Possible Involvement of a Small G-Protein Sensitive to Exoenzyme C3 of *Clostridium botulinum* in the Regulation of Myofilament Ca^{2+} Sensitivity in β-Escin Skinned Smooth Muscle of Guinea Pig Ileum

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ABSTRACT—The effects of exoenzyme C3 of *Clostridium botulinum* on Ca^{2+}- and drug-induced tension developments were investigated in β-escin skinned smooth muscle of guinea pig ileum to test the involvement of a small G-protein in the regulation of myofilament Ca^{2+} sensitivity. C3 is known to ADP-ribosylate the *rho* p21 family of small G-proteins. Treatment with C3 (0.35 μg/ml, for 30 min) shifted the pCa-tension curve rightward along the Ca^{2+} concentration axis, indicating a decrease in Ca^{2+} sensitivity of the contractile elements. The inhibitory effect of C3 was not preserved after treatment with GDPβS (1 mM), an antagonist of GTP for the binding to G-proteins. Stimulation of muscarinic receptors with carbachol (CCh, 100 μM) shifted the pCa-tension curve leftward, indicating Ca^{2+} sensitization of tension development. The Ca^{2-}-sensitizing effect of CCh was not observed after C3 treatment. When GTPγS (10 μM), an activator of G-proteins, was applied at a plateau of tension development produced by a moderate concentration of Ca^{2+}, further increase in tension was elicited and the effect of GTPγS was inhibited by C3 treatment. The results suggest the possible involvement of a *rho* p21-like small G-protein in the regulation of Ca^{2+} sensitivity of smooth muscle myofilaments.

Keywords: Carbachol, Ca^{2+}-sensitization, Smooth muscle, GTP-binding protein, Contraction

The primary mechanism underlying smooth muscle contraction is phosphorylation of the 20-kD myosin light chain (MLC20) by MLC20 kinase that is activated by Ca^{2+}-calmodulin (1). It is well known that various receptor agonists can produce a greater tension development than high KCl solution even if cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is increased to the same level in intact smooth muscle (2, 3). This phenomenon was clearly demonstrated in $[Ca^{2+}]_i$-clamped, permeabilized smooth muscle preparations (4, 5). The greater tension development is associated with an increase in MLC20 phosphorylation (6-8), and thus an increase in the ratio of MLC20 kinase activity to MLC phosphatase activity has been suggested (9, 10). Heterotrimetric G-proteins have been suggested to serve as transmembrane signal transducers for receptors by which the Ca^{2+}-sensitizing effect is mediated (5, 7), but signaling pathways responsible for the receptor/G-protein-mediated Ca^{2+}-sensitizing effect have not been clarified yet.

Recent studies indicate that C3 and EDIN, exoenzymes of *Clostridium botulinum* and *Staphylococcus aureus*, respectively, selectively ADP-ribosylate the *rho* p21 family of a superfamily of *ras* p21 and its related small G-proteins (11-13). Hirata et al. (14) found that EDIN and C3 blocked GTPγS-induced Ca^{2+} sensitization of tension development and caused ADP-ribosylation of a *rho* p21-like protein in saponin-skinned arterial smooth muscle, and they suggested involvement of a member of the *rho* p21 family, *rho* A p21, in the Ca^{2+} sensitization. Current knowledge suggests that it would be attractive to test the possibility that Ca^{2+}-sensitizing effects of agonists can be blocked after ADP-ribosylation of the *rho* p21 small G-protein, since GTPγS, which is a non-selective activator of G-proteins (15, 16), can mimic some of the agonist receptor-mediated effects (5, 17, 18)

In the present study, the effect of C3 on carbachol (CCh)-induced Ca^{2+} sensitization of tension development was examined in β-escin-skinned smooth muscle of

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guinea pig ileum in which receptor-coupled signal transduction systems are well retained (18–20).

MATERIALS AND METHODS

Muscle strips (5 mm in length, 0.3 mm in width) were dissected from the longitudinal smooth muscle layer of the ileum of male guinea pigs (350–450 g). The muscle strip was mounted horizontally in a 0.3-ml organ bath, with one end of the strip fixed on the black rubber bottom of the bath with a pin and the other attached to the thin lever of an isometric force transducer (TB-612T; Nihon Kohden, Tokyo), as previously described (18). The organ bath was filled with a physiological salt solution (PSS, composition given below) kept at 23℃, and the muscle strip was equilibrated for 30–60 min under a tension of 150–180 mg. The bath solution was then replaced with a relaxing solution (composition given below); and 15–20 min later, it was changed to a relaxing solution containing 1 μM Ca^{2+}, 40 μM β-escin and 10 μM A23187. The incubation was carried out for 20–30 min until a gradually-developed tension reached a plateau level. β-Escin was used to permeabilize the muscle cells, and A23187 was used to remove their Ca^{2+} storage function. After the incubation, the skinned muscle strip was washed several times with fresh relaxing solution to remove Ca^{2+}, β-escin and A23187.

Skinned muscle strips were treated with C3 by incubating with C3 (0.35 μg/ml) and 10 μM NAD in the relaxing solution for 30 min. To evaluate the effects of C3, skinned smooth muscles incubated without C3 but otherwise handled in the same manner as the C3-treated preparations were used as control preparations.

PSS was a HEPES-buffered, modified Krebs solution: 126 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 14 mM glucose and 10.5 mM HEPES (pH adjusted to 7.2 with NaOH). The composition of the high K^+ relaxing solution was 130 mM potassium propionate, 4 mM $MgCl_2$, 4 mM Na_2ATP, 10 mM creatine phosphate, 20 mM tris maleate, 3.3 units/ml creatine phosphokinase and 10 mM EGTA (pH adjusted to 6.8 with KOH). Solutions containing various Ca^{2+} concentrations were prepared by adding appropriate amounts of $CaCl_2$ to the relaxing solution. The Ca^{2+} concentration in these solutions was computed according to Oiki et al. (21) under the following conditions: ionic strength of 0.16; pH 6.8; temperature of 23℃; in the presence of 4 mM ATP, 4 mM $MgCl_2$ and 10 mM EGTA.

Drugs used were guanosine 5′-[γ-thio]triphosphate (GTPγS), guanosine 5′-[β-thio]diphosphate (GDPβS), β-escin, β-nicotinamide adenine dinucleotide (NAD), which were all purchased from Sigma (St. Louis, MO, USA); carbachol chloride (CCh) (from Tokyo Kasei, Tokyo); and A23187 (from Wako, Tokyo). The exoenzyme C3 was purified from *Clostridium botulinum* type C as previously described (22). All other reagents were of the highest grade commercially available.

Data are expressed as the mean±one S.E.M., and statistical significance was determined by Student's *t*-test. Probabilities less than 5% ($P<0.05$) were considered significant.

RESULTS

Effect of C3 on the relationship between Ca^{2+} concentration and tension development

The concentration of Ca^{2+} in terms of pCa was increased stepwise by replacing the bath medium with solutions containing Ca^{2+} at various concentrations (pCa 7–5, see Fig. 1A), and the magnitude of the tension responses was expressed as a percentage of the magnitude of the tension response to Ca^{2+} applied at a single concentration (pCa 5), which had been obtained 30–40 min beforehand (also see Fig. 1A). Figure 1B illustrates the pCa-tension relationships in C3-untreated (control) and -treated preparations, in which the relative magnitudes of tension responses are plotted against Ca^{2+} concentration in terms of pCa. The pCa-tension curve was shifted after treatment with C3 rightward along the axis of Ca^{2+} concentration and gave the concentration of Ca^{2+} required for half maximum tension response, EC_{50}, as pCa 5.86±0.04 (n=6). The mean value for EC_{50} was statistically different from the mean value for the EC_{50} of 6.03±0.02 (n=12) in control preparations ($P<0.05$). The maximum relative tension was also decreased from 56.8±1.1% (n=12) to 49.4±2.8% (n=6). These findings suggest an apparent reduction by C3 of the Ca^{2+} sensitivity of contractile elements.

When application of Ca^{2+} at a single pCa 5 was repeated twice at an interval of 30 or 40 min in three different preparations, the second tension response was 82±2% of the first response. The mean value was much greater than the corresponding value obtained after successive applications of Ca^{2+} (56.8±1.1%, n=12) (see above). The difference was considered to result from a time- and Ca^{2+}-dependent desensitization to Ca^{2+} of the muscle preparation (7) that developed during successive applications of Ca^{2+} with a certain concentration increment. To eliminate any errors arising from this mechanism, the protocol for exposure to the different solutions was kept constant.

Tension responses to application of Ca^{2+} at a single pCa of 6.2 for 6 min reached a peak and then declined with time to a level that was still elevated. In C3-treated preparations, the peak tension was attained in 61±7 sec (n=10), and the tension level measured at the end of the Ca^{2+} application was 75±5% (n=10) of the peak ten-

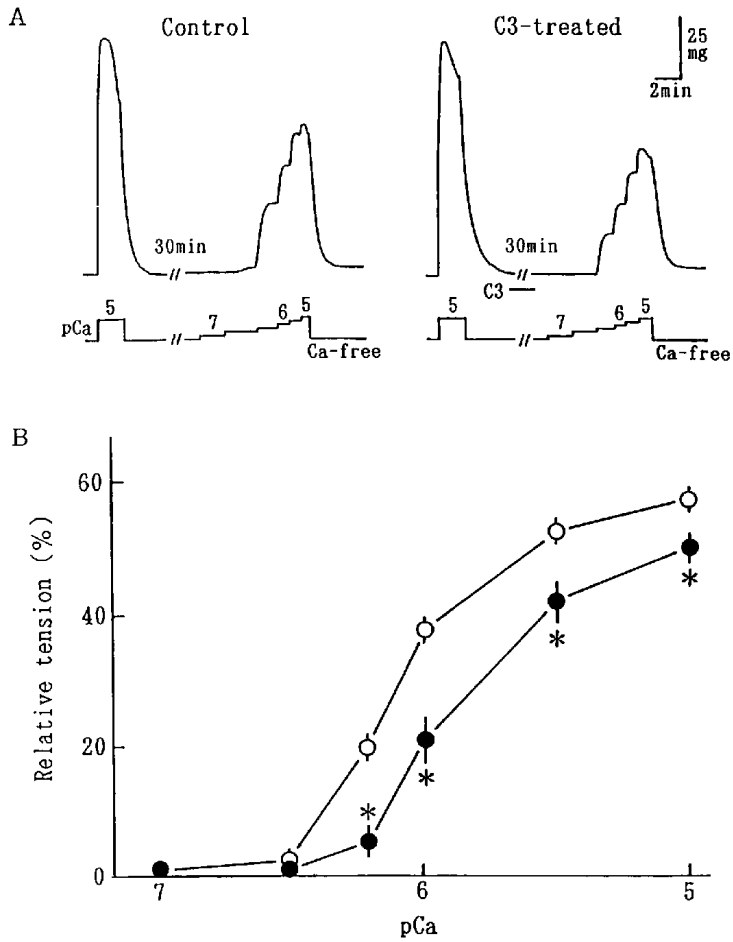

Fig. 1. Effect of C3 on the pCa-tension relationship in β-escin-skinned smooth muscle of guinea pig ileum. Functional removal of internal Ca^{2+} stores was made by application of A23187 (10 μM). Skinned- and Ca^{2+} store deprived-muscle strips were treated with C3 by incubating them in Ca^{2+}-free solution (no Ca^{2+} and 10 mM EGTA were added) with C3 (0.35 μg/ml) and NAD (10 μM) for 30 min. A, a tension response to Ca^{2+} applied at a single concentration (pCa 5.0) followed by a graded tension response to Ca^{2+} applied successively in six doses from lower to higher concentrations (pCa 7.0, 6.5, 6.2, 6.0, 5.5 and 5.0) at varied intervals shorter than 2 min. Left, in a control preparation that was handled in the same manner as the C3-treated preparation except for no application of C3; right, in a C3-treated preparation. B, pCa-tension relationships in the control (○) and C3-treated preparations (●). Magnitudes of tension responses to Ca^{2+} at individual concentrations applied successively were expressed as percentages of that of the tension response to a single dose application of Ca^{2+} (pCa 5.0). Each point is the mean ± S.E.M. (indicated by vertical bars) of 12 experiments in the control preparations and 6 experiments in the C3-treated preparations. *, a significant difference ($P<0.05$) between the mean values for the control and C3-treated preparations at each pCa.

sion. These mean values were not significantly different from the corresponding values in control preparations (66±5 sec and 81±3%, n=12).

Effect of C3 on the pCa-tension relationship in the presence of GDPβS

GDPβS (1 mM), an antagonist of GTP for the binding to G-proteins, was applied at the beginning of incubation of the muscle preparations with or without C3 (see Fig. 1A); and 30 min later, the concentration of Ca^{2+} in the bath medium was increased stepwise in the same way as in Fig. 1. During the successive applications of Ca^{2+}, GDPβS continued to be present. As shown in Fig. 2, no significant shift of the pCa-tension curve occurred after treatment with C3. The value for EC_{50} was 5.97±0.05 (n=6) in the control preparations and 5.94±0.03 (n=6) in the C3-treated preparations. Thus, GDPβS abolished the inhibitory effect of C3 on the Ca^{2+} sensitivity. This suggests the involvement of a G-protein, probably a *rho* p21-like small G-protein (11-13), in producing the C3-sensitive component of the Ca^{2+} response. In other words, the mechanism enhancing the Ca^{2+} sensitivity of the contractile elements seems likely to be operated without stimulation of muscarinic receptors in β-escin-skinned smooth muscle.

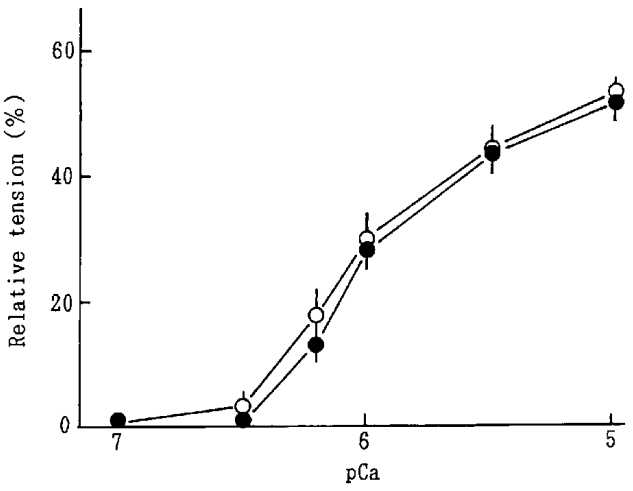

Fig. 2. Effect of C3 on the pCa-tension relationship in the presence of GDPβS. GDPβS (1 mM) was applied 30 min before successive applications of Ca^{2+} (see Fig. 1A). Magnitudes of tension responses to Ca^{2+} at individual concentrations applied successively were expressed as percentages of that of the tension response to a single dose application of Ca^{2+} (pCa 5.0), which was obtained before application of GDPβS. pCa-tension curves with the symbols ○ and ● show the data from the control and C3-treated preparations, respectively. Each point indicates the mean±S.E.M. (shown by vertical bars) of 6 experiments in both the control and C3-treated preparations. No significant shift of pCa-tension relationship by C3 treatment was observed.

Effect of C3 on carbachol-mediated Ca^{2+} sensitization of tension development

Carbachol (CCh, 100 μM), when applied during the tension development induced by Ca^{2+} at pCa 6.2, produced an additional increase in tension (Fig. 3A). The increased tension reached a peak within 1.5 min after CCh application and then declined to a sustained level in a few minutes. In preparations treated with C3, the tension responses to CCh developed with a time course substantially similar to those in the control preparations, but were significantly reduced in peak tension (Fig. 3B). The mean peak tension of the CCh-induced responses was 235.8±22.5% (n=5) of that of the Ca^{2+} alone-induced tension responses, and this was reduced to 138.2±7.1% (n=6) by C3 treatment (Fig. 3C).

The pCa-tension relationship in the presence of CCh (100 μM) was obtained in the control and C3-treated preparations in the same way as those shown in Fig. 1A. CCh was applied 5 min before the application of Ca^{2+} and continued to be present throughout the application period. Figure 4A shows that CCh shifted the pCa-tension curve leftward along the Ca^{2-} concentration axis in the control preparations. The mean value for EC_{50} in the presence of CCh (pCa 6.13±0.02, n=12) was significantly greater than that in the absence of CCh (pCa 6.03±0.02, n=12) ($P<0.05$). In C3-treated preparations, the pCa-tension curve remained almost unaltered after the application of CCh (Fig. 4B). The mean value for EC_{50} in the presence of CCh was 5.90±0.03 (n=7),

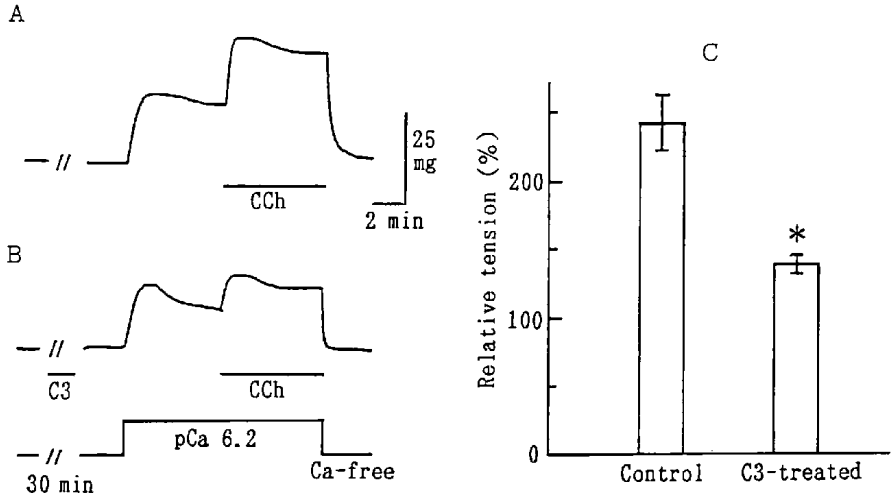

Fig. 3. Inhibitory effect of C3 on tension response to carbachol (CCh). CCh (100 μM), applied as indicated by bars below the tension traces, induced further tension development in the presence of Ca^{2+} at pCa 6.2 (see the line below B). A and B, tension records in the control and C3-treated preparations, respectively. C, summarized potentiating effects of CCh (100 μM) on Ca^{2+} (pCa 6.2)-induced tension development. Ordinate, the CCh-induced peak tension expressed as a percent of the peak tension induced by Ca^{2+} alone. Each column indicates the mean±S.E.M. (shown by vertical bars) of 5 experiments in the control preparations and 6 experiments in the C3-treated preparations. *, $P<0.05$. A smaller effect of CCh was observed in the C3-treated preparation.

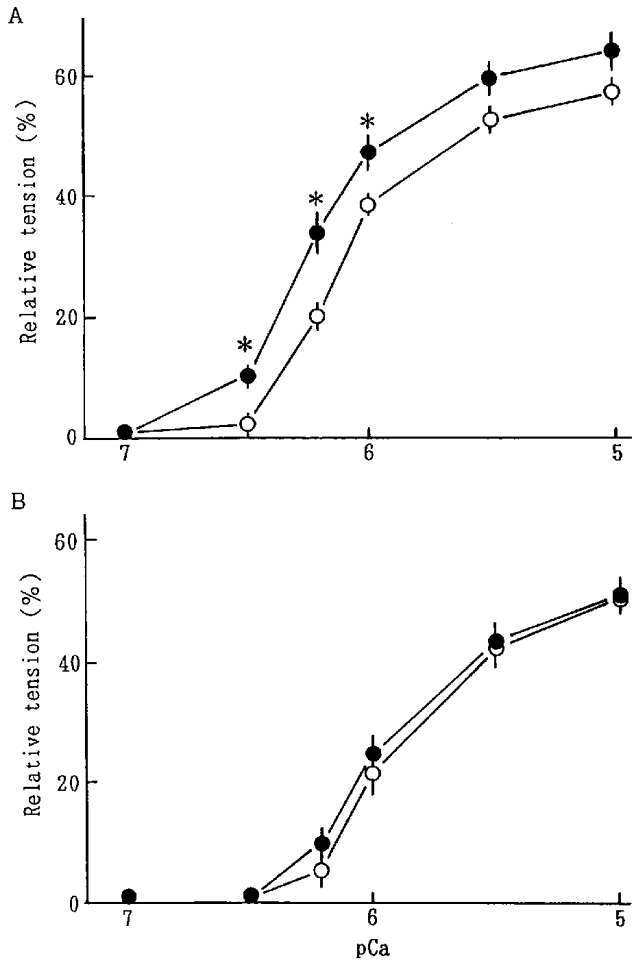

Fig. 4. Abolition by C3 of the carbachol (CCh)-induced leftward shift of the pCa-tension relationship. The pCa-tension relationships were obtained in the same way as described in Fig 1. CCh (100 μM) was applied 5 min before successive applications of Ca^{2+} and continued to be present throughout the experiment. A, pCa-tension curves in the absence (○) and presence (●) of CCh in the control preparations. B, corresponding pCa-tension curves in the C3-treated preparations. The pCa-tension curves in the absence of CCh are the same as those shown in Fig. 1B. Each point of the pCa-tension curves in the presence of CCh is a mean±S.E.M. (shown by vertical bars) of 7 experiments in the control preparations and 6 experiments in the C3-treated preparations. *, a significant difference ($P<0.05$) between the mean values in the presence and absence of CCh at each pCa.

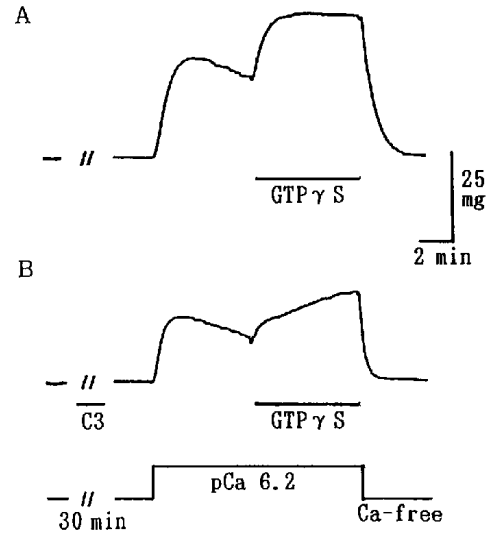

Fig. 5. Inhibitory effect of C3 on the GTPγS-induced tension development. Experiments were carried out using the same protocol as described for CCh in Fig. 3. GTPγS (10 μM), applied as indicated by the bars below the tension traces, induced further tension development in the presence of Ca^{2+} at pCa 6.2 (see the line below B). A and B, representative tension records in 5 control and 6 C3-treated preparations, respectively. We observed a slowly-developing rise in tension after application of GTPγS in the C3-treated preparation.

which was not significantly different from that in the absence of CCh (5.86±0.04, n=6).

These results suggest that a G-protein sensitive to C3, a *rho* p21-like small G-protein, may be involved in the CCh-induced Ca^{2+} sensitization of tension development.

Effect of C3 on GTPγS-mediated Ca^{2+} sensitization of tension development

Figure 5 shows that GTPγS (10 μM), an activator of G-proteins, elicited an additional rise in tension after tension development induced by Ca^{2+} (pCa 6.2), and this reached a sustained level in preparations treated with C3 or without it. However, in C3-treated preparations, tension development after application of GTPγS occurred with a slow time course and 6 min later, attained 140.0±4.3% (n=6) of the peak tension of the responses to Ca^{2+} alone. In the control preparations, the rise in tension produced by GTPγS reached a peak of 137.8±5.6% (n=5) within 1.5 min, which persisted until GTPγS was removed. The results suggest the possible role of a *rho* p21-like small G-protein as a modifier at least in the early phase of GTPγS-induced Ca^{2+} sensitization of tension development.

DISCUSSION

The present study shows that in β-escin-skinned and Ca^{2+} storage function-free smooth muscle of guinea pig ileum, C3 can inhibit the CCh-induced Ca^{2+}-sensitization of tension development (Figs. 3 and 4). The present results taken together with previous evidence that C3 causes ADP-ribosylation of the *rho* p21 family of small G-proteins by its specific action on them (11, 12, 23) suggests that CCh can cause Ca^{2+} sensitization of tension development of the smooth muscle by stimulation of a *rho* p21-like G-protein and the small G-protein-dependent modification of some intracellular processes. This is sup-

ported by the findings that GTPγS exerted a similar Ca^{2+}-sensitizing effect, and the effect was also inhibited by C3. GTPγS has been demonstrated to act on G-proteins that are implicated in muscarinic signaling mechanisms and mimic some muscarinic effects in intestinal smooth muscle such as an increase in myofilament Ca^{2+} sensitivity (5), release of internal Ca^{2+} stores (17, 18, 24) and activation of nonselective cationic current (17, 25). To elaborate on the idea that muscarinic Ca^{2+}-sensitization of tension development involves the small G-protein, further studies are needed to demonstrate that C3 induced ADP-ribosylation of the G-protein correlates with its inhibitory effect on CCh-induced tension development. In addition, it remains to be clarified how stimulation of muscarinic receptors is coupled to the small G-protein and how the G-protein modifies the activities of MLC20 kinase and/or MLC phosphatase, determinants of the extent of MLC20 phosphorylation (7).

It has been suggested that there are two pathways for Ca^{2+} sensitization in vascular smooth muscle: one is protein kinase C (PKC)-dependent and activated by phorbol esters, and the other is PKC-independent and activated by receptor agonists (26). Thus, it also remains to be clarified whether PKC is involved in the muscarinic Ca^{2+}-sensitizing effect in intestinal smooth muscle.

The GTPγS-induced rise in tension, but not the CCh-induced one, was very slow in C3-treated preparations (cf. Figs. 3B and 5B). Since GTPγS can activate different G-proteins nonselectively, the GTPγS-induced rise in tension may comprise C3-sensitive and C3-resistant components for which a G-protein-linked pathway is separately provided. In skinned vascular smooth muscle, arachidonic acid (AA) applied externally produces a slowly-developed rise, in the presence of a low and constant level of Ca^{2-}, through direct inhibition of MLC phosphatase (10). The C3-resistant component is characteristic of a slow development, and it might be due to stimulation of phospholipase A_2 (PLA_2), accumulation of AA and AA-dependent modification of the Ca^{2+} sensitivity of contractile elements. A major component of the rise in Ca^{2+} sensitivity evoked by stimulation of muscarinic receptors is not brought about by this PLA_2/AA pathway, because the C3-resistant component was virtually absent in the Ca^{2-}-sensitizing effect of CCh.

An interesting finding is that C3 reduced the Ca^{2+} sensitivity of tension development in the absence of CCh and GTPγS, and its effect was abolished by GDPβS. This indicates that some Ca^{2} sensitization has already been caused even under CCh- and GTPγS-free conditions by a mechanism in which a C3-sensitive G-protein is involved (resting Ca^{2+} sensitization). However, Hirata et al. (14) reported that in arterial smooth muscle permeabilized with saponin, C3 had no such effect. It is well known that skinning with β-escin does allow receptor-coupled signal transduction systems to be retained. Thus, the resting Ca^{2+} sensitization may be produced by some ingredients of the bath medium, such as ATP and its metabolites, which act on their respective receptors to enhance Ca^{2+} sensitivity. Another possibility is that under normal conditions, the Ca^{2+} sensitizing mechanism is in a partially-operated state and preservation of some membrane and/or cytosol components may be required for the operation.

REFERENCES

1 Hartshorne DJ: Biochemistry of the contractile process in smooth muscle. *In* Physiology of the Gastrointestinal Tract, Edited by Johnson LR, pp 423–482, Raven Press, New York (1987)

2 Karaki H: Ca^{2+} localization and sensitivity in vascular smooth muscle. Trends Pharmacol Sci **10**, 320–325 (1989)

3 Somlyo AP and Himpens B: Cell calcium and its regulation in smooth muscle. FASEB J **3**, 2266–2276 (1989)

4 Nishimura J, Kolber M and van Breemen C: Norepinephrine and GTPγS increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. Biochem Biophys Res Commun **157**, 677–683 (1988)

5 Kitazawa T, Kobayashi S, Horiuti K, Somlyo AV and Somlyo AP: Receptor-coupled, permeabilized smooth muscle. J Biol Chem **264**, 5339–5342 (1989)

6 Rembold CM and Murphy RA: $[Ca^{2+}]_i$-Dependent myosin phosphorylation in phorbol ester stimulated smooth muscle contraction. Am J Physiol **255**, C719–C723 (1988)

7 Kitazawa T, Gaylinn BD, Denny GH and Somlyo AP: G-protein mediated Ca^{2+} sensitization of smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem **266**, 1708–1715 (1991)

8 Hori M, Sato K, Ozaki H, Takano-Ohmuro H, Tsuchiya T, Sugi H, Kato I and Karaki H: Receptor agonists induce myosin phosphorylation-dependent and phosphorylation-independent contrations in vascular smooth muscle. J Pharmacol Exp Ther **261**, 506–512 (1992)

9 Stull JT, Hsu LC, Tansey MG and Kamm KE: Myosin light chain kinase phosphorylation in tracheal smooth muscle. J Biol Chem **265**, 16683–16690 (1990)

10 Gong MC, Fuglsang A, Alessi D, Kobayashi S, Cohen P, Somlyo AV and Somlyo AP: Arachidonic acid inhibits myosin light chain phosphatase and sensitizes smooth muscle to calcium. J Biol Chem **267**, 21492–21498 (1992)

11 Kikuchi A, Yamamoto K, Fujita T and Takai Y: ADP-ribosylation of the bovine brain *rho* protein by botulinum toxin type C1. J Biol Chem **263**, 16303–16308 (1988)

12 Narumiya S, Sekine A and Fujiwara M: Substrate for botulinum ADP-transferase, Gb, has an amino acid sequence homologous to a putative *rho* gene product. J Biol Chem **263**, 17255–17257 (1988)

13 Sugai M, Hashimoto K, Kikuchi A, Inoue S, Okumura H, Matsumoto K, Goto Y, Ohgai H, Moriishi K, Syuto B, Yoshikawa K, Suginaka H and Takai Y: Epidermal cell differentiation inhibitor ADP-ribosylates small GTP-binding proteins and induces hyperplasia of epidermis. J Biol Chem **267**,

2600–2604 (1992)

14 Hirata K, Kikuchi A, Sasaki T, Kuroda S, Kaibuchi K, Matsuura Y, Seki H, Saida K and Takai Y: Involvement of *rho* p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. J Biol Chem **267**, 8719–8722 (1992)

15 Gilman AG: G-Proteins: transducers of receptor-generated signals. Annu Rev Biochem **56**, 615–649 (1987)

16 Bourne HR, Sanders DA and McCormick F: The GTPase superfamily: a conserved switch for diverse cell functions. Nature **348**, 125–132 (1990)

17 Komori S, Kawai M, Takewaki T and Ohashi H: GTP-binding protein involvement in membrane currents evoked by carbachol and histamine in guinea pig ileal muscle. J Physiol (Lond) **450**, 105–126 (1992)

18 Fukami K, Itagaki M, Komori S and Ohashi H: Contractile responses to histamine and GTPγS in β-escin-treated skinned smooth muscle of guinea pig ileum. Jpn J Pharmacol **63**, 171–179 (1993)

19 Kobayashi S, Kitazawa T, Somlyo AV and Somlyo AP: Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. J Biol Chem **264**, 17997–18004 (1989)

20 Uyama Y, Imaizumi Y and Watanabe M: Effects of cyclopiazonic acid, a novel Ca^{2+}-ATPase inhibitor, on contractile responses in skinned ileal smooth muscle. Br J Pharmacol **106**, 208–214 (1992)

21 Oiki S, Yamamoto T and Okada Y: A simultaneous evaluation method of purity and apparent stability constant of Ca-chelating agents and selectivity of coefficient of Ca-selective electrodes. Cell Calcium **15**, 199–208 (1993)

22 Moriichi K, Syuto B, Yokosawa N, Oguma K and Saito M: Purification and characterization of ADP-ribosyltransferases (exoenzyme C3) of *Clostridium botulinum* type C and D strain. J Bacteriol **173**, 6025–6029 (1991)

23 Braun U, Habermann B, Just I, Aktories K and Vandekerckhove J: Purification of the 22kDa protein substrate of botulinum ADP-ribosyltransferase C3 from porcine brain cytosol and its characterization as a GTP-binding protein highly homologous to the *rho* gene product. FEBS J **243**, 70–76 (1989)

24 Komori S and Bolton TB: Calcium release induced by inositol 1,4,5-trisphosphate in single rabbit intestinal smooth muscle cells. J Physiol (Lond) **433**, 495–517 (1991)

25 Inoue R and Isenberg G: Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. Am J Physiol **258**, C1173–C1178 (1990)

26 Hori M, Sato K, Miyamoto S, Ozaki H and Karaki H: Different pathways of calcium sensitization activated by receptor agonists and phorbol esters in vascular smooth muscle. Br J Pharmacol **110**, 1527–1531 (1993)