been suggested to be correlated to distinct motor functions. In the esophagus, contractile protein content and isoforms, as well as excitation–contraction coupling mechanisms, differ between "tonic" and "phasic" smooth muscle [1,2]. In the intestine, the increase in cytosolic Ca^{2+} necessary to activate the contractile machinery has been reported to be IP_3-dependent in circular, but Ca^{2+}-dependent in longitudinal smooth muscle cells (SMC) [3]. Regional heterogeneity in smooth muscle relaxation has been studied less extensively. Relaxation of gastrointestinal SMC is essentially mediated by either cAMP and/or cGMP-dependent signalling pathways. These two latter inhibitory pathways are differentially activated by NO and VIP. NO selectively activates the cGMP-dependent

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pathway while VIP, by interacting with different subtypes of receptors, activates both the cAMP- and cGMP-dependent pathways. The sympathetic agonists, through β-adrenergic receptors, activate only the cAMP-dependent pathway [4].

The large intestine, namely the colon, is peculiar for regional heterogeneity in that it presents two districts with distinct physiological functions that may be related to differences in motor activity [5]. The proximal colon is considered the primary site of storage for stools whereas the distal colon mainly promotes the expulsion of the faecal bolus. Several differences have been reported between these two regions. As far as the neural apparatus is concerned, differences between proximal ascending and distal descending colon has been observed in the nerve cell number [6] and in the number of NO containing neurons [7]. Regional different patterns of motor activities have also been reported in the basal contractile activity and in the neural cholinergic response [8] as well as in the mediators involved in smooth muscle relaxation [9]. The above functional studies have, however, been carried out on muscle strips, implying that the differences observed on motor activity could either be due to the neural and/or to the muscular apparatus.

The aim of the present study was to investigate the presence of regional intrinsic myogenic differences in the colon by evaluating, on smooth muscle cells isolated separately from the circular muscle layer of proximal and distal rabbit colon, the effect exerted by several contractile and relaxant regulatory agonists on biological activity (i.e., contraction and relaxation) and Ca^{2+} release. The inter-relationships between receptor and signalling transduction pathways coupling were also investigated.

2. Materials and methods

2.1. Chemicals

VIP and [βAla^8]-NKA (4-10) were obtained from Bachem (Bubendorf, Switzerland). Forskolin (FORSK), sodium nitroprusside (SNP), 1-[2-(carboxyoxazol-2yl]-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid, sodium salt (Fura-2-AM), were purchased from Calbiochem (La Jolla, CA). Collagenase CLS Type II and soybean trypsin inhibitor were obtained from Worthington (Freehold, NJ, USA); Eagle's minimum essential amino acid mixture was obtained from GIBCO (Paisley, UK). Carbachol (Cch), dibutyryl guanosine 3′,5′-cyclic monophosphate (DBcGMP), dibutyryl adenosine 3′,5′-cyclic monophosphate (DBcAMP), isoproterenol (ISOP), HEPES and other reagents were obtained from Sigma (St. Louis, MO).

2.2. Animals

Male New Zealand white rabbits (2–3 Kg, body weight) were purchased from Charles River Italia (Milan, Italy).

2.3. Isolation of dispersed muscle cells from the rabbit proximal and distal colon

Rabbits were sacrificed by stunning and bleeding. 10-cm segments of distal and proximal colon were excised with the aboral end cut nearly 1 cm above the pubis symphysis and the caecum, respectively. Both segments were immediately placed separately in ice-cold 25 mM HEPES buffer (pH 7.4) bubbled with oxygen. Mucosa and submucosa were removed by scraping from each sample. The circular muscle was then separated from the longitudinal muscle using a Stadie-Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA, USA) in the distal segment and by peeling in the proximal segment. The slices of circular muscle were then minced into pieces of 1 cm^2, and incubated at 31°C for two successive 60-min periods. The medium was (mM): NaCl (115), KCl (5.8), $KHPO_4$ (2.1), $CaCl_2$ (2.0), $MgCl_2$ (0.6), HEPES (25), glucose (14) plus 0.1% (w/v) collagenase (150 U/ml), 2.1% (v/v) Eagle's essential amino acid mixture and 0.01% (w/v) soybean trypsin inhibitor, pH 7.4. At the end of the second incubation period, the partly digested muscle strips were washed with enzyme-free medium on 500-μm Nitex mesh and resuspended in the same medium for 30 min to allow the cells' dispersion under slow mechanical agitation. The cells then were harvested through a 500-μm Nitex mesh. Trypan blue exclusion assay showed that 90%, or even more, of cells in suspension were viable.

2.4. Measurement of response in isolated SMC

Contraction and relaxation were measured in suspension of muscle cells as previously described [10, 11]. For contraction studies, 0.5 ml of cell suspension was added to 0.2 ml of medium containing the agent to be tested, and the reaction was stopped after a fixed interval, with acrolein (final concentration 1%). Relaxation was measured by pre-incubation of cell suspension with the relaxant agent for a fixed time, depending upon the agent to be tested, after which a maximal dose of a contractile agent was added and the reaction stopped with acrolein after the time required to elicit peak contraction. For measurement of control cell length, the agent was omitted and an equivalent volume of medium was added. The length of 50 cells in sequential microscopic fields was measured by image-scanning micrometry both in the control samples and upon addition of tested agents (Lasico, Los Angeles, CA). Contraction was expressed as the mean percentage decrease in cell length from control taken as 100%, while relaxation was expressed as percent inhibition of maximal contraction.

2.5. Measurements of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in SMC according to [12] by using the fluorescent Ca^{2+} dye Fura-2-AM. Dispersed cells were resuspended in medium containing (mM): NaCl (125), KCl (5), HEPES (10), $MgSO_4$ (0.5), $CaCl_2$ (1), glucose (5), taurine (20), sodium pyruvate (5) and creatine (5), pH 7.4. 0.5 ml of cell suspension (10^6 cells/ml) was incubated with FURA-2-AM (2 μM) for 30 min at 31°C, centrifuges at 350*g* for 5 min, and resuspended in fresh HEPES medium. Fluorescence was measured within 5 min. Maximal fluorescence was determined after addition of 100 μg/ml digitonin (81 μM) and minimal fluorescence was determined after addition of 7.5 mM EGTA plus 60 mM Tris-HCl, pH 10. Fluorescence was monitored at an emission wavelength of 510 nm using a Perkin-Elmer LS50 B luminescence spectrometer. Autofluorescence of unloaded cells was determined in each suspension and subtracted from fluorescence values of Fura-2-loaded cells. Intracellular Ca^{2+} levels were calculated from the ratio of observed minimal and maximal fluorescence intensities at 340 and 380 nm, using a dissociation constant of 224 nM, as described by [13].

2.6. Statistical analysis

Results were expressed as mean ± S.E. of *n* separate experiments and the data were calculated with Student's *t*-test ($p < 0.05$ was considered statistically significant). Cells for each experiment were obtained from different animals. The agonist concentration causing half-maximal response, indicated as ED_{50}, was obtained by linear regression analysis.

3. Results

SMC isolated from the proximal and the distal circular muscle layer of rabbit colon showed similar mean resting cell length (93.3 ± 3.4 and 88.8 ± 3.1 μm, respectively); the corresponding basal $[Ca^{2+}]_i$ levels were 105.3 ± 19.4 nM and 119.0 ± 12.7 nM. No regional differences were observed in maximal contractile response to the synthetic cholinergic agonist Cch (30 nM) and to the synthetic tachykinergic agonist [βAla^8]-NKA (4-10) (10 nM). These two agonists induced a similar maximal contraction, of about 20%, of proximal and distal colonic SMC irrespective of the agonist used and of the colon region considered. In fact Cch induced a decrease in cell length of 17.3 ± 0.9% in the proximal and 18.8 ± 1.1% in the distal whereas the responses when [βAla^8]-NKA (4-10) is used were 19.9 ± 1.0% and 20.7 ± 1.0%, respectively.

Dose–response curves were constructed with [βAla^8]-NKA (4-10) to investigate if the similar regional efficacy (i.e., maximal contraction) was associated to a similar potency. Dose–response curves displayed similar shapes

