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Role of Rho proteins in carbachol-induced contractions in intact and permeabilized guinea-pig intestinal smooth muscle

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1. The aim of this study was to determine whether the low molecular mass GTPase RhoA or related proteins are involved in carbachol- and high-K^+-induced contractions in *intact* intestinal smooth muscle as well as the carbachol-induced increase in Ca^{2+} sensitivity of the myofilaments in *permeabilized* preparations.

2. The carbachol-induced increase in the Ca^{2+} sensitivity of force production in β-escin-permeabilized intestinal smooth muscle was enhanced in preparations that were loaded with the constitutively active mutant of RhoA, ^{Val14}RhoA, and was inhibited by exoenzyme C3 from *Clostridium botulinum*, which ADP-ribosylates and inactivates small GTPases of the Rho family. The effect of C3 on Ca^{2+} sensitivity in the absence of the agonist was negligible, while the maximal Ca^{2+}-activated force was inhibited by about 20%.

3. Inhibition of carbachol-induced force was associated with an increase in ADP-ribosylation of a protein band with a molecular mass of ~22 kDa, corresponding to Rho, and was partially reversed in the presence of ^{Ile41}RhoA, which is not a substrate for C3. ^{Val14}RhoA did not restore carbachol-induced Ca^{2+} sensitization in C3-treated smooth muscle.

4. In intact intestinal smooth muscle, toxin B from *Clostridium difficile*, which monoglucosylates members of the Rho family, inhibited high-K^+-induced contractions and the initial phasic response to carbachol by about 30%. The delayed contractile response to carbachol was completely inhibited.

5. In smooth muscle preparations that were permeabilized with β-escin after treatment with toxin B, carbachol- and GTPγS-induced Ca^{2+} sensitization was significantly inhibited.

6. These findings are consistent with a role for Rho or Rho-like proteins in agonist-induced increase in Ca^{2+} sensitivity of force production in *intact* and *permeabilized* intestinal smooth muscle.

Neurohumoral stimulation of smooth muscle (pharmacomechanical coupling) leads to an increased responsiveness of the myofilaments to Ca^{2+} (reviewed in Somylo & Somlyo, 1994). The agonist-induced increase in Ca^{2+} sensitivity is mimicked by the poorly hydrolysed GTP analogue, GTPγS (Fujiwara, Itoh, Kubota & Kuriyama, 1989), and is inhibited by GDPβS (e.g. Satoh, Kreutz, Wilm, Ganten & Pfitzer, 1994), indicating that the intracellular signalling cascade leading to Ca^{2+} sensitization requires the activation of at least one GTP-binding protein. There are two major superfamilies of GTP-binding proteins: (1) the heterotrimeric G proteins serving as transducers for membrane receptors; and (2) the superfamily of Ras-related low molecular mass GTPases (Bourne, Sanders & McCormick, 1990). A heterotrimeric G protein appears to be involved in Ca^{2+} sensitization (Rembold, 1990; Fujita, Takeuchi, Nakajima, Nishio & Hata, 1995; Gong *et al.* 1996) but its nature has not yet been identified. It was also proposed that low molecular weight GTPases may be involved because loading of permeabilized smooth muscle with constitutively active mutants of Ras (Satoh, Rensland & Pfitzer, 1993) or Rho (Gong *et al.* 1996) or wild-type Rho activated with GTPγS (Hirata *et al.* 1992) increased the Ca^{2+} sensitivity of force production. However, the effects of the mutated proteins

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may differ from their normal counterparts. The mutant proteins may have higher levels of GTP loading than their normal counterparts, they may be active permanently rather than temporarily, and may be abnormally localized, permitting them to interact with effectors they do not normally contact (Vojtek & Cooper, 1995), while the effects of wild-type Rho may be due to release of free GTPγS (Satoh *et al.* 1993).

A different approach to elucidate the physiological role of Rho in intracellular signalling cascades relies on bacterial toxins. Rho is ADP-ribosylated at Asn-41, which is located in the putative effector domain by the staphylococcal toxin, epidermal differentiation inhibitor (EDIN), and by exoenzyme C3 from *Clostridium botulinum*. This modification most probably inhibits Rho (Sugai *et al.* 1992; Aktories, Mohr & Koch, 1992). These toxins prevent the GTPγS- and the agonist-induced increase in Ca^{2+} sensitivity in permeabilized vascular and intestinal smooth muscle (Hirata *et al.* 1992; Itagaki, Komori, Unno, Syuto & Ohashi, 1995; Kokubu, Satoh & Takayanagi, 1995; Pfitzer, Otto, Steusloff, Just & Aktories, 1995; Gong *et al.* 1996). However, it was recently proposed that C3 at high concentrations acts less specifically and may not only inhibit the function of Rho but also that of other related proteins (Ridley, Comoglio & Hall, 1995); for example, *in vitro* C3 inefficiently ADP-ribosylates the Rho-related GTPases Rac and Cdc42H (Ridley & Hall, 1992). One aim of this study was therefore to determine whether the extent of ADP-ribosylation catalysed by C3 correlates with the extent of its inhibitory effect on the agonist-induced increase in Ca^{2+} sensitivity of the myofilaments. We also tested whether the inhibitory effect of C3 on force can be reversed by loading the permeabilized smooth muscle preparations with the mutant ^{Ile41}RhoA, which is not a substrate for C3.

These experiments carried out in permeabilized smooth muscle suggest, but do not prove, that Rho proteins participate in excitation–contraction coupling in *intact* smooth muscle. This important question can be addressed by using toxin B from *C. difficile* as a tool. Toxin B, which is internalized into intact cells (Florin & Thelestam, 1983), is a monoglucosyltransferase which glucosylates Rho in the putative effector domain at Thr-37 (Just, Selzer, Wilm, von Eichel-Streiber, Mann & Aktories, 1995). In cultured cells, toxin B disrupts the actin cytoskeleton and causes cell rounding much in the same way as does microinjected C3 (Just *et al.* 1995). This suggests that the intracellular targets of toxin B are members of the Rho family. Here, we report that incubation of *intact* intestinal smooth muscle with toxin B inhibits the delayed contractile response to carbachol which was suggested to be due to Ca^{2+} sensitization (Himpens & Somlyo, 1988), while the inhibitory effect on the initial phasic response was small. The inhibition of force in the intact smooth muscle by this toxin was associated with a loss of the Ca^{2+}-sensitizing effect of carbachol in permeabilized smooth muscle. Preliminary reports of our results have been published in abstract form (Otto, Troschka, Just, Aktories & Pfitzer, 1995; Pfitzer *et al.* 1995).

METHODS

Force measurements

Guinea-pigs of either sex (200–300 g) were killed by cervical dislocation and exsanguination. The small intestine was rapidly removed and flushed with physiological salt solution (PSS) containing (mM): NaCl, 118; KCl, 5; Na_2HPO_4, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 1·6; Hepes, 24; and glucose, 10 (pH 7·4 at room temperature, 20–21 °C). The longitudinal muscle layer was separated from the circular layer under a dissecting microscope. For isometric tension recordings, small strips of the longitudinal muscle (5–7 mm long and 200–250 μm wide) were mounted horizontally on a myograph with an AM 801 force transducer (Akers, Horton, Norway). Strips subjected to treatment with the toxins or Rho proteins and control strips were taken from adjacent pieces of tissue. All experiments were carried out at room temperature. Following an equilibration period of about 30 min, a test contraction with carbachol was elicited. The strips were then permeabilized with β-escin according to the protocol of Kobayashi, Kitazawa, Somlyo & Somlyo (1989). In brief, the strips were incubated for 10 min in Ca^{2+}-free PSS containing, in addition, 2 mM EGTA, and this was followed by treatment with 50 μM β-escin in relaxing solution for 35 min. Relaxing solution consisted of (mM): imidazole, 20; EGTA, 10; magnesium acetate, 10; ATP, 7·5; creatine phosphate, 10; NaN_3, 5; pH 7·0 at room temperature. Ionic strength was adjusted to 150 mM with potassium methanesulphonate. The contracting solution contained in addition 10 mM $CaCl_2$. Alterations in the free Ca^{2+} concentration were obtained by mixing relaxing and contracting solution in the appropriate ratio (see Satoh *et al.* 1993). To allow addition of proteins and guanosine nucleotides without diluting the ionic composition of the solutions, solutions were made up doubly concentrated and stored in aliquots at −20 °C. This concentrate was diluted 2-fold by the addition of (final concentrations) dithioerythritol (2 mM), leupeptin (1 μM) and calmodulin (1·5 μM), as well as C3 or Rho proteins or the appropriate protein buffers as indicated. To the solutions containing C3 or C3 buffer, 100 μM NAD was added. Solutions were contained in 0·15 or 1 ml cups. In some experiments, the tissues were treated with 10 μM A23187 for 20 min in relaxing solution to deplete Ca^{2+} from the intracellular store sites (Kobayashi *et al.* 1989). Treatment with A23187 did not affect the responses to Ca^{2+} or carbachol.

ADP-ribosylation

To confirm qualitatively that C3 ADP-ribosylates an endogenous protein with a molecular mass of ~22 kDa, single permeabilized strips were held isometrically and subjected to the same protocol as fibres used for force measurements except that only one sensitization with carbachol was elicited. Fibres were then incubated for 15 min at room temperature at pCa 6·27 with 0·67 μg ml^{-1} C3 and 10 μCi [^{32}P]NAD (1–2 μM); unlabelled NAD was omitted from the solutions. Control experiments confirmed that these conditions were sufficient to induce complete inhibition of the contractile response to carbachol. The reaction was terminated by incubating the strips in 10 mM NAD for 5 min on ice followed by 5 min in boiling SDS (5%). The tissues were homogenized in Laemmli buffer (Tris–HCl, 50 mM, pH 6·8; urea, 4 M; SDS, 1% w/v; glycerol, 15% v/v; DTE, 20 mM) and the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE); 40 μg of protein

was loaded on each lane. The protein concentration was determined using the method of Bradford (1976) with immunoglobulin G (IgG) as the standard. [^{32}P] incorporation was detected by autoradiography.

To quantify ADP-ribosylation of Rho in the smooth muscle strips, the method of differential ADP-ribosylation was used. ADP-ribosylation in the permeabilized smooth muscle decreases subsequent [^{32}P]ADP-ribosylation of Rho in homogenates of the strips. After force measurement experiments, the strips were mechanically homogenized followed by sonication on ice. After adjusting the samples to identical protein concentration, C3-catalysed ADP-ribosylation was performed. The lysates were incubated in a buffer (containing 10 mM thymidine, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM [^{32}P]NAD (0·25 μCi) and 50 mM triethanolamine-HCl, pH 7·5) with 1 μg ml^{-1} C3 exoenzyme for 15 min at 37 °C. The proteins were separated by 12·5% SDS-PAGE followed by evaluation of the [^{32}P] incorporation by the phosphorimager (Molecular Dynamics).

Protein purifications

Calmodulin was prepared by the method of Gopalakrishna & Anderson (1982) except for the use of bovine testicles instead of bovine brain. *C. botulinum* C3 exoenzyme (Aktories, Rösener, Blaschke & Chatwal, 1988) and *C. difficile* toxin B (Eichel-Streiber, Harperath, Bosse & Hadding, 1987) were purified as described. The buffers of the protein stock solutions of C3 and toxin B had the following compositions (mM): NaCl, 100; sodium pyrophosphate, 100; pH 7·2 (C3); and NaCl, 600; Tris–HCl, 50, pH 7·5 (toxin B). Anti-C3 IgG (10 mg ml^{-1} in phosphate-buffered saline) was prepared by affinity purification of polyclonal anti-C3 rabbit antiserum by eluting from exoenzyme C3 immobilized to Sepharose beads. ^{Ile41}RhoA and ^{Val14}RhoA proteins were prepared from an *Escherichia coli* pGEX-2T expression system. The glutathione S-transferase RhoA fusion protein immobilized to beads was loaded with either ^{Ile41}RhoA·GTPγS or ^{Val14}RhoA·GTP in the presence of EDTA. After washing, RhoA proteins were released from the fusion protein by thrombin treatment. Thrombin was removed with benzamidine-Sepharose. The buffer of the Rho proteins contained (mM): NaCl, 150; triethanolamine, 50, pH 7·5; $CaCl_2$, 2·3; $MgCl_2$, 2·5.

Statistics

Values are shown as means ± S.E.M. (n = number of experiments). Difference of responsiveness among groups was tested by Student's t test. Significance was determined when $P < 0.05$.

RESULTS

Effect of Rho proteins on carbachol-induced Ca^{2+} sensitization

Following permeabilization, the strips were maximally activated (F_{max}) at pCa 4·85, which yielded 113 ± 11% ($n = 12$) of the tension elicited by 10 μM carbachol in the intact preparation. The fibres were then relaxed at pCa > 8 followed by submaximal activation with pCa 6·27. Addition of carbachol in the presence of GTP at constant pCa 6·27 reversibly increased force from 3 ± 0·3 to 14 ± 1% ($n = 39$) of the maximal force (F_{max}) elicited by pCa 4·85 (Fig. 1*A*). This corresponds to a 4·7-fold increase in force induced by carbachol. Carbachol also increased force when the strips were pre-activated with higher submaximal concentrations of Ca^{2+}. However, F_{max} was not affected by carbachol. This indicated that carbachol induced an increase in the Ca^{2+}

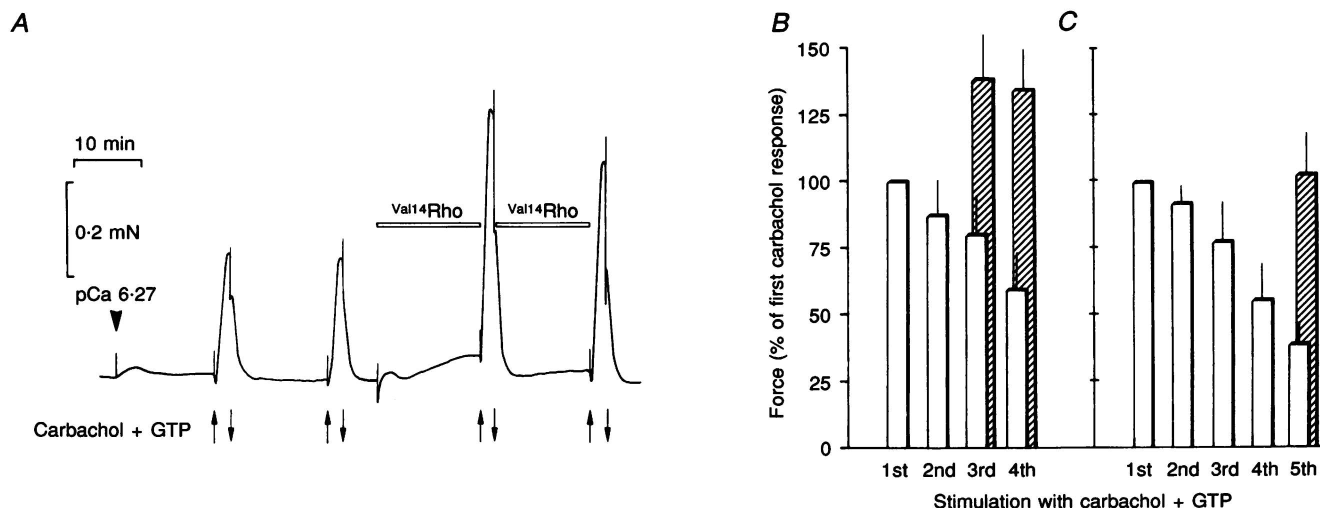

Figure 1. Effect of the constitutively active mutant of RhoA (^{Val14}RhoA) on carbachol-induced increase in Ca^{2+} sensitivity of smooth muscle contraction

Intestinal smooth muscle strips, which were permeabilized with β-escin, were stimulated submaximally with pCa 6·27 (arrowhead), which was then maintained at this constant level. Carbachol and GTP (10 μM each) were added (↑) and washed out immediately after the peak of the contraction was reached (↓). The strips were incubated with 0·23 mg ml^{-1} ^{Val14}Rho·GTP (▨) or with protein buffer (□) at pCa 6·27 for 15 min before the 3rd and 4th application of carbachol + GTP (*A* and *B*, $n = 6$) or before the 5th application (*C*, $n = 5$). Bars and vertical lines represent means ± S.E.M.

sensitivity of force production at constant [Ca^{2+}] similar to what has been reported previously (Kitazawa, Gaylinn, Denney & Somlyo, 1991*a*). However, as shown in Fig. 1*C*, the response to carbachol decreased with repeated applications.

We then tested whether the constitutively active form of RhoA (^{Val14}Rho·GTP, 0·23 mg ml^{-1}) would affect Ca^{2+}-activated force and reverse the run-down of carbachol-induced Ca^{2+} sensitization. In a first series of experiments, ^{Val14}Rho·GTP was added at pCa 6·27 after the 2nd and 3rd stimulation with carbachol for 15 min each (Fig. 1*A* and *B*). The pCa 6·27 solutions of the control fibres contained 12 μM GTP (corresponding to the concentration of ^{Val14}Rho·GTP). The Rho proteins had a variable effect on Ca^{2+}-activated force in the absence of the agonist. The small and slowly developing increase in force shown in Fig. 1*A* was observed in only four out of eleven experiments. In contrast, force elicited by carbachol at constant pCa was enhanced in all preparations. Thus, in six strips loaded with Rho, carbachol-induced force was 138 ± 18 and 135 ± 16% of the initial response (3rd and 4th application of carbachol, respectively), which compares with 80·5 ± 15 and 59·3 ± 13·7% in six control preparations (Fig. 1*B*).

In a second series of experiments, we tested whether the carbachol response may be recovered after a significant run-down was established. In these experiments, ^{Val14}Rho·GTP was added after the 4th challenge with carbachol and thereafter, a test contraction was elicited. While the response in the control strips ($n = 3$) amounted to 39 ± 7·5% of the initial response to carbachol, the force production of the strips incubated with ^{Val14}Rho·GTP was fully recovered and was 102·5 ± 14·8% ($n = 6$) of the initial response (Fig. 1*C*). The effect of ^{Val14}Rho·GTP was reversible. After washing out Rho for 15 min at pCa 6·27 the carbachol response dropped by 53 ± 6% ($n = 9$) but only by 7 ± 4% ($n = 11$) in the presence of RhoA.

To clarify the role of Rho in carbachol-induced Ca^{2+} sensitization further, we determined whether the response

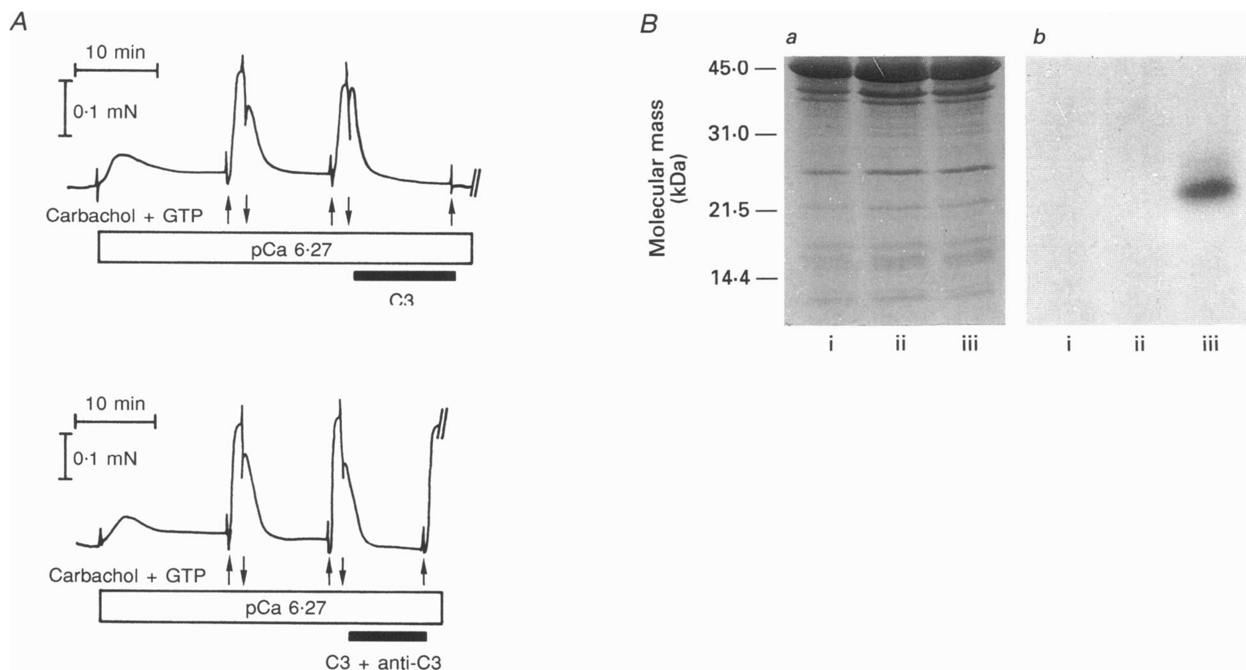

Figure 2. The exoenzyme C3 from *C. botulinum* inhibits carbachol-induced Ca^{2+} sensitization in β-escin-permeabilized smooth muscle, which is associated with ADP-ribosylation of a protein band of molecular mass of ~22 kDa

In *A* the strips were pre-activated with pCa 6·27 and carbachol + GTP (10 μM each) was added (↑) and removed (↓). The response to carbachol was completely inhibited after incubation with C3 (0·67 μg ml^{-1}) in the presence of 100 μM NAD (upper trace) but not when C3 was pre-incubated with the affinity-purified anti-C3 antibody (0·1 mg ml^{-1}) on ice for 1 h (C3 + anti-C3, lower trace). The strips were shock frozen in liquid nitrogen at the end of the experiment (at //) for determination of ADP-ribosylation (Fig. 3). The traces show typical examples of 6 (upper trace) and 8 strips (lower trace). *B*, ADP-ribosylation in single permeabilized smooth muscle strips. The smooth muscle strips were incubated with [^{32}P]NAD and analysed by SDS-PAGE and autoradiography as described in Methods. *a*, Coomassie-stained gel and *b*, the corresponding autoradiogram: i, control fibre; ii, fibre incubated with C3 + anti-C3 antibodies; iii, fibre incubated with C3. Note, radioactivity was only incorporated in the C3-treated fibres into a protein band with a molecular mass of ~22 kDa. The results shown are representative of 4 independent experiments.

to carbachol is prevented by C3. Unlike toxin B from *C. difficile* (see below), C3 did not inhibit tension development in intact strips. In strips permeabilized with β-escin, two test contractions with carbachol at pCa 6·27 were elicited. The strips were then incubated for 12 min with either C3 or buffer at constant calcium (pCa 6·27) and then stimulated with carbachol again (Fig. 2*A*). The response to carbachol was completely inhibited after incubation with 0·67 μg ml^{-1} C3. No tension response was observed even when the strips were incubated for 20 min with carbachol. C3 (0·67 μg ml^{-1}) also completely inhibited the Ca^{2+}-sensitizing effect of carbachol at a higher level of Ca^{2+} activation (pCa 5·9). The contractile response to carbachol was not inhibited by C3 when the toxin was pre-incubated with an inactivating antibody (0·1 mg ml^{-1}) for 1 h on ice (Fig. 2*A*).

The inhibitory action of C3 was associated with ADP-ribosylation of a polypeptide with a molecular mass of about 22 kDa (Fig. 2*B*). In the absence of C3 there was no incorporation of radioactivity, indicating that there is no endogenous ADP-ribosylating activity under our experimental conditions. There was also no incorporation of radioactivity when the strips were incubated with anti-C3-treated C3.

The inhibitory action of C3 was concentration dependent and correlated with the extent of ADP-ribosylation. About 50% inhibition of force was observed at 0·2 μg ml^{-1} C3 (Fig. 3*A*). At this concentration of C3, 60% of the endogenous Rho was ADP-ribosylated (Fig. 3*B*). Increasing the concentration of C3 did not enhance the extent of ADP-ribosylation while force was now completely inhibited (Fig. 3*A* and *B*).

The concentration of C3 required to obtain 50% inhibition of carbachol-induced increase in Ca^{2+} sensitivity depended on the method used to obtain the dose–response curve. When the dose–response curve was obtained in a cumulative manner, whereby the strips were incubated with each concentration of C3 for 12 min, about 50–90% inhibition of force was observed at 0·07 and 0·2 μg ml^{-1} C3, respectively. The apparent higher potency of C3 under this condition may be due to the fact that there was more time for diffusion of C3 into the strips and for the enzymic reaction to proceed.

The effect of C3 on carbachol-induced Ca^{2+} sensitization was irreversible. However, the response to carbachol and GTPγS could be partially restored by loading the strips with recombinant ^{Ile41}RhoA·GTPγS (Fig. 4*A*), which is resistant to ADP-ribosylation (Paterson, Self, Garret & Just, 1990). After incubating the strips for 45 min with ^{Ile41}RhoA about 50% of the initial response to carbachol was recovered (Fig. 4*B*). Extending the incubation period to 60 min did not further increase the response to carbachol (data not shown). There was also some recovery of the force response to GTPγS (Fig. 4*B*). The effect of ^{Ile41}Rho·GTPγS was not due to activation of endogenous GTP binding proteins by free GTPγS, since GTPγS alone was unable to induce a contraction after incubation with C3 (Fig. 4*A*). Moreover, ^{Ile41}Rho·GTPγS itself in the absence of the agonist had no contractile action (Fig. 4*A*). ^{Val14}RhoA·GTP did not restore the response to carbachol: this was unexpected. The most likely explanation is that ^{Val14}RhoA, which is a substrate for C3, may be inactivated in the intestinal smooth muscle strips loaded with C3.

Effect of *C. difficile* toxin B on intact and permeabilized intestinal smooth muscle

Incubation of intact smooth muscle strips with 10 μM carbachol produced a biphasic tension response: a rapid

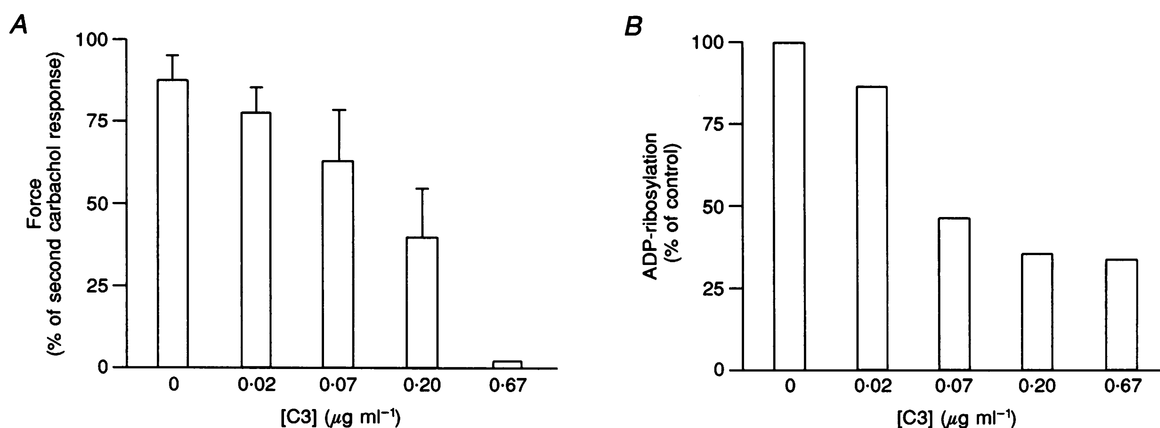

Figure 3. Dose–response relation of C3-induced inhibition of carbachol-induced force at constant pCa 6·27 (*A*) and ADP-ribosylation (*B*)

Experimental protocol as in Fig. 2*A*. Note that each strip was subjected to a single concentration of C3. Fibres subjected to isometric tension measurement were shock frozen. The lysates of the strips were subjected to C3-catalysed [^{32}P]ADP-ribosylation. Incorporation into control fibres was set at 100%. Bars and vertical lines represent means ± S.E.M. (n = 5–8).

increase in force to a first maximum (peak 1) followed by a partial relaxation and a second contraction (peak 2), after which force declined to nearly basal levels. This response was reproducible for many hours. After eliciting three test contractions, the average of which was taken as 100%, the strips were incubated with either toxin B (6 fibres with 20 ng ml^{-1} and 4 fibres with 40 ng ml^{-1}) or protein buffer for up to 14 h and challenged with carbachol at different times (Fig. 5*A*). In the control fibres, force increased during the first 3 h to 111 ± 13% (peak 1) and 162 ± 24% (peak 2) of the initial response. This tension level was then maintained in the control preparations (Fig. 5*B*). In the fibres incubated with toxin B, peak 2 was nearly completely inhibited after 5 h incubation (Fig. 5*B*). The effect of the toxin on peak 1 was variable. In three out of six fibres treated with 20 ng ml^{-1} toxin for 3–5 h, force at peak 1 was less than 34% of the control force, while in the remaining strips peak 1 amounted to 78% of control force. In an attempt to obtain a more complete inhibition, we incubated another four fibres with 40 ng ml^{-1} for more than 7 h. Under these conditions, peak 2 was completely inhibited (Fig. 5*B*) while peak 1 amounted to about 76% of the control force. Depolarization with high-K^+ (100 mM) solution induced a transient contraction. After an initial phasic rise, force declined to about 10–20% of the initial peak. Figure 5*C* shows the effect of toxin B on peak force. After 3 h incubation, force was inhibited by about 30% (means of tension values: 144 ± 8% for control strips and 101 ± 6% in toxin B-treated strips, $P < 0.05$). The inhibitory effect of the toxin was not enhanced when the fibres were incubated for up to 13 h irrespective of the concentration of the toxin.

In a second series of experiments, incubation with toxin B was followed by permeabilization with β-escin. After a submaximal activation (pCa 6·27), the strips were incubated with carbachol and then with GTPγS (Fig. 6*A*). In control strips, both carbachol and GTPγS induced an increase in Ca^{2+} sensitivity. In contrast, in the strips treated with toxin B, the response to carbachol was nearly completely inhibited. The response to GTPγS was inhibited by about 80% ($P < 0.05$; Fig. 6*B*).

Effect of C3 and toxin B on Ca^{2+}-activated force in permeabilized smooth muscle in the absence of an agonist

To test whether C3 has a direct effect on the contractile apparatus, two cumulative force–pCa relations were obtained in β-escin-permeabilized intestinal smooth muscle strips. Before the second force–pCa response curve, the fibres were incubated with either C3 (0·67 μg ml^{-1}) or buffer at pCa > 8. Compared with the control strips, C3 significantly decreased the amplitude of maximal force elicited by pCa 4·85 (Fig. 7*B*). Ca^{2+} sensitivity was, however, barely affected (pCa_{50} values for control and C3-treated preparations were 5·93 and 5·89, respectively, cf. Fig. 7*C*). The effect of

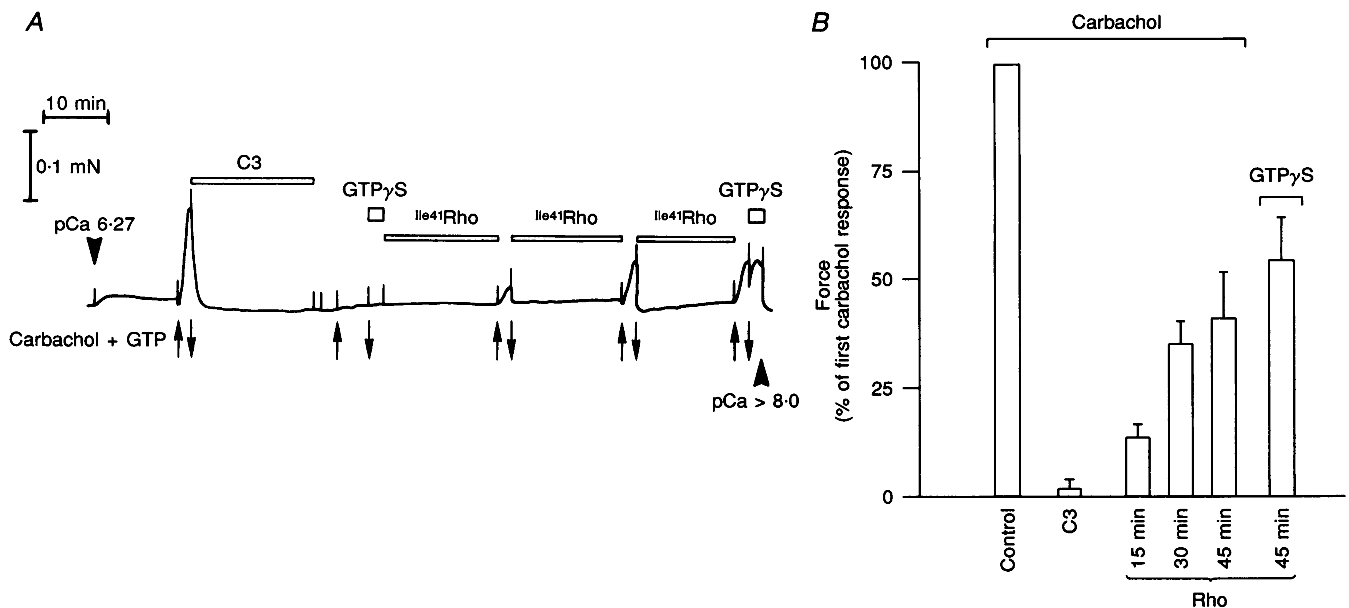

Figure 4. The inhibitory effect of C3 is reversed with recombinant ^{Ile41}Rho activated with GTPγS

A, fibres were pre-activated with pCa 6·27 (arrowhead) as described in Fig. 1, which yielded 3·7 ± 0·8% of F_{max} ($n = 5$). After a test response to carbachol and GTP (10 μM each; Control in *B*), the strips were incubated with C3 (1 μg ml^{-1}) for 15–20 min, washed twice in contracting solution (pCa 6·27) and were then stimulated with carbachol + GTP (C3 in *B*) and GTPγS (10 μM). The responses were completely inhibited (cf. *B*). Fibres were then incubated with ^{Ile41}Rho·GTPγS (0·1 mg ml^{-1}) for 45 min interrupted by challenges with carbachol + GTP at intervals of 15 min (*B*). After the 3rd carbachol challenge the solutions were changed to GTPγS, which in some experiments produced a further increase in force (*B*). Bars and vertical lines represent means ± S.E.M. for 5 experiments.

Figure 5. Effect of toxin B from *C. difficile* on contractions in intact intestinal smooth muscle strips

A shows the experimental protocol. After mounting, the strips were activated 3 times with carbachol (only 1 response shown); the mean was taken as 100%. The strips were then incubated with toxin B or buffer and subjected to repeated test contractions with carbachol. *B*, effect of toxin B on the delayed contraction (peak 2), at concentrations of 20 ng ml^{-1} (●; $n = 6$), 40 ng ml^{-1} (▲; $n = 4$) and control fibres (open symbols; $n = 11$). *C*, original force tracing of K^+-induced contractions. *D*, summary of the results obtained with toxin B on high-K^+-induced contractions. Force is expressed as the percentage of the carbachol-induced contraction; 20 ng ml^{-1} (●; $n = 2$), 40 ng ml^{-1} (▲; $n = 4$) and control fibres (open symbols; $n = 6$).

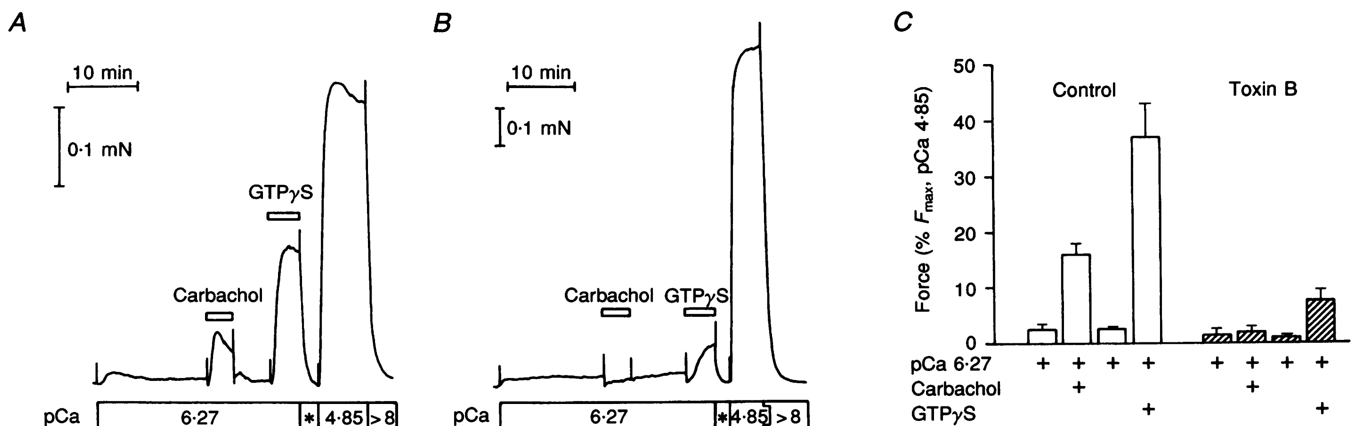

Figure 6. Effect of toxin B treatment on carbachol- and GTPγS-induced increase in Ca^{2+} sensitivity

The strips were permeabilized with β-escin after incubation with toxin B (40 ng ml^{-1}) for 10 h (*B*). *A* (control) and *B* show representative force tracings; GTPγS was washed out in relaxing solution, pCa > 8 (*). *C* summarizes the results shown in *A* and *B*. Bars and vertical lines represent means ± S.E.M. for 4 toxin B-treated and 4 control fibres.

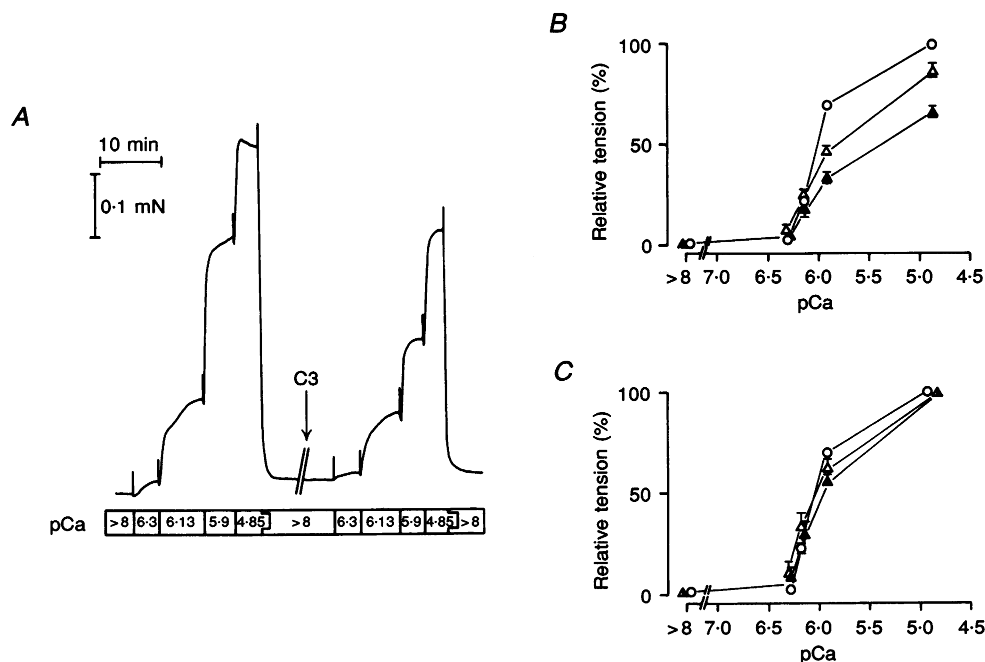

Figure 7. Effect of C3 on Ca^{2+}-activated force

A shows the experimental protocol. Fibres were activated by cumulatively increasing [Ca^{2+}] (○ in *B* and *C*). After relaxation, they were either incubated with C3, 0·67 μg ml^{-1} (▲ in *B* and *C*; $n = 6$), or with buffer (△ in *B* and *C*; $n = 6$) for 20 min and then a second force–pCa relation was obtained. *B*, force–pCa relation whereby force is normalized to the first maximal force. Note the decline of maximal force is larger in the C3-treated fibres (▲) than in the control fibres (△). *C*, force is normalized to the respective maximal activation. Note that there is only a small shift in pCa_{50} values. Symbols represent means ± S.E.M.

toxin B on maximal Ca^{2+}-activated force could not be determined because the toxin had no effect when incubated with β-escin-permeabilized fibres. We therefore incubated intact strips with toxin B as described above and then determined the effect on Ca^{2+} sensitivity after permeabilization in the absence of the agonist. As shown in Fig. 8, the force–pCa relationship in toxin-treated fibres was similar to that in control preparations.

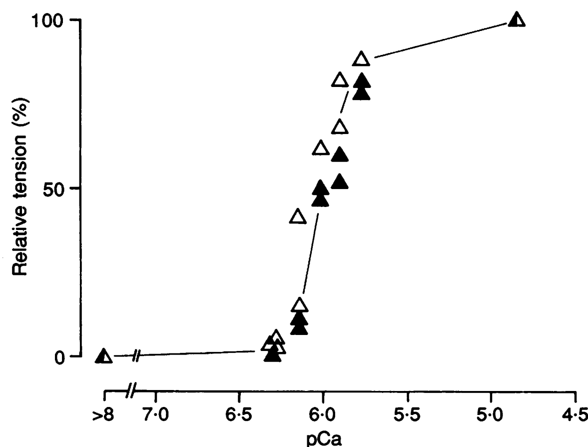

Figure 8. Effect of toxin B on Ca^{2+}-activated force

Intact smooth muscle strips were treated for 8 h with toxin B (40 ng ml^{-1}) or buffer and then permeabilized with β-escin. A force–pCa relation was obtained by cumulatively increasing [Ca^{2+}]. Control fibres, △; toxin B-treated fibres, ▲. Symbols represent individual force values obtained in 4 fibres in each group.

DISCUSSION

Recombinant Rho proteins and agonist-induced increase in Ca^{2+} sensitivity in permeabilized smooth muscle

As has been shown in a number of studies (see Pfitzer, 1996 for review), the receptor–effector coupling in β-escin-permeabilized smooth muscle is functionally intact, as indicated by the fact that carbachol increases force at

constant submaximal [Ca^{2+}]. However, the response to carbachol declines with repeated stimulations. This decline in responsiveness may, at least in part, be due to loss of Rho proteins from the permeabilized smooth muscle. When the permeabilized smooth muscle strips were incubated with the constitutively active mutant of Rho (^{Val14}RhoA·GTP) early in the course of the experiment, the response to carbachol was even larger than the initial response elicited in the absence of the Rho proteins (cf. Fig. 1*A* and *B*). Addition of the Rho proteins after the contractile response to carbachol had declined to about 50% of the initial response restored force to the initial level, while force in the control preparations declined further (Fig. 1*C*). These experiments indicate that Rho, which in resting cells is localized mainly in the cytosol, diffuses out of the fibres during or shortly after permeabilization. Thus, the determination of the agonist-induced sensitization in β-escin-permeabilized smooth muscle may underestimate the actual contribution of Ca^{2+} sensitization of myofilaments to contraction in intact smooth muscle. Because run-down was not completely prevented by the exogenous Rho proteins (Fig. 1), additional soluble components appear to be required for Ca^{2+} sensitization, which act either downstream or in parallel with Rho.

We used the mutant ^{Val14}RhoA, which is permanently active in the GTP-bound form due to its very low GTPase activity, rather than the wild-type of Rho, which has to be activated with GTPγS. As discussed by Satoh *et al.* (1993), there is some uncertainty concerning the stability of nucleotide binding to low molecular weight GTPases since the nucleotides may be released within the smooth muscle cells by exchange factors and may then bind to and activate endogenous GTP-binding proteins. Thus, sensitizing effects ascribed to small GTPases may in fact be due to the activation of endogenous GTP-binding proteins by released nucleotides. This problem is particularly severe when GTPases are activated with GTPγS because the Ca^{2+}-sensitizing effect of GTPγS is much larger than that of GTP (Kitazawa *et al.* 1991*a*). It should be noted that in this study GTP alone did not reverse the run-down but required the presence of Rho proteins. However, we cannot exclude the possibility that the effect of the mutant Rho may differ from its normal counterpart. Therefore, we attempted to elucidate the contribution of Rho proteins to agonist-induced Ca^{2+} sensitization by inactivating endogenous Rho with exoenzyme C3. C3 inhibited the carbachol- and GTPγS-induced increase in Ca^{2+} sensitivity when added to permeabilized intestinal smooth muscle as previously reported (Hirata *et al.* 1992; Itagaki *et al.* 1995; Pfitzer *et al.* 1995). Unlike Itagaki *et al.* (1995), we found only a very small effect on the pCa_{50} value in the absence of carbachol, while maximal Ca^{2+}-activated force was inhibited by about 20%.

Inhibition of carbachol-induced Ca^{2+} sensitization was associated with an increase in ADP-ribosylation of a protein band with a molecular mass of about 22 kDa, corresponding to that of Rho proteins. In an attempt to address the question of whether the inhibitory effect of C3 is specifically related to the ADP-ribosyltransferase activity of C3, we incubated the permeabilized smooth muscle strips with C3 but in the absence of exogenous NAD. Under this condition C3 still inhibited the carbachol-induced Ca^{2+} sensitization (cf. Fujita *et al.* 1995). Because the K_m value of C3 for NAD is lower than 1 μM (Just *et al.* 1992), we assume that the endogenous NAD concentration is high enough to sufficiently ADP-ribosylate endogenous Rho. However, the following experiments indicate that at least part of the effect of C3 is due to ADP-ribosylation of Rho. When C3 was pre-incubated with an inactivating antibody both the effect on force and the ADP-ribosylation of the 22 kDa protein were blocked. Furthermore, about 50% of the initial response to carbachol was recovered when C3-treated strips were loaded with ^{Ile41}RhoA, which is not a substrate of C3. Because the GTPase activity of ^{Ile41}RhoA might be activated in the permeabilized smooth muscle by GTPase-activating proteins, ^{Ile41}RhoA was activated with the poorly hydrolysed GTP analogue, GTPγS. As discussed above, there is the possibility that GTPγS dissociates from ^{Ile41}RhoA in the smooth muscle strips and activates endogenous GTP-binding proteins. However, this cannot account for the observed recovery of Ca^{2+} sensitization for the following reasons: (i) in C3-treated fibres, GTPγS in the absence of exogenous Rho proteins has only a very small Ca^{2+}-sensitizing effect; and (ii) ^{Ile41}RhoA·GTPγS itself, i.e. in the absence of carbachol or unbound GTPγS, has no effect on force (Fig. 4). No force recovery was observed when the strips were loaded with ^{Val14}RhoA·GTP. Inactivation by C3, which may diffuse only very slowly out of the strips, is the most likely explanation for this somewhat surprising result.

Unlike ^{Ile41}RhoA·GTPγS, ^{Val14}RhoA·GTP induced a slow increase in Ca^{2+}-activated force in the absence of the agonist in four out of eleven experiments. This increase in Ca^{2+} sensitivity was small and slow in onset compared with the effects of carbachol or GTPγS, indicating that activation of Rho proteins is necessary but not sufficient for agonist-induced increase in Ca^{2+} sensitivity of smooth muscle myofilaments. Thus, an additional signal may be required to induce an increase in Ca^{2+} sensitivity. Because ^{Val12}H-Ras can induce a rapid increase in Ca^{2+} sensitivity in the absence of an agonist (Satoh *et al.* 1993) which is not further enhanced by addition of GTPγS, Ras may provide this second signal and one possibility is that activation of the mitogen-activated protein (MAP) kinase pathway may be required (Leevers & Marshall, 1992; Adam, Franklin, Raff & Hathaway, 1995). Our result that Rho proteins for themselves did not increase Ca^{2+} sensitivity in the majority of preparations differs from that of Hirata *et al.* (1992), who found that Rho·GTPγS enhanced the Ca^{2+}-activated force. Whether this is due to a confounding effect of GTPγS as discussed above or due to post-translational modification of Rho as suggested by a study published after completion of our experiments (Gong *et al.* 1996) is not clear at present. It should be noted that neither the effect of ^{Ile41}RhoA·GTPγS

or ^{Val14}RhoA·GTP on carbachol-induced Ca^{2+} sensitization (this study) nor the direct Ca^{2+}-sensitizing effect of ^{Val12}H-Ras (Satoh *et al.* 1992) required post-translational modification. Our results are consistent with the finding that for certain types of cell motility, activation of Rho is a necessary but not sufficient event (Takaishi *et al.* 1994).

Is carbachol-induced Ca^{2+} sensitization exclusively mediated by Rho?

If this were the case then we would expect that the extent of ADP-ribosylation and inhibition of force by C3 were stoichiometrically related and that loading the fibres with recombinant Rho proteins completely restored tension response to carbachol. This was not the case. Maximal ADP-ribosylation was obtained at 0·2 μg ml^{-1} C3, which produced 50% inhibition of force. Increasing the concentration of C3 produced complete inhibition of force but did not further enhance the extent of ADP-ribosylation. This suggests that part of the inhibitory action of C3 may not be due to ADP-ribosylation of Rho. C3 at high concentrations may act less specifically and inactivate other Rho-related proteins in addition to Rho (Ridley *et al.* 1995). The fact that carbachol-induced force could be restored only partially by loading the fibres with Rho proteins also supports the idea that only part of the inhibitory action of C3 is due to inactivation of Rho.

Only about 50% of endogenous Rho is ADP-ribosylated under conditions where force is completely inhibited, suggesting that only a fraction of the cellular Rho is involved in regulation of contraction. Rho is associated with different proteins and the phosphorylation state of these proteins influences ADP-ribosylation of Rho (Fritz & Aktories, 1994). Thus, some of the Rho may not be accessible to C3. However, it could be argued that both partial ADP-ribosylation of endogenous Rho and the incomplete force recovery in the presence of exogenous Rho could be due to incomplete permeabilization of the smooth muscle or due to inhomogeneous loading of the strips with the proteins. Incomplete permeabilization appears unlikely because force elicited by maximal [Ca^{2+}] was as large as carbachol-induced contractions in the intact preparations. Incomplete loading appears to be unlikely because C3 completely inhibited force, and extending the incubation period with Rho proteins beyond 45 min did not improve force recovery. Incomplete recovery of force could also be due to the fact that the Rho proteins used in this study were not post-translationally modified, which may be an important prerequisite for full activity. Also, we cannot exclude the possibility that the concentration of Rho may have been too low. However, the concentration used in our study was higher than that used by Hirata *et al.* (1992). They found that recombinant RhoA·GTPγS could reverse the inhibitory effect of the staphylococcal toxin EDIN on the GTPγS-induced Ca^{2+} sensitization of myofilaments. Unfortunately, it is not known whether force recovery was complete in their study. Taken together, our results raise the interesting possibility that carbachol-induced sensitization is mediated by more than one pathway, which merits further investigation.

Effect of toxin B on contractions in intact and β-escin-permeabilized smooth muscle

To address the question of whether the findings in permeabilized smooth muscle are of relevance for excitation–contraction coupling in *intact* smooth muscle, we incubated *intact* intestinal longitudinal smooth muscle with toxin B from *C. difficile*, the causative agent of antibiotic-associated enterocolitis. Unlike C3, toxin B is internalized into intact cells (Florin & Thelestam, 1983). It induces a similar change in cellular morphology as does microinjected C3, i.e. cell rounding and disruption of stress fibres that is correlated with a decrease in C3-catalysed ADP-ribosylation of Rho proteins (Just *et al.* 1994), indicating that the intracellular target of toxin B is Rho or Rho-like proteins. This was confirmed when the mechanism of action of toxin B was identified. Toxin B is a monoglucosyl-transferase which specifically catalyses the glucosylation of low molecular weight GTPases of the Rho subfamily (Just *et al.* 1995). Microinjection of monoglucosylated Rho into cultured cells induced disruption of actin filaments accompanied by morphological features identical to those caused by microinjection of toxin B (Just *et al.* 1995). Thus, toxin B can serve as a tool to probe the participation of members of the Rho subfamily in excitation–contraction coupling of intact smooth muscle.

In intact smooth muscle, carbachol induced a biphasic contractile response: an initial rapid increase in force (peak 1) which was followed by a slow second rise in force (peak 2) after partial relaxation of the initial phasic response, as has previously been reported (Himpens & Somlyo, 1988). Activation by depolarization with high-K^+ solutions induced only a phasic response. Toxin B completely inhibited the delayed rise in force (peak 2) of the carbachol-induced force while peak 1 and the high-K^+-induced force were only partially inhibited. As shown by Himpens & Somlyo (1988), the initial phasic response is associated with a transient, large rise in intracellular [Ca^{2+}], which was proposed to have its origin in intracellular release of Ca^{2+}. Rho proteins have been implicated in regulatory mechanisms that affect intracellular Ca^{2+} handling (Chong, Traynor-Kaplan, Bokoch & Schwartz, 1994) although this may be cell type dependent (Jalink, van Corven, Hengeveld, Morii, Narumiya & Moolenaar, 1994). Toxin B has been reported to induce an increase in intracellular calcium (Gilbert, Pothoulakis, Lamont & Yakubovich, 1995). It is, therefore, unlikely that the inhibitory effect of toxin B on the initial phase of carbachol- and high-K^+-induced force, which was on average only about 30%, is due to a decrease in intracellular Ca^{2+}. The small inhibition of force could be the expression of a direct inhibitory action on the contractile apparatus, as discussed below.

Himpens & Somlyo (1988) reported that the delayed response (peak 2) was associated with no or only a

disproportionately small increase in cytosolic [Ca^{2+}]. As discussed by the authors, several mechanisms could account for this phenomenon. In the context of our study the most interesting possibility is the occurrence of a second slower regulatory mechanism leading to an increase in the sensitivity of the myofilaments for Ca^{2+} (reviewed by Somlyo & Somlyo, 1994). Thus, the complete inhibition of peak 2 of the carbachol-induced contraction by toxin B could be due to inhibition of agonist-induced increase in the Ca^{2+} sensitivity of the myofilaments. To test this possibility, the intestinal smooth muscle strips were permeabilized with β-escin after treatment with toxin B. Incubation of intact smooth muscle strips with toxin B for 8 h had only a small effect on submaximal Ca^{2+}-activated force and on pCa_{50} in permeabilized smooth muscle strips. Whether maximal force was inhibited as with C3 could not be determined because the cross-sectional area could not be determined with sufficient accuracy. In contrast, the carbachol-induced increase in Ca^{2+} sensitivity was almost completely inhibited by treatment with toxin B much in the same way as it was inhibited by C3. The GTPγS-induced Ca^{2+} sensitization was inhibited by 80%. These findings suggest that toxin B modifies intracellular signalling pathways involved in agonist-induced Ca^{2+} sensitization in a manner which survives the permeabilization procedure, most probably by a covalent modification of one or more proteins. Given the fact that toxin B is a monoglucosyltransferase with proteins of the Rho subfamily as substrate, we suggest that the inhibition of Ca^{2+} sensitization is due to glucosylation of one or more of these proteins. The incomplete inhibition of GTPγS-induced Ca^{2+} sensitization is in line with previous observations with C3 (Itagaki *et al.* 1995), suggesting that there are additional GTP-dependent pathways leading to Ca^{2+} sensitization which do not require the activation of Rho or Rho-like proteins.

The signal leading to activation of Rho is not known but it may involve activation of the protein kinase C cascade (Takaishi *et al.* 1994). This is an interesting possibility because it has been suggested that activation of protein kinase C increases Ca^{2+} sensitivity of smooth muscle contraction (Collins, Walsh & Morgan, 1992; Khalil, Lajoie, Resnick & Morgan, 1992). The downstream effectors of Rho in smooth muscle are also not known. The major determinant of the Ca^{2+} sensitivity of smooth muscle contraction is the ratio of the activities of myosin light chain kinase to phosphatase. Inhibition of myosin light chain phosphatase was suggested to underlie agonist-induced Ca^{2+} sensitization (Kitazawa, Masuo & Somlyo, 1991*b*; Kubota, Nomura, Kamm, Mumby & Stull, 1992). Since Ca^{2+} sensitivity of contraction in the absence of an agonist is not affected by C3 or toxin B (cf. Hirata *et al.* 1992), it appears unlikely that Rho has a direct effect on the activities of these enzymes. It may, however, affect the activity of myosin light chain phosphatase indirectly (Noda *et al.* 1995) whereby the intermediate steps are not known. Another putative target is the microfilaments, since both toxin B (Just *et al.* 1995) and C3, when injected into cells, disrupt the actin cytoskeleton (see Aktories *et al.* 1992 for review). So far, we have no evidence for a major effect of C3 on the actin filaments of smooth muscle (M.-F. Wendt-Gallitelli, B. Otto & G. Pfitzer, unpublished observations) but we cannot exclude minor changes, e.g. at the sites of the dense plaques where actin filaments are attached to the plasma membrane. Alternatively, C3 and toxin B could have some direct effect on the actin–myosin interaction as was proposed by Jalink *et al.* (1994). Such a direct effect on the actin–myosin interaction or on force-transmitting structures at the plasma membrane could account for the inhibition of maximal force in the presence of C3 (Fig. 7) and could be responsible for part of the inhibition of force induced by toxin B in intact preparations. In this context it is interesting that C3 inhibits tyrosine phosphorylation of paxillin, a protein of adhesion plaques (Rankin, Morii, Narumiya & Rozengurt, 1994) which is phosphorylated on tyrosine residues in stimulated smooth muscle (Pavalko, Adam, Wu, Walker & Gunst 1995). We have shown that agonist-induced smooth muscle contraction (Di Salvo, Steusloff, Semenchuk, Satoh, Kolquist & Pfitzer, 1993) as well as Ras- (Satoh *et al.* 1993) and carbachol-induced Ca^{2+} sensitization of permeabilized smooth muscle are inhibited by tyrosine kinase inhibitors (Steusloff, Paul, Semenchuk, Di Salvo & Pfitzer, 1995) and are associated with an increase in tyrosine phosphorylation of several proteins (Di Salvo, Pfitzer & Semenchuk, 1994). Thus, events downstream from activation of Rho could involve activation of a tyrosine kinase cascade (Kumagi, Morii, Fujisawa, Nemoto & Nyrumiya, 1993).

In conclusion, our results are consistent with the hypothesis that Rho or Rho-like proteins participate in the agonist-induced Ca^{2+} sensitization. However, future studies have to show whether GTPases other than Rho are involved in this. To the best of our knowledge this is also the first study demonstrating that activation of Rho or Rho-like proteins is involved in pharmacomechanical coupling in intact smooth muscle.

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