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Mechanisms involved in carbachol-induced Ca^{2+} sensitization of contractile elements in rat proximal and distal colon

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1 Mechanisms involved in Ca^{2+} sensitization of contractile elements induced by the activation of muscarinic receptors in membrane-permeabilized preparations of the rat proximal and distal colon were studied.

2 In α-toxin-permeabilized preparations from the rat proximal and distal colon, Ca^{2+} induced a rapid phasic and subsequent tonic component. After Ca^{2+}-induced contraction reached a plateau, guanosine 5′-triphosphate (GTP) and carbachol (CCh) in the presence of GTP further contracted preparations of both the proximal and distal colon (Ca^{2+} sensitization).

3 Y-27632, a rho-kinase inhibitor, inhibited GTP plus CCh-induced Ca^{2+} sensitization more significantly in the proximal colon than in the distal colon. Y-27632 at 10 μM had no effect on Ca^{2+}-induced contraction or slightly inhibited phorbol-12,13-dibutyrate-induced Ca^{2+} sensitization in either proximal or distal colon. Chelerythrine, a protein kinase C inhibitor, inhibited GTP plus CCh-induced Ca^{2+} sensitization in the distal colon, but not in the proximal colon. The component of Ca^{2+} sensitization that persisted after the chelerythrine treatment was completely inhibited by Y-27632.

4 In β-escin-permeabilized preparations of the proximal colon, C3 exoenzyme completely inhibited GTP plus CCh-induced Ca^{2+} sensitization, but PKC(19–31) did not. In the distal colon, C3 exoenzyme abolished GTP-induced Ca^{2+} sensitization. It inhibited CCh-induced sensitization by 50 % and the remaining component was inhibited by PKC(19–31).

5 These results suggest that both protein kinase C and rho pathways in parallel mediate the Ca^{2+} sensitization coupled to activation of muscarinic receptors in the rat distal colon, whereas the rho pathway alone mediates this action in the proximal colon.
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Keywords: Rat; proximal colon; distal colon; carbachol; Ca^{2+} sensitization; rho; protein kinase C

Abbreviations: CCh, carbachol; GTP, guanosine 5′-triphosphate; NAD, nicotinamide adenine dinucleotide; PKC(19–31), PKC inhibitor peptide; PDBu, phorbol-12,13-dibutyrate

Introduction

The contents of the rat proximal colon contain considerable amounts of water, whereas those of the distal colon are pellet-like feces containing little water. The primary role of the proximal colon is to mix colonic contents and absorb fluid, and that of the distal colon is to propel and eliminate feces (Mizuta *et al*., 1999). Phasic to- and -fro movements are dominant in the proximal colon, and propulsive movements and giant contractions are often observed in the mid- and distal colon (Sarna, 1993). We previously showed that circular (Hata *et al*., 1990) and longitudinal (Suthamnatpong *et al*., 1993) muscle of the rat proximal and distal colon exhibited different patterns of spontaneous contractile activities and different time-dependent changes of resting tone. Responses of the segments prepared from both regions to exogenously added nitric oxide were also different: nitric oxide induced significant relaxation in the proximal colon, but only slight relaxation in the distal colon (Maehara *et al*., 1994). In addition, the relaxant effect of cyclic GMP in α-toxin-permeabilized preparations was significantly greater in the proximal region than in the distal region (Takeuchi *et al*., 1997). Snape *et al*. (1989) also reported that responses of the rabbit proximal and distal colon to electrical field stimulation were different. These results suggest that intracellular mechanisms regulating contraction and relaxation differ between the proximal and distal colon.

In a variety of smooth muscle types, stimulatory agonists produce contraction through an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), which activates myosin light chain (MLC) kinase, with a simultaneous augmentation of the sensitivity of the contractile apparatus to Ca^{2+} (Ca^{2+} sensitization) (Somlyo & Somlyo, 1994). The Ca^{2+} sensitization of smooth muscle by agonists is well demonstrated in preparations made permeable with α-toxin or β-escin. In such plasma membrane-permeabilized smooth muscle tissues, agonists can increase contractile amplitude while $[Ca^{2+}]i$ is clamped (Kitazawa *et al*., 1989).

Intracellular mechanisms linking receptor activation to Ca^{2+} sensitization have been investigated in several laboratories. The results obtained suggest the existence of at least two main

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pathways. One, the rho pathway, involves a small guanosine 5′-triphosphate (GTP)-binding protein. Stimulation of receptor leads to activation of rho and, in turn, the activated rho interacts with rho-associated kinase (rho kinase), leading to its activation (Fukata *et al*., 2001). The other is a protein kinase C pathway (Li *et al*., 1998; Kitazawa *et al*., 1999). The activation of protein kinase C activates CPI-17, a smooth muscle-specific protein kinase inhibitor of MLC phosphatase (Kitazawa *et al*., 2000). Both protein kinases, rho kinase and CPI-17, induce Ca^{2+} sensitization in smooth muscle by inhibiting the activity of MLC phosphatase, resulting in an increase in the level of MLC phosphorylation (Somlyo & Somlyo, 2000). However, the extent of the contribution of the two pathways in Ca^{2+} sensitization differs with the type of smooth muscle and the species of animals. Utilizing C3 exoenzyme, a specific inhibitor of rho protein, and Y-27632 and HA 1077, rho kinase inhibitors, the involvement of a rho-rho kinase pathway in agonist-induced Ca^{2+} sensitization was suggested in the rabbit aorta (Kokubu *et al*., 1995; Uehara *et al*., 1997), pulmonary artery (Fu *et al*., 1998), portal vein (Fu *et al*., 1998) and trachea (Iizuka *et al*., 1999), guinea pig vas deferens (Fujita *et al*., 1995) and ovine cerebral artery (Akopov *et al*., 1998). In contrast, a protein kinase C pathway was proposed to mediate agonist-induced Ca^{2+} sensitization in the rabbit femoral (Gailly *et al*., 1997), rabbit mesenteric artery (Nishimura *et al*., 1992), rabbit portal vein (Brozovich, 1995), ferret portal vein (Lee *et al*., 1999) and rat tail artery (Weber *et al*., 2000), as based on the inhibitory effects of protein kinase C inhibitors, GF109203X and chelerythrine, and an inhibitory peptide of protein kinase C. However, only one report, in canine tracheal smooth muscle, indicates the involvement of both pathways in agonist-induced Ca^{2+} sensitization in the same tissue (Iizuka *et al*., 1997).

In the gastrointestinal tract, acetylcholine is a neurotransmitter that contracts intestinal smooth muscle through activation of muscarinic receptors. We previously demonstrated in the rat proximal colon that carbachol (CCh), a muscarinic agonist, induced significant contraction with only a slight increase in $[Ca^{2+}]i$, suggesting the existence of a Ca^{2+} sensitizing mechanism in colonic smooth muscle (Takeuchi *et al*., 2001). In β-escin-permeabilized preparations of the guinea pig ileum, CCh induced Ca^{2+} sensitization that was abolished by Y-27632 and HA 1077, whereas phorbol 12,13-dibutyrate (PDBu), an activator of protein kinase C, failed to induce Ca^{2+} sensitization (Otto *et al*., 1996; Sward *et al*., 2000). Similar results were obtained in the rabbit (Itagaki *et al*., 1995) and rat (Loirand *et al*., 1999) ileum. These results suggest that a rho-mediated pathway contributes to Ca^{2+} sensitization by activation of muscarinic receptors in ileal smooth muscle. Sato *et al*. (1994) showed Ca^{2+} sensitization in canine colonic smooth muscle induced by acetylcholine, histamine and neurokinin A. However, the intracellular mechanism that connects activation of muscarinic receptors to Ca^{2+} sensitization in colonic smooth muscle remains unclear. In this study, we examined the intracellular mechanism(s) of CCh-induced Ca^{2+} sensitization in permeabilized preparations of longitudinal muscle of the rat proximal and distal colon.

Methods

Male Wistar rats (200–300 g) were lightly anesthetized with ether and then stunned by a blow on the head and bled *via* the carotid. The proximal and distal colon were removed and placed into Tyrode solution containing (in mM) 127 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 1.05 $MgCl_2$, 11.9 $NaHCO_3$, 0.4 NaH_2PO_4 and 5.6 glucose. Longitudinal muscles of the proximal and distal colon were prepared as described previously (Maehara *et al*., 1994). Small strips ($0.1–0.2 \times 1$ mm) of longitudinal muscle were prepared using an inverted microscope. Only one preparation was made from each animal. The strips were tied with monofilament silk to the fine tips of two tungsten needles, one of which was connected to a force transducer. They were then placed in a well on a plate (Horiuti, 1988) kept at 25°C by circulating water. To change solutions, an adjacent well was moved by sliding the plate to the position of the tissue preparation. Isometric tension was measured with a force displacement transducer (AE801, SensoNor, Horten, Norway) and recorded with a recorder (LR4110, Yokogawa, Japan) with a preamplifier (EF601G, Nihon Koden, Japan). Preparations were permeabilized by treatment with α-toxin ($167\ \mu g\ ml^{-1}$) for 30 min in a relaxing solution containing 111 mM potassium-methanesulfonic acid, 4 mM ATP-2Na, 4 mM Mg-methanesulfonic acid, 4 mM EGTA, 20 mM Tris maleic acid (pH 6.8) and 5 mM phosphocreatine (Takeuchi *et al*., 1995). In the case of β-escin, after measuring steady contractions induced by 100 mM K^+, strips were incubated in a relaxing solution for 5 min. Permeabilization with β-escin at 40 μM was achieved by incubation at 25°C for 40 min. To prevent deterioration of Ca^{2+}-induced contraction, 1 μM calmodulin was added to the bathing solution throughout the experiments as described by Fujita *et al*. (1995). After treatment with α-toxin or β-escin, for 10 min in relaxing solution, responses of the permeabilized muscle to various concentrations of Ca^{2+} at 10-min intervals were recorded in activating solution. Activating solutions containing Ca^{2+} were prepared using a computer program (a gift from Dr Kitazawa) resulting in a desired set of free ion concentrations adjusted for both temperature and ionic strength (Kitazawa *et al*., 1989). All solutions had an ionic strength of 200 mM, and experiments were performed at 25°C. 4 mM EGTA was used to clamp free Ca^{2+} concentrations, and an appropriate amount of Ca-methanesulfonic acid (0.1 M) was added to give the desired concentration of free Ca^{2+} (Horiuti, 1988). Concentrations of ATP and phosphocreatine in the relaxing and activating solutions were expressed as total ATP and total phosphocreatine concentrations, respectively.

Statistical analysis

Data were expressed as mean ± s.e.m. of n experiments using tissues obtained from different animals. Differences between values were evaluated by paired t-test or by ANOVA and thereafter assessed by Student's t-test or Welch test (if significant differences were indicated by ANOVA). P-values <0.05 were considered significant.

Drugs

Chelerythrine, *Staphylococcus aureus* α-toxin, PDBu, β-escin and calmodulin were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Protein kinase C inhibitor peptide (PKC19–31) was purchased from Seikagaku Kogyo (Tokyo, Japan). [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) was purchased from Dojin (Kumamoto, Japan). ATP, GTP,

GTPγS and nicotinamide adenine dinucleotide (NAD) were supplied by Boehringer Mannheim (Mannheim, Germany). ADP, phosphocreatine and methane sulfonate were purchased from Wako Pure Chemicals (Osaka, Japan). Maleic anhydride, magnesium hydroxide, calcium carbonate and potassium hydroxide were purchased from Koso Co. (Tokyo, Japan). Y-27632 (R−(+)−*trans*−*N*−(4−pyridyl)−4−(1−aminoethyl)−cyclohexanecarboxamide) was a gift from the Welfide Corporation (Osaka, Japan). C3 exoenzyme was kindly provided by Dr Kozaki (Department of Veterinary Science, Osaka Prefecture University, Sakai, Japan). All other chemicals used were of analytical grade.

Results

To study Ca^{2+} sensitizing mechanisms induced by carbachol in longitudinal muscle of the rat proximal and distal colon, we first used α-toxin-permeabilized colonic preparations. Treatment with α-toxin results in the formation of pores of 2- to 3-nm diameter in the cell membrane, from which important soluble proteins, such as calmodulin, do not leak out (Nishimura *et al*., 1988). In α-toxin-permeabilized preparations of the proximal and distal colon, Ca^{2+}-induced contraction consisted of two components (Figure 1a), a rapid phasic and subsequent tonic component. The contractile effect of Ca^{2+} was greater in the distal colon than in the proximal colon (Figure 1a). In subsequent experiments, the effects of GTP and CCh were examined on the tonic contraction induced by 1 μM Ca^{2+}, which was about 40–50% of 100 μM Ca^{2+}-induced tonic contraction, in preparations of both regions of the colon (Figure 1b). After the tonic phase of 1 μM Ca^{2+}-induced contraction reached a plateau (6.02±0.53 mg in the proximal colon, $n=12$; 14.0±2.74 mg in the distal colon, $n=6$), the addition of GTP enhanced the tonic tension in preparations of both regions of the colon (GTP-induced Ca^{2+} sensitization) (Figures 2 and 3a). GTP (1–10 μM) induced an increase in tonic contraction concentration-dependently in both tissues, but the responsiveness to GTP was more potent in the proximal colon (94.1±6.8% increase at 10 μM GTP) than in the distal colon (51.4±8.4% increase at 10 μM GTP) (Figure 2). In the presence of GTP at 10 μM, subsequent addition of CCh (1–100 μM) further enhanced the tension in preparations of both regions (CCh-induced Ca^{2+} sensitization) (Table 1), although no sensitization was induced in the absence of GTP. The effects of CCh were moderate in the proximal and marked in the distal colon (Table 1). CCh (100 μM)-induced Ca^{2+} sensitization was induced depending on the concentration of GTP in preparations of both regions (Figure 2). In all, 100 μM CCh in the presence of 10 μM GTP increased the 1 μM Ca^{2+}-induced tonic contraction in the proximal and distal colon (Figure 2). A net increase in contraction induced by 100 μM CCh was 26.6±8.2% (% of 1 μM Ca^{2+}-induced tonic contraction; $n=5$) and 106.8±22.2% ($n=4$), respectively (Figure 2). In the following studies, CCh at a concentration of 100 μM was used to induce clear sensitization in the proximal colon, although 10 μM CCh was used to obtain a comparable sensitizing effect in the distal colon (Table 1). Ca^{2+}-induced contractions and the effects of GTP without or with CCh remained unchanged after Ca^{2+} in the sarcoplasmic reticulum was depleted by treatment of the preparations with 1 μM A23187 for 20 min ($n=3$, data not shown).

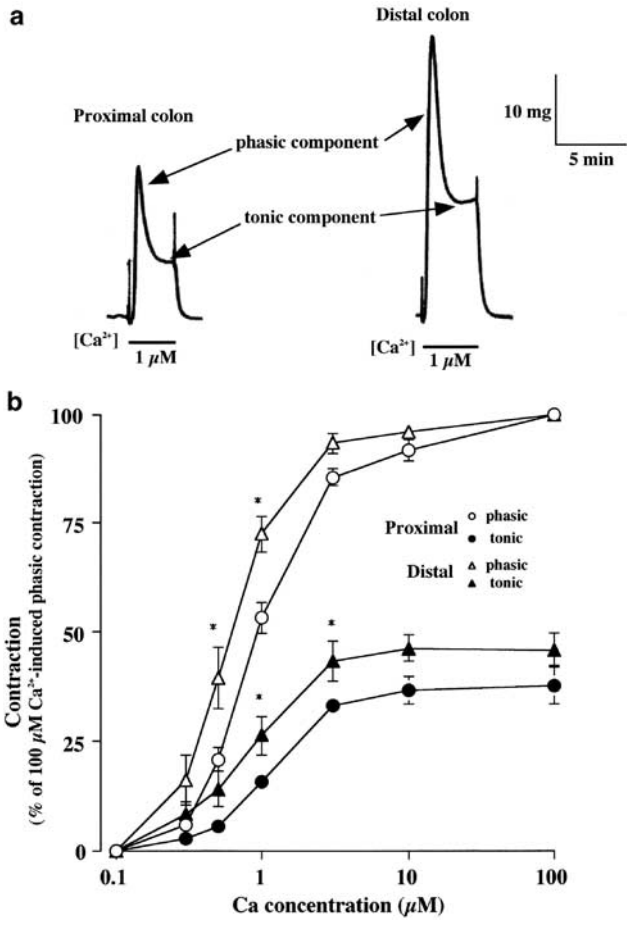

Figure 1 Ca^{2+}-induced contraction in α-toxin-permeabilized preparations of the rat proximal and distal colon. (a) Representative traces of Ca^{2+}-induced contraction in the proximal and distal colon. Note that 1 μM Ca^{2+} induced the contraction consisting of phasic and tonic components in the proximal and distal colon. (b) Concentration–response curves of Ca^{2+}-induced contraction. Values are plotted as percentages of the maximal phasic contraction induced by 100 μM Ca^{2+} in each region. Points and bars are the means and s.e.m. for 4–5 experiments. Only one preparation was made from each animal. *Significantly different from the value of contraction in the proximal colon induced by each Ca^{2+} concentration indicated, $P<0.05$.

In the proximal colon, Y-27632, an inhibitor of rho-associated kinase, at concentrations up to 10 μM had no effect on Ca^{2+}-induced contraction. However, Y-27632 inhibited GTP- and CCh-induced Ca^{2+} sensitization in a concentration-dependent manner. At 10 μM, the enhancement of tonic contraction induced by GTP plus CCh was inhibited by 79.3±4.5% ($n=8$, Figure 3). In contrast, Y-27632 (10 μM) inhibited GTP plus CCh-induced Ca^{2+} sensitization only 38.8±5.0% in the distal colon ($n=5$, Figure 3). Y-27632 (10 μM) also inhibited GTPγS-, a nonhydrolyzable GTP analoge, or GTP-induced Ca^{2+} sensitizations in the proximal (89.7±9.2% inhibition, $n=4$) and distal (85.6±6.5% inhibition, $n=4$) colon (Figure 4a). However, Y-27632 (10 μM) only slightly inhibited PDBu-, a protein kinase C activator, induced Ca^{2+} sensitization in preparations of both regions of the colon (13.0±1.1%, $n=4$, and 11.1±0.9% inhibition, $n=3$, in the proximal and distal colon, respectively, Figure 4b). PDBu-induced Ca^{2+} sensitization was 651.0±59.0 ($n=4$) and

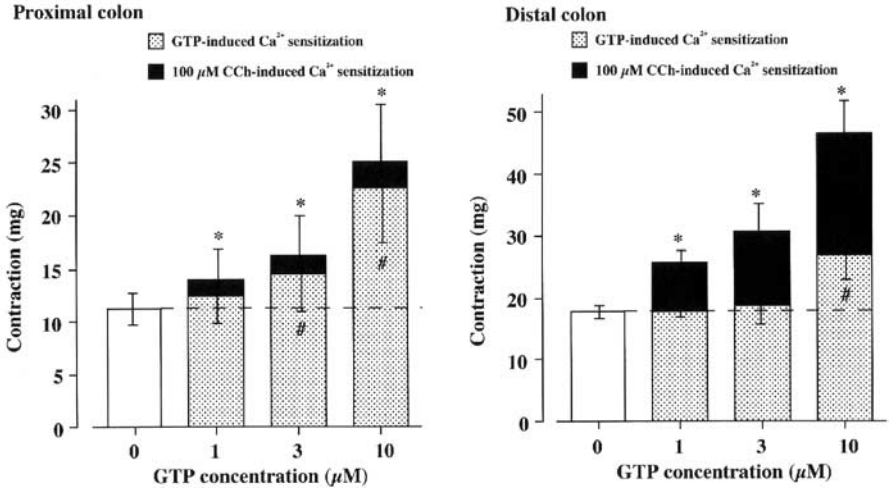

Figure 2 Effects of GTP without or with CCh on Ca^{2+}-induced contraction in α-toxin-permeabilized preparations of the rat proximal and distal colon. The preparations were first contracted by 1 μM Ca^{2+} (open bar). After the tonic phase reached a plateau, effects of GTP at the concentration of 1, 3 and 10 μM were examined (hatched bars). After GTP-induced contraction reached the maximum, 100 μM CCh was further added (CCh-induced Ca^{2+} sensitization, closed bar). Columns and bars are the means and s.e.m. for 4–5 experiments. Only one preparation was made from each animal. #Significantly different from the value of 1 μM Ca^{2+}-induced tonic contraction, $P<0.05$ (by paired t-test). *Significantly different from the value of GTP-induced Ca^{2+} sensitization, $P<0.05$ (by paired t-test).

487.5±66.2% ($n=3$) (% change from 1 μM Ca^{2+}-induced tonic contraction) in the proximal and distal colon, respectively. Y-27632 at a high concentration of 30 μM significantly inhibited Ca^{2+}-induced contraction itself in both the proximal and distal colon, suggesting that it exhibits some nonselective action on contractile mechanism at this concentration. Therefore, we could not examine the effect of a high concentration of Y-27632 on Ca^{2+} sensitization in the distal colon.

Pretreatment with chelerythrine, an inhibitor of protein kinase C, at concentrations up to 10 μM did not affect Ca^{2+}-induced contraction in preparations isolated from both regions. Chelerythrine at 3 μM did not inhibit GTP- and CCh-induced Ca^{2+} sensitization in the proximal colon, and at 10 μM induced a slight inhibition but it was not significant (Figure 5a). In the distal colon, chelerythrine inhibited GTP plus CCh-induced Ca^{2+} sensitization concentration-dependently, and at 3 μM resulted in inhibition of this combined component of Ca^{2+} sensitization of about 30% (Figure 5b). Chelerythrine had a slight inhibitory effect on GTP-induced Ca^{2+} sensitization, but the changes were not significant (Figure 5b, lower panel). GTP- and CCh-induced Ca^{2+} sensitization, which persisted after the chelerythrine treatment, was completely inhibited by Y-27632 (Figure 6). PDBu- (1 μM) induced Ca^{2+} sensitization in the distal colon was significantly inhibited by chelerythrine (374.0±45.5% increase in tension was inhibited to 194.5±38.9%, $n=4$).

The effects of C3 exoenzyme, which inactivates GTPase of the rho subfamily of ras-related low-molecular-mass GTPases, and a protein kinase C inhibitor peptide, PKC(19–31), on GTP- and CCh-induced Ca^{2+} sensitization were examined. For this study, β-escin was used to permeabilize the preparations because the procedure makes it possible for C3 exoenzyme and PKC(19–31) to permeate the plasma membrane of preparations while α-toxin treatment does not. In β-escin-permeabilized preparations of the proximal colon, Ca^{2+} (1 μM) induced contraction exhibiting a similar pattern to that in α-toxin-permeabilized preparations (Figure 7). GTP (10 μM) induced Ca^{2+} sensitization (Figure 7a, Table 2). In the presence of GTP, CCh-induced Ca^{2+} sensitization was also shown (Figure 7a, Table 2). Pretreatment of the proximal colonic preparations with 250 ng ml^{-1} C3 exoenzyme in the presence of 10 μM NAD resulted in very significant inhibition in GTP- and CCh-induced Ca^{2+} sensitization, whereas Ca^{2+}-induced contraction was only slightly inhibited (Figure 7a, Table 2). Under the same conditions, however, microcystin-LR, an inhibitor of MLC phosphatase, induced enhancement of the contraction (Figure 7a), and PDBu-induced Ca^{2+} sensitization remained unchanged, excluding the possibility of a nonselective inhibitory effect of C3 exoenzyme on Ca^{2+} sensitization. PKC(19–31) at 10 μM induced only a slight inhibition in GTP plus CCh-induced Ca^{2+} sensitization (Figure 7b, $n=6$) and in GTPγS-induced enhancement of Ca^{2+}-induced tonic contraction (Figure 7c, $n=9$). PKC(19–31) significantly inhibited PDBu-induced Ca^{2+} sensitization (Figure 7b, $n=9$).

In β-escin-permeabilized preparations of the distal colon, 1 μM Ca^{2+} also induced a phasic-type contraction with a large tonic phase (Figure 8). After the tonic phase of contraction reached a plateau, addition of GTP at 10 μM induced Ca^{2+} sensitization (Figure 8, Table 2). Subsequent addition of CCh (100 μM) induced further Ca^{2+} sensitization (Figure 8, Table 2). However, Ca^{2+} sensitization was low in magnitude in both cases. Pretreatment of preparations with 500 ng ml^{-1} C3 exoenzyme in the presence of 10 μM NAD abolished GTP-induced Ca^{2+} sensitization, but inhibited only 50 % of CCh-induced Ca^{2+} sensitization (Figure 8, Table 2). PKC(19–31) significantly inhibited the CCh-induced Ca^{2+} sensitization that persisted after treatment of C3 exoenzyme (Figure 8, Table 2).

Discussion

In the present study, α-toxin-permeabilized longitudinal muscle preparations obtained from both the rat proximal

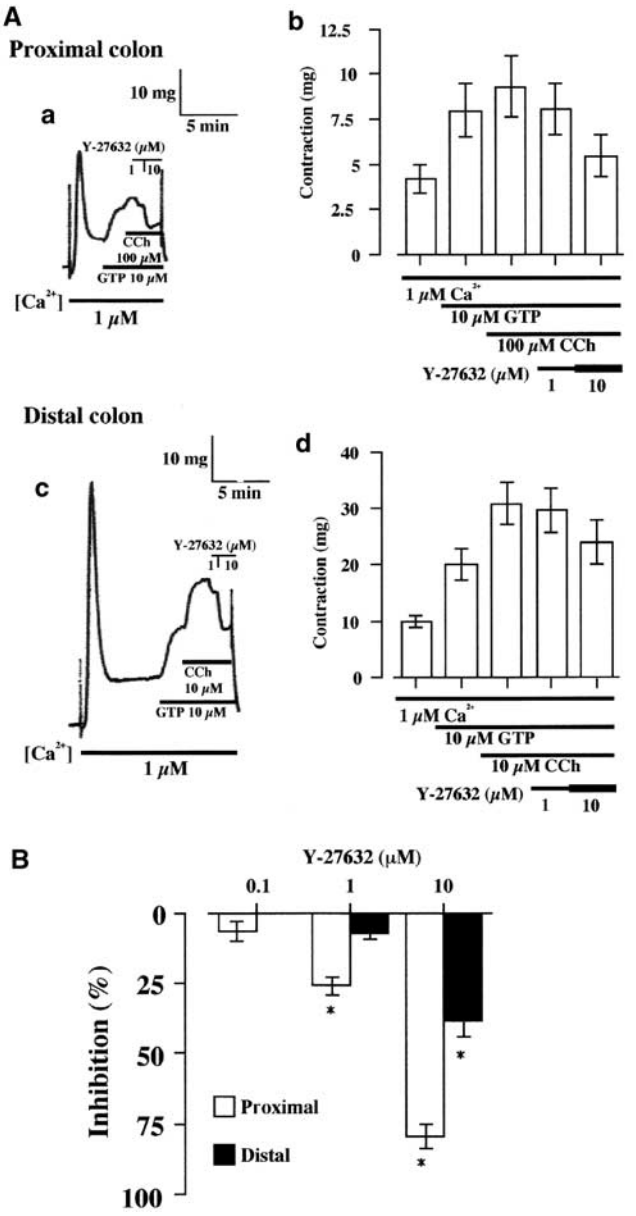

Figure 3 Inhibitory effects of Y-27632 on Ca^{2+} sensitization induced by CCh in α-toxin-permeabilized preparations of the rat proximal and distal colon. (A) The preparations were first contracted by 1 μM Ca^{2+}. After the tonic phase reached a plateau, 10 μM GTP was added. After GTP-induced contraction reached the maximum, 100 (proximal, a) or 10 (distal, c) μM CCh was further added. Effects of Y-27632 (1 and 10 μM) on GTP plus CCh-induced Ca^{2+} sensitization were examined. Lines indicate the presence of the drugs indicated. The results are summarized in (b) (proximal colon, $n=8$) and (d) (distal colon, $n=5$). (B) Summarized results of inhibitory effects of Y-27632 on GTP plus CCh-induced Ca^{2+} sensitizations. Inhibitions are expressed as a percentage of the contractions induced by GTP and CCh above the Ca^{2+}-induced contraction (GTP plus CCh-induced Ca^{2+} sensitization). Columns and bars are the means and s.e.m. for 3–9 experiments. Only one preparation was made from each animal. *Significantly different from the value of contraction induced by GTP and CCh, $P<0.05$.

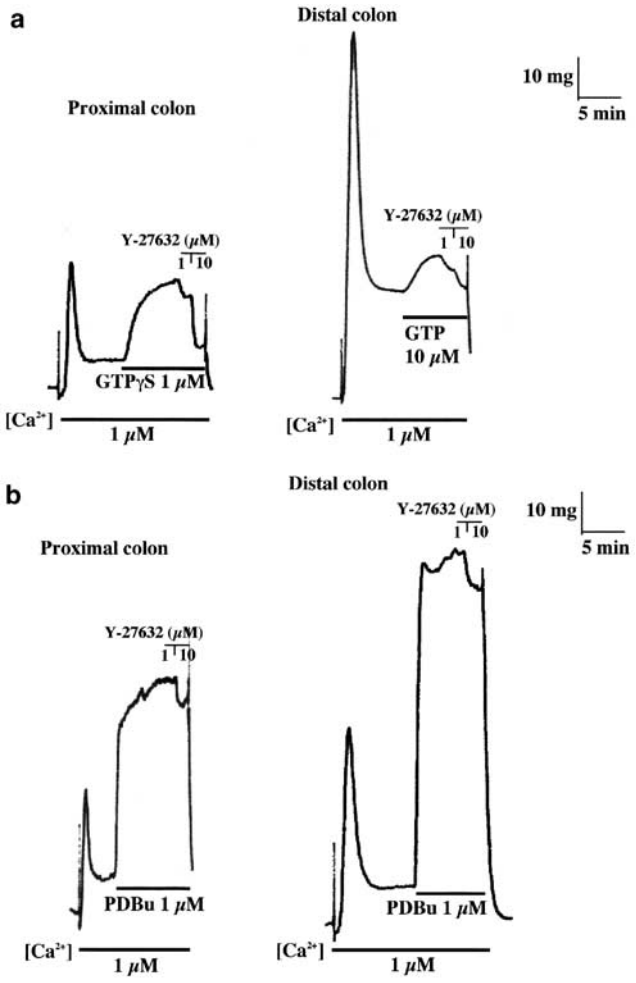

Figure 4 Effects of Y-27632 on Ca^{2+} sensitization induced by PDBu or GTP in α-toxin-permeabilized preparations of rat proximal and distal colon. The preparations were first contracted by 1 μM Ca^{2+}. After the tonic phase reached a plateau, 1 μM GTPγS or 10 μM GTP (a), or 1 μM PDBu (b) was added in the proximal and distal colon, respectively. After GTPγS-, GTP- or PDBu-induced contraction reached the maximum, effects of Y-27632 (1 and 10 μM) were examined. Lines indicate the presence of the drugs indicated.

and distal colon exhibited Ca^{2+}-induced contraction that was biphasic in nature, that is, the response consisted of a rapid transient contraction (phasic phase) followed by a sustained contraction (tonic phase). During the tonic phase of contraction, addition of GTP resulted in an increase in the force level (Ca^{2+} sensitization) and subsequent addition of CCh increased the force level further, highlighting a CCh-induced component of Ca^{2+} sensitization. However, the intracellular mechanism of CCh-induced Ca^{2+} sensitization was different between both regions.

In the proximal colon, GTP plus CCh-induced Ca^{2+} sensitization was very significantly inhibited by Y-27632 (Figure 3) and completely by C3 exoenzyme (Figure 7a), specific inhibitors of the rho pathway, whereas chelerythrine, an inhibitor of protein kinase C, had no effect (Figure 5a). PKC(19–31), an inhibitor of protein kinase C, had a slight inhibitory effect on GTP plus CCh-induced Ca^{2+} sensitization (Figure 7b). However, the effect seems to be not significant on CCh-induced sensitization, since PKC(19–31) also exerted a similar magnitude inhibitory effect on GTPγS-induced Ca^{2+} sensitization (Figure 7c), although it completely inhibited PDBu-induced sensitization (Figure 7b). C3 exoenzyme, at a concentration of 250 ng ml^{-1} used in the present study, ADP-ribosylated 60% of endogenous rho in the guinea pig ileum

Table 1 Comparison of Ca^{2+} sensitization of induced by GTP without or with CCh in the presence of GTP in α-toxin-permeabilized of rat proximal and distal colon

	Ca^{2+} senitization (% change from 1 μM Ca^{2+}-induced tonic contraction)			
	GTP 10 μM	1 μM	*+ CCh* 10 μM	100 μM
Proximal colon	94.1±6.8 (31)		102.8±14.8* (13)	122.2±10.4* (22)
Distal colon	51.4±8.4 (5)	74.0±10.8* (5)	108.1±19.8* (5)	156.2±31.1* (5)

Contraction were induced by 1 μM Ca^{2+} in the absence or presence of 10 μM GTP without or with CCh at indicated concentrations. Ca^{2+} sensitization induced by GTP without or with CCh is expressed as the % change from 1 μM Ca^{2+}-induced tonic contraction. Values are the means±s.e.m. for the number of experiments shown in parentheses. Only one preparation was made from each animal. *Significantly different from the value induced by GTP, $P<0.05$.

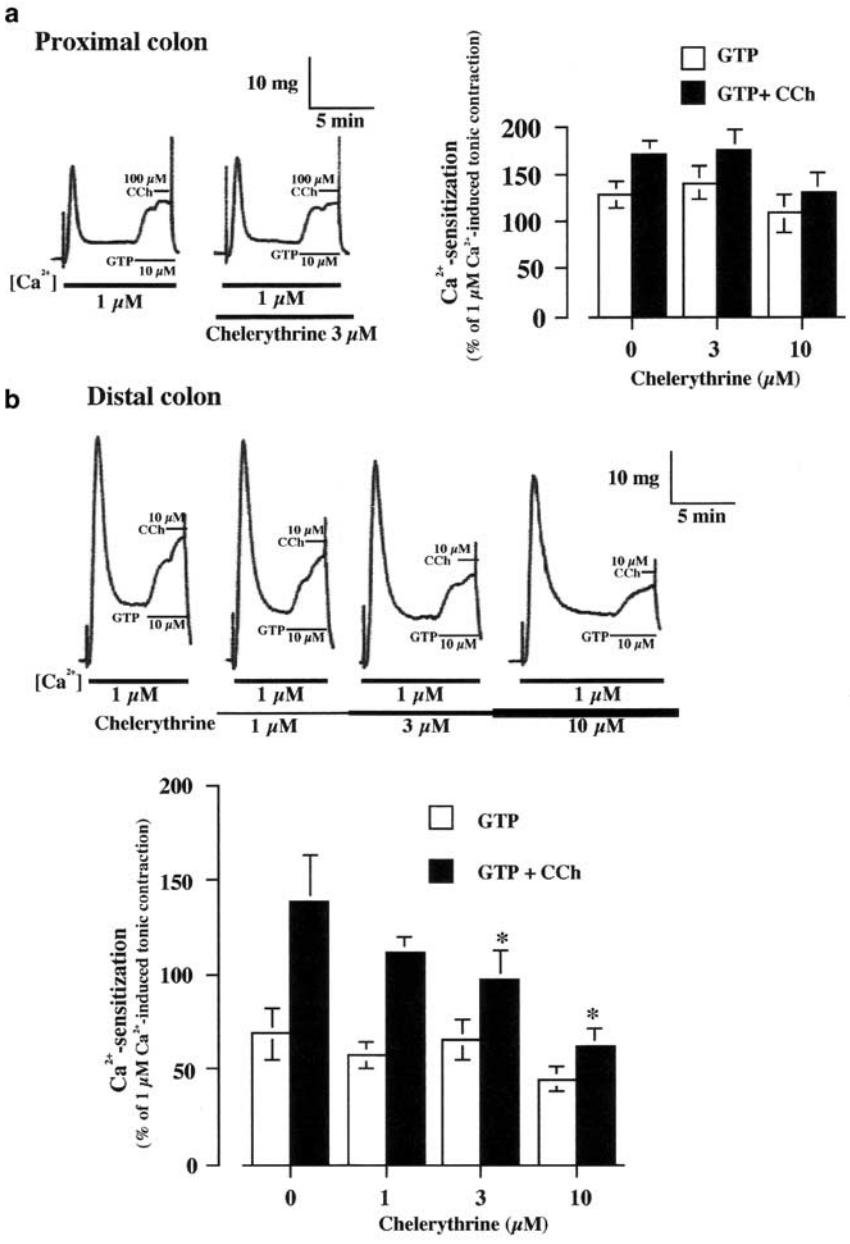

Figure 5 Effects of chelerythrine on Ca^{2+} sensitization induced by CCh in α-toxin-permeabilized preparations of rat proximal and distal colon. (a) Representative traces showing the effects of chelerythrine on Ca^{2+} sensitizations induced by either GTP or CCh plus GTP in the proximal colon. Summarized data are also shown (right panel). Lines in the tracings indicate the presence of the drugs indicated. Chelerythrine was added 10 min before the second Ca^{2+}-induced contraction. Columns and bars are the means and s.e.m. for four experiments. Only one preparation was made from each animal. (b) Representative traces showing the effects of various concentrations of chelerythrine in the distal colon. Summarized data are also shown (lower panel). Columns and bars are the means and s.e.m. for five experiments. Only one preparation was made from each animal. *Significantly different from the values without chelerythrine, $P<0.05$.

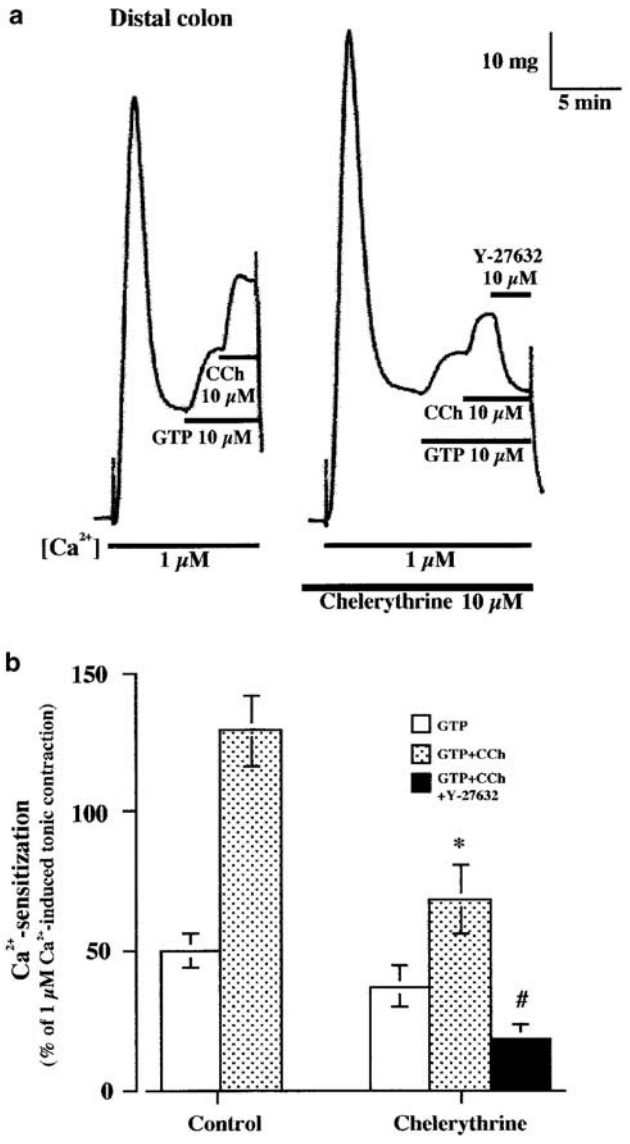

Figure 6 Effects of Y-27632 on CCh-induced Ca^{2+} sensitization in the presence of chelerythrine in α-toxin-permeabilized preparations of rat distal colon. (a) Representative traces showing inhibitory effects of Y-27632 on CCh-induced Ca^{2+} sensitizations in the presence of chelerythrine in the distal colon. After GTP plus CCh-induced Ca^{2+} sensitization reached the maximum, Y27632 was added. Chelerythrine was added 10 min before Ca^{2+}-induced contraction. Lines indicate the presence of the drugs indicated. (b) Summary of the effects of Y-27632 in the presence of chelerythrine. Note the significant decrease in GTP plus CCh-induced Ca^{2+} sensitizations in the presence of chelerythrine. Columns and bars are the means and s.e.m. for five experiments. Only one preparation was made from each animal. *Significantly different from the value without chelerythrine, $P<0.05$. #Significantly different from the value of CCh-induced Ca^{2+} sensitization in the presence of chelerythrine, $P<0.05$.

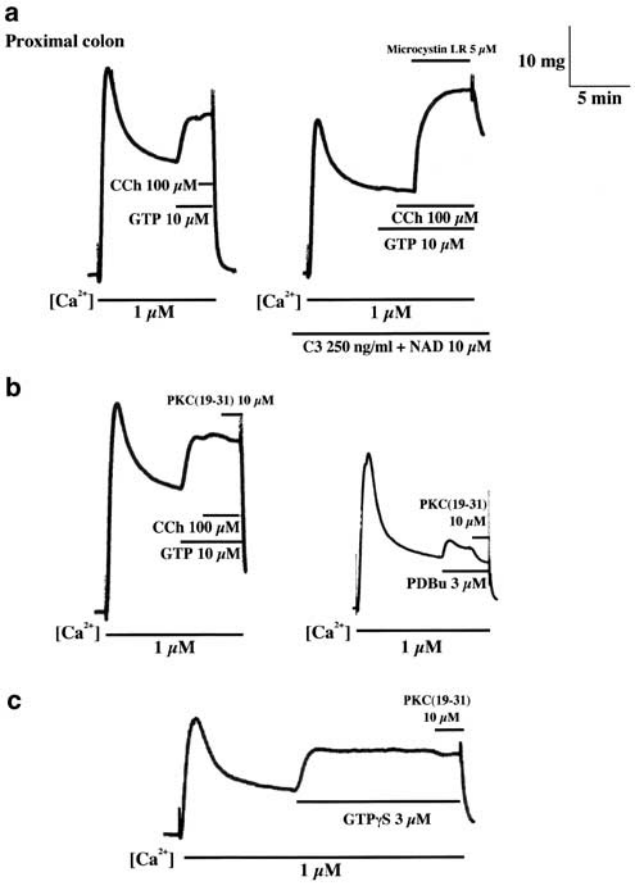

Figure 7 Effects of C3 exoenzyme and PKC(19–31) on Ca^{2+} sensitization in β-escin-permeabilized preparations of rat proximal colon. (a) Inhibition of GTP- and CCh-induced Ca^{2+} sensitization by C3 exoenzyme. GTP- and CCh-induced Ca^{2+} sensitizations were abolished after pretreatment of the proximal colonic preparations with 250 ng ml^{-1} of C3 exoenzyme and 10 μM NAD for 20 min. Note that microcystin-LR induced Ca^{2+} sensitization, even after the C3 exoenzyme treatment. (b) Effects of PKC(19–31) on PDBu- and GTP plus CCh-induced Ca^{2+} sensitization. Note that PKC(19–31) at 10 μM slightly inhibited GTP plus CCh-induced Ca^{2+} sensitization and completely inhibited PDBu-induced Ca^{2+} sensitization. (c) Effect of PKC(19–31) on GTPγS-induced Ca^{2+} sensitization. Note that PKC(19–31) at 10 μM had only a slight inhibitory effect on GTPγS-induced Ca^{2+} sensitization.

(Otto *et al*., 1996) and completely inhibited agonist-induced Ca^{2+} sensitization in the guinea pig vas deferens (Fujita *et al*., 1995) and ileum (Otto *et al*., 1996). Therefore, it seems likely that C3 exoenzyme inhibited CCh-induced Ca^{2+} sensitization in both regions of the rat colon *via* inhibition of rho protein. Y-27632 was reported to inhibit rho kinase specifically; its Ki values on the activity of rho-associated protein kinase, p160-ROCK, and protein kinase C were 0.14 and 26 μM, respectively (Uehara *et al*., 1997). Indeed, Y-27632 only slightly inhibited PDBu-induced Ca^{2+} sensitization in the present study (Figure 4b). PKC(19–31) is a synthetic peptide corresponding to the pseudosubstrate region of protein kinase C. It was previously reported that it did not inhibit GTPγS-induced Ca^{2+} sensitization in the guinea pig vas deferens (Fujita *et al*., 1995). Chelerythrine inhibited protein kinase C activity by interacting with the catalytic domain of the enzyme, whereas it did not inhibit activity of tyrosine protein kinase, calcium/calmodulin-dependent protein kinase or cyclic AMP-dependent kinase (Herbert *et al*., 1990). Chelerythrine inhibited PDBu-induced Ca^{2+} sensitization in canine tracheal smooth muscle (Bremerich *et al*., 1998) and porcine pulmonary artery (Kutz *et al*., 1998). In the present study, both PKC(19–31) and chelerythrine significantly inhibited PDBu-induced Ca^{2+} sensitization, but not GTP-induced Ca^{2+} sensitization in preparations of both regions of the colon, indicating the specificity of these compounds to protein kinase C. Thus, these

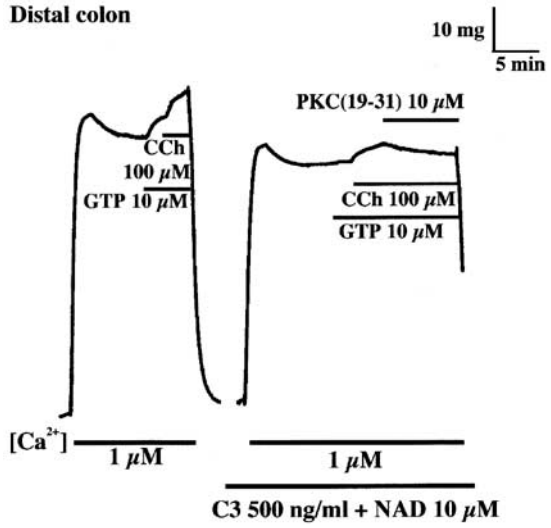

Figure 8 Inhibitory effects of C3 exoenzyme and PKC(19–31) on Ca^{2+} sensitization induced by CCh in β-escin-permeabilized preparations of rat distal colon. CCh (100 μM) was added in the presence of 10 μM GTP during 1 μM Ca^{2+}-induced contraction. Note a complete inhibition of GTP-induced Ca^{2+} sensitization and a partial inhibition of CCh-induced Ca^{2+} sensitization after pretreatment of the preparations with 500 ng ml^{-1} of C3 exoenzyme with 10 μM NAD for 20 min. The CCh-induced Ca^{2+} sensitization, which persisted after the treatment of C3 exoenzyme, was further inhibited by PKC(19–31).

results suggested that CCh-induced Ca^{2+} sensitization was solely through the rho pathway in the rat proximal colon.

In the distal colon, GTP plus CCh-induced Ca^{2+} sensitization was partially inhibited by rho inhibitors, Y-27632 (Figure 3) and C3 exoenzyme (Figure 8). Since GTP-induced Ca^{2+} sensitization was almost completely inhibited by the treatment with Y-27632 (Figure 4a) or C3 exoenzyme (Figure 8), the sensitization, which persisted after these treatments appeared to be CCh-induced sensitization. The simultaneous application of Y-27632 and chelerythrine in α-toxin-permeabilized preparations or C3 exoenzyme and PKC(19-31) in β-escin-permeabilized preparations resulted in an additive effect on GTP plus CCh-induced Ca^{2+} sensitization (Figures 6 and 8). These results suggest that CCh-induced Ca^{2+} sensitization is induced by both the rho and protein kinase C pathways *via* activation of muscarinic receptors in the distal colon. A similar result was reported in smooth muscle of the rabbit colon, where acetylcholine induces the translocation of rho A and protein kinase Cα to the membrane fraction (Bitar *et al*., 2002). Further study is needed to clarify the mechanisms whereby the muscarinic receptor is able to activate the two second-messenger systems.

The CCh-induced Ca^{2+} sensitization in the distal colon was more potent than that in the proximal colon. Muscarinic receptors associated with the contractile response in the gastrointestinal smooth muscle are M2 and M3 subtypes (Eglen, 2001). An antagonist of the M3 muscarinic receptor, 4-DAMP, significantly inhibited CCh-induced Ca^{2+} sensitization in preparations of both regions, whereas AFDX116, an inhibitor of the M2 muscarinic receptor, did not (data not shown). Thus, the difference shown between both regions may not be due to different receptor subtypes. The existence of the protein kinase C pathway contributes partially, at least, to the potent CCh-induced sensitization in the distal colon, although the question as to why intracellular mechanisms of Ca^{2+} sensitization induced by the same type of muscarinic receptor differ between both regions remained.

GTP is required for activation of numerous receptors and intracellular GTP-binding proteins (Pfitzer & Arner, 1998). In the present study, GTP by itself induced Ca^{2+} sensitization. GTP-induced Ca^{2+} sensitization was almost completely inhibited by Y-27632 and C3 exoenzyme, but not by chelerythrine and PKC(19–31) in the proximal and distal colon. These results suggest that GTP augments the Ca^{2+} sensitivity of contractile elements by directly activating the rho pathway in both regions of colon. However, the GTP-induced Ca^{2+} sensitization was more potent in the proximal than in the distal colon (Figure 2). It was reported that the amount of contractile proteins, the composition of their isoforms and the extent of their phosphorylation are different between phasic and tonic smooth muscle tissues (Lorenz *et al*., 2002; Szymanski *et al*., 2002). Ca^{2+}-induced contractions in the proximal colon were smaller than those in the distal colon. Therefore, the amount of contractile proteins that are involved in GTP-induced sensitization may differ between the proximal and distal colon.

A protein kinase C activator, PDBu, induced Ca^{2+} sensitization in the proximal colon that was inhibited by PKC(19–31) and chelerythrine. Thus, although smooth muscle of the rat proximal colon possesses a mechanism of Ca^{2+} sensitization through the activation of protein kinase C, this mechanism seems not to be linked to activation of muscarinic receptors in the proximal colon. Similar results in adrenergic receptors were reported in the rabbit mesenteric artery (Yoshida *et al*., 1994) and the guinea pig vas deferens (Fujita *et al*., 1995). On the other hand, the linkage between the muscarinic receptor and protein kinase C in the distal colon is evident in the present study. The activating mechanism of protein kinase C in muscarinic receptor-mediated Ca^{2+} sensitization of the rat distal colon is of great critical interest and requires further study.

In the rat distal colon, a small component of CCh-induced Ca^{2+} sensitization still remained after both the rho and protein kinase C pathways were inhibited. Ca^{2+} sensitizing mechanisms mediated through arachidonic acid (Somlyo & Somlyo, 1998) and MAP kinase (Cain *et al*., 2002) have been reported. Recently, it was reported that arachidonic acid-induced Ca^{2+} sensitization in α-toxin-permeabilized rabbit femoral artery was inhibited by Y-27632 (Araki *et al*., 2001), suggesting that arachidonic acid activates the rho pathway. MAP kinase was shown to be activated by acetylcholine in canine colonic smooth muscles (Gerthoffer *et al*., 1996). Therefore, MAP kinase is another candidate as a mediator in Ca^{2+} sensitization induced by CCh in the rat distal colon.

In the present study, there were some intriguing results, which were even thought clearly beyond the scope of the present study. In membrane-permeabilized preparations of the distal colon treated with β-escin, Ca^{2+} induced greater responses (tonic contraction) than those treated with α-toxin. Treatment of the preparations with β-escin causes formation of pores in the plasma membrane, from which 30–40 kDa molecules are permeable (Ohtsuki *et al*., 1987). In contrast, treatment with α-toxin forms pores of 2–3 nm from which molecules smaller than 1 kDa leak out, but important soluble proteins, such as calmodulin (18 kDa), do not leak out (Nishimura *et al*., 1988). Calponin (31–32 kDa) is known to

Table 2 Effects of C3 exoenzyme and PKC(19–31) on CCh-induced Ca^{2+}-sensitization in β-escin-permeabilized preparations of rat proximal and distal colon

				C3 + NAD treatment			
Tissue	*1 μM Ca^{2+}-induced tonic contraction*	*GTP (10 μM)*	*GTP + CCh (100 μM)*	*1 μM Ca^{2+}-induced tonic contraction*	*GTP (10 μM)*	*GTP + CCh (100 μM)*	*GTP + CCh (100 μM) + PKC(19–31)*
Proximal colon	9.5 ± 0.8 (7)	12.4 ± 1.2* (7)	13.7 ± 1.2$^{\#}$ (7)	7.3 ± 1.5 (7)	7.5 ± 1.6 (7)	7.8 ± 1.6 (7)	
Distal colon	55.0 ± 6.8 (5)	57.2 ± 6.9* (5)	62.4 ± 7.7$^{\#}$ (5)	42.6 ± 3.9 (5)	43.3 ± 3.7 (5)	46.3 ± 4.1$^{\#}$ (5)	44.5 ± 3.9† (5)

Contractions were induced by 1 μM Ca^{2+} in the absence or presence of 10 μM GTP without or with 100 μM CCh, before and after treatment of the preparations with 250 or 500 ng ml^{-1} C3 exoenzyme and 10 μM NAD for 20 min. Values are the means ± s.e.m. for the number of experiments shown in parentheses. Only one preparation was made from each animal. Contractions were expressed as tension in mg. *Significantly different from the values of 1 μM Ca^{2+}-induced tonic contraction, $P<0.05$ (by paired t-test). $^{\#}$Significantly different from the values in the presence of GTP, $P<0.05$ (by paired t-test). †Significantly different from the values in the presence of GTP plus carbachol, $P<0.05$(by paired t-test).

attenuate Ca^{2+}-induced contraction (Winder *et al*., 1998). Telokin (17 kDa) is also known to induce relaxation of permeabilized ileum smooth muscle at a constant Ca^{2+} concentration (Somlyo *et al*., 1998). So, some such regulatory element(s), probably inhibitory in nature, might leak out in β-escin-treated preparations. Another difference between the results obtained in both permeabilized preparations is the effect of PDBu, that is, PDBu induced significantly greater sensitization in α-toxin-permeabilized preparations (Figure 4) than that in β-escin-permeabilized preparations (Figure 7). Although leakage of the cofactor(s) necessary for activation of protein kinase C with PDBu is suggested in β-escin-permeabilized preparations, the precise mechanism also remained unclarified.

In conclusion, the results of the present study strongly suggest that both the protein kinase C and rho pathways mediate independently Ca^{2+} sensitization induced by activation of muscarinic receptors in the rat distal colon, whereas the rho pathway is solely responsible for the action in the proximal colon.

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