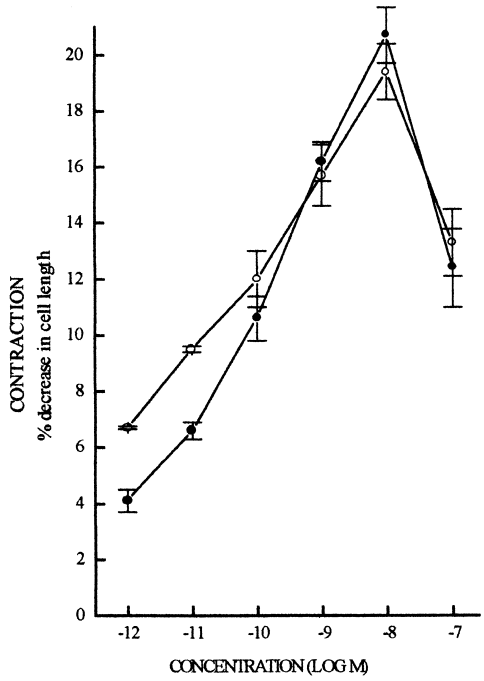

Fig. 1. Concentration–response curves for the contractile response to [βAla^8]-NKA(4-10) of SMC isolated from the proximal (○) and distal (●) rabbit colon. Data are the mean ± S.E. of three to five experiments.

in colonic proximal and distal colonic muscle cells, the maximal response being obtained at 10 nM of the agonist and a decrease in contraction appearing at supramaximal doses (Fig. 1). The tachykinergic agonist, however, resulted in being five times more potent on proximal, ED_{50}: 12.4 ± 4.6 pM, than on distal, ED_{50}: 63.3 ± 6.6 pM, colonic SMC ($p < 0.01$).

To investigate the relationship between contraction and intracellular Ca^{2+} increase, the concentration–response curve of [βAla^8]-NKA (4-10)-induced Ca^{2+} increase was constructed and compared to the contraction curve (Fig. 2). The tachykinergic agonist induce in both proximal (a) and distal (b) colonic SMC a dose-dependent Ca^{2+} increase whose concentration curves were parallel to that of contraction, being however shifted to the right. In both segments, maximal contraction was in fact observed in response to 10 nM of [βAla^8]-NKA (4-10) while maximal Ca^{2+} release was obtained in response to 1 μM of the agonist. The effect on calcium release resulted then to be shifted of two orders of magnitude with respect to the maximal contractile response. The shift in [βAla^8]-NKA (4-10) ED_{50} for contraction observed between the two segments was parallel to a shift in ED_{50} for intracellular Ca^{2+} increase. The tachykinergic agonist indeed resulted again in being more potent in inducing Ca^{2+} increase in proximal, ED_{50}: 3.3 ± 0.33 nM, than in distal, ED_{50}: 5.8 ± 0.76 nM, SMC ($p < 0.05$). Similar shifts in maximal concentration for contraction and Ca^{2+} increase were observed with Cch

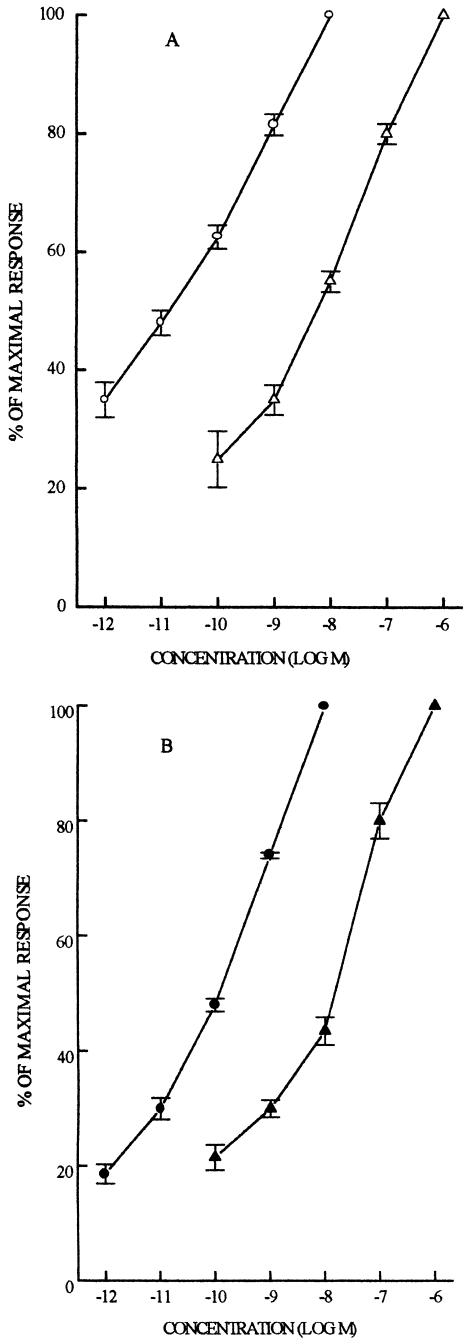

Fig. 2. Normalized concentration–response curves for contraction (○) and Ca^{2+} (△) increase to [βAla^8]-NKA(4-10) of SMC isolated from the proximal (A) and distal (B) rabbit colon. Data are the mean ± S.E. of three to seven experiments.

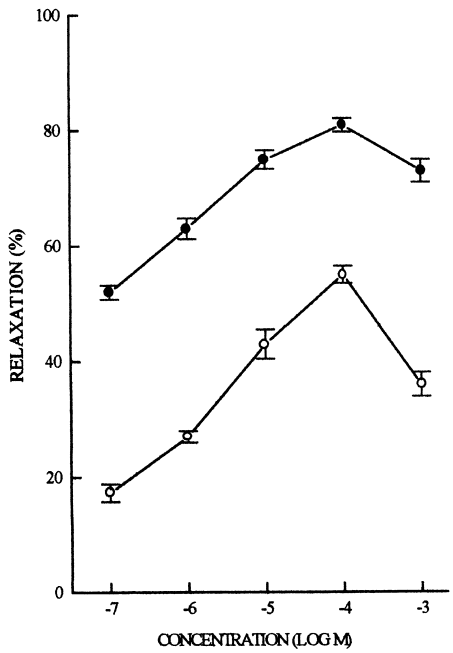

Fig. 3. Concentration–response curves for relaxation to ISOP of SMC isolated from the proximal (○) and distal (●) rabbit colon. Data are the mean ± S.E. of three to six experiments.

(data not shown). On both proximal and distal colonic circular SMC, Cch induced a maximal contraction at a dose of 30 nM, whereas maximal intracellular Ca^{2+} increase was at 3 μM.

Major regional differences between proximal and distal colon were observed in relaxation of SMC. VIP as well as the β-adrenergic agonist ISOP, two agonists activating preferentially the cAMP-dependent signalling pathway, presented a higher efficacy in inducing relaxation of distal than proximal muscle cells being the maximal relaxation 1.5 times higher in distal SMC. Indeed, the relaxation induced by VIP (1 μM) was 73.0 ± 5.6% in the distal and 40.0 ± 2.5% in the proximal ($p < 0.01$), whereas when ISOP 0.1 mM was used the values were 81.0 ± 2.5% and 55.0 ± 1.5%, ($p < 0.01$) respectively.

To better analyse the differences between the two colon segments, dose–response curves were constructed for ISOP-induced relaxation (Fig. 3). In both segments, 0.1 mM ISOP induced a maximal relaxation, while supramaximal doses caused a decrease in response. By comparing the ED_{50} values obtained from the linear regression analysis of the two dose–response curves, it appeared that ISOP, besides its higher efficacy, was also more potent on distal, ED_{50}: 0.79 μM, than proximal, ED_{50}: 5.5 nM, colonic muscle cells. Similar differences were observed in VIP-induced relaxation (data not shown) suggesting a prominent role of cAMP as an intracellular mediator of distal colon relaxation.

Nevertheless, the observed differences could be due to a different expression of receptors on the membrane of proximal and distal colonic SMC or to different post-receptor regulatory mechanisms. This means that relaxation of proximal colon, which is scarcely stimulated by the cAMP-dependent agonists, could be maximally activated by another transduction mechanisms, namely the cGMP-dependent signalling pathway. To explore this hypothesis and to determine whether the distinct regional relaxant behaviours were due to differences in the inhibitory intracellular signalling pathways, relaxation and Ca^{2+} increase were evaluated in response to

Table 1
Inhibition of Cch-induced contraction and intracellular Ca^{2+} increase by FORSK, DBcAMP, SNP and DBcGMP on SMC isolated from the proximal and distal rabbit colon.

	Proximal		Distal	
	Inhibition of contraction (%)	Inhibition of Ca^{2+} increase (%)	Inhibition of contraction (%)	Inhibition of Ca^{2+} increase (%)
FORSK 0.1 mM	30.9 ± 9.5	53.7 ± 13.0	73.8 ± 8.5	93.7 ± 6.3
DBcAMP 1 mM	20.9 ± 8.3	59.5 ± 9.6	63.8 ± 4.3	83.7 ± 8.8
SNP 1 μM	54.4 ± 7.4	85.3 ± 7.5	30.8 ± 5.3	36.3 ± 3.7
DBcGMP 1 mM	54.4 ± 8.3	66.3 ± 3.1	33.0 ± 7.5	37.5 ± 5.2

Note: Data are the mean ± S.E. of four to seven experiments.

agents that selectively activate the different steps of the cAMP- and cGMP-dependent intracellular cascades. For this purpose, a direct activator of adenylate cyclase, (FORSK), and a direct activator of guanylate cyclase, (SNP), as well as the permeant analogues of the second messengers cAMP and cGMP (DBcAMP and DBcGMP, respectively), were used (Table 1). Both c-AMP-dependent agonists, as already observed for the receptor agonists VIP and ISOP, were more efficacious in causing relaxation of distal than proximal SMC. In the former cells, relaxation induced by FORSK and DBcAMP were 2.5–3 times higher than in proximal SMC, further supporting the primary role of cAMP-mediated mechanisms in causing relaxation of distal colon. When compounds involved in the cGMP transduction pathway, namely SNP and DBcGMP, were used, an opposite trend was observed. Both the cGMP-dependent agents were more efficacious in inducing relaxation of proximal colonic muscle cells, supporting a prominent role for cGMP-dependent signalling pathway in causing relaxation of proximal colon. The distinct regional efficacy of cAMP- and cGMP-dependent agents in inducing relaxation of distal and proximal smooth muscle, respectively, was paralleled to their distinct efficacy in inhibiting Cch-induced Ca^{2+} increase (Table 1). FORSK and DBcAMP induced a higher inhibition of Ca^{2+} increase in the distal colon whereas the effect of SNP and DBcGMP was more pronounced on proximal SMC. Indeed the extent of the inhibition induced by these four agents on intracellular Ca^{2+} increase was higher than that on contraction, irrespective of the agonist used and of the colon region considered.

4. Discussion

In this study SMC isolated from the proximal and distal circular muscle of rabbit colon were used to highlight the hypothesis that intrinsic myogenic properties contribute to regional heterogeneity in colonic motor responses. Proximal and distal rabbit colonic SMC differ in sensitivity to contractile agonists and above all in the inhibitor intracellular transduction pathways mediating their relaxation. In the proximal colon, relaxation appears to be mainly mediated through the cGMP-dependent signalling pathway whereas in the distal colon the role of the cAMP signalling pathway appears to be prominent. Furthermore, this study shows that the stoichiometric relation between contraction and intracellular Ca^{2+} increase exhibit some peculiarities with respect to those previously observed in SMC isolated from different species and regions of the gastrointestinal tract.

Colonic motility depends on the interaction between the neural apparatus and the colonic smooth muscle. Regional heterogeneity between proximal and distal colonic muscles [8,14,15] and nerves [7] have been observed in different species, the different contribution of the two components in the overall motor activity, however, needing to be elucidated. In this context, the use of suspension of SMC isolated from the two colonic regions helps clarify the role played by the muscular component. Furthermore, in this cellular preparation, the regional differences in potency of contractile agonists can be studied more extensively because the isolated muscle cells as compared to strips show a higher sensitivity to agonists. This is due to the presence of high-affinity receptors on isolated cells; in fact, it has been suggested [16] that muscle cells in intact strips are exposed to a background of neurohumoral agents that interact preferentially with high-affinity receptors causing their desensitization [17]. The procedure of cell dispersion, by eliminating the neurohumoral environment, allows the re-expression of high-affinity receptors on the membrane. No major regional differences were observed between proximal and distal contraction in response to Cch, a synthetic cholinergic agonist, and [βAla^8]-NKA, a synthetic tachykinergic agonist. The choice of these two contractile agonists was determined by the prominent role played by cholinergic and tachykinergic motoneurons in inducing contraction of circular smooth muscle layer [18]. Differences in contractile behaviour were only observed in sensitivity to contractile agonists. [βAla^8]-NKA induced similar maximal contraction in both segments but was shown to be around five times more potent on proximal than distal colon. The proximal colon presented the higher sensitivity to the tachykinergic agonist since the ED_{50} of [βAla^8]-NKA

(4-10) on distal colon was similar to that reported for guinea pig gastric SMC [19] and three times higher than ED_{50} on circular SMC isolated from rat intestine [20].

The stoichiometric relation between contraction and intracellular calcium increase in colonic SMC differs from that previously observed in guinea pig gastric muscle cells [21], further supporting the heterogeneity of SMC throughout the gastrointestinal tract. In both colonic segments, concentration–response curve for contraction was to the left of the curve for Ca^{2+} increase, suggesting that contraction could be started by only a small fraction of the increased intracellular Ca^{2+}. Similar shift between agonist-induced contraction and intracellular Ca^{2+} has been reported in pancreatic acinar cells [22,23]. The observation that picomolar concentrations of [βAla^8]-NKA (4-10) cause contraction without a significant intracellular Ca^{2+} increase could be interpreted in several ways. This might be due to a low sensitivity and/or to the buffering capability of chelators like Fura 2 that could hide small increases in intracellular Ca^{2+} [24]. Besides, the methodology used might not allow observation of either calcium oscillations, known to be stimulated by low doses of agonists [25], or localized changes in Ca^{2+} concentrations [26]. It could in fact be possible that low concentrations of agonist mobilise superficial intracellular Ca^{2+} stores in rapid exchange with the extracellular solution [27], with only net Ca^{2+} changes in the whole cytoplasm being detected. The further increase in Ca^{2+} observed with supramaximal doses of agonist associated to a decline of contractile response also deserves some comment. It is possible that the increase in Ca^{2+} induced by supramaximal doses arises from a non-contractile compartment [28]. It is possible that the inhibition of contraction observed at supramaximal doses of agonist might be independent of intracellular Ca^{2+} or that high intracellular Ca^{2+} concentrations are inhibitory to the contractile response. The entity of the shift between dose–response curves for contraction and Ca^{2+} increase in colonic SMC is higher than that previously observed in intestinal circular SMC between IP_3-induced contraction and Ca^{2+} efflux [29], suggesting that other factors might be important in producing optimal contraction of colonic circular SMC [30].

The involvement of different inhibitory intracellular signalling mechanisms in mediating proximal and distal smooth muscle relaxation have been hypothesised by previous studies carried out on rat smooth muscle strips. These studies highlighted an essential role of NO in non-adrenergic non-cholinergic relaxation only of the proximal colon [9,31]. The peculiarities of the ENS innervating the proximal colon were ascribed to be responsible for this distinct relaxant behaviour since an increase in number of NO synthase-containing neurons and in NO synthase activity was observed in the myenteric plexus of the proximal colon, compared with that of the distal colon [7]. Our study demonstrates that, in rabbit, relaxation of the proximal colon is mainly cGMP-dependent due to a biochemical intrinsic myogenic property. Besides, our data indicate that relaxation of the distal colon is mainly cAMP-dependent, as suggested by the higher responsiveness of distal muscle cells to ISOP and to VIP. The regional differences between colonic SMC did not lie in the characteristics of the receptors for these two agonists and/or in their coupling mechanisms, since the distinct relaxant behaviours were still observed when relaxation was induced by direct activation of adenylate cyclase with FORSK or by DBcAMP. Differences in inhibitory signalling transduction mechanisms have already been reported between circular and longitudinal SMC isolated from guinea pig ileum [32]. Their distinct effects in inhibiting intracellular Ca^{2+} increase paralleled the distinct effects of cGMP- and cAMP-dependent agents in inducing relaxation of proximal and distal SMC. SNP and DBcGMP induced a higher inhibition of Ca^{2+} increase in proximal SMC, whereas the two cAMP-dependent agents displayed the higher inhibition of Ca^{2+} increase in distal colonic muscle cells. The respective degree of inhibition induced by cGMP- and cAMP-dependent agents in proximal and distal muscle cells, respectively, was however similar, suggesting that, as in gastric SMC [12], cGMP and cAMP appear to have converging mechanisms in inhibiting contraction, namely in inducing relaxation. Finally, the higher degree of inhibition induced by these agents on Ca^{2+} increase with respect to that on contraction is in agreement with the shift between dose–response curves for contraction and Ca^{2+} increase previously observed in this study.

In conclusion, this study highlights the heterogeneity of SMC throughout the gastrointestinal tract and indicates that regional intrinsic myogenic properties contribute to the overall regional patterns of motor activity. The distinct biochemical characteristics intrinsic to proximal and distal colonic SMC appear to influence the motor patterns of these two regions that are characterised by distinct physiological functions.

Acknowledgments

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