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Inhibition of RhoA Translocation and Calcium Sensitization by In Vivo ADP-Ribosylation with the Chimeric Toxin DC3B

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Pretreatment of intact rabbit portal vein smooth muscle with the chimeric toxin DC3B (10^{-6} M, 48 h; Aullo *et al.*, 1993; Boquet *et al.* 1995) ADP-ribosylated endogenous RhoA, including cytosolic RhoA complexed with rhoGDI, and inhibited the tonic phase of phenylephrine-induced contraction and the Ca^{2+}-sensitization of force by phenylephrine, endothelin and guanosine triphosphate (GTP)γS, but did not inhibit Ca^{2+}-sensitization by phorbol dibutyrate. DC3B also inhibited GTPγS-induced translocation of cytosolic RhoA (Gong *et al.*, 1997a) to the membrane fraction. In DC3B-treated muscles the small fraction of membrane-associated RhoA could be immunoprecipitated, even after exposure to GTPγS, which prevents immunoprecipitation of non-ADP–ribosylated RhoA. Dissociation of cytosolic RhoA–rhoGDI complexes with SDS restored the immunoprecipitability and ADP ribosylatability of RhoA, indicating that both the ADP-ribosylation site (Asn 41) and RhoA insert loop (Wei *et al.*, 1997) are masked by rhoGDI and that the long axes of the two proteins are in parallel in the heterodimer. We conclude that RhoA plays a significant role in G-protein-, but not protein kinase C-mediated, Ca^{2+} sensitization and that ADP ribosylation inhibits in vivo the Ca^{2+}-sensitizing effect of RhoA by interfering with its binding to a membrane-associated effector.

INTRODUCTION

The role of the Ras-related monomeric guanosine triphosphate (GTP)-binding protein RhoA in regulation of protein phosphorylation is increasingly recognized (reviewed in Lim *et al.*, 1996), and contraction of vertebrate smooth muscle stands out among mechanisms acutely regulated by protein phosphorylation: phosphorylation of the regulatory light chains of smooth muscle myosin (MLC_{20})[1] by Ca_4-calmodulin-dependent myosin light chain kinase leads to contraction, whereas dephosphorylation of MLC_{20} by the smooth muscle myosin phosphatase (SMPP-1 M) causes relaxation (reviewed in Hartshorne, 1987; Kamm and Stull, 1989; Somlyo and Somlyo, 1994). Furthermore, MLC_{20} phosphorylation can also be modulated, independently of changes in $[Ca^{2+}]_i$, by a receptor-mediated, G-protein-coupled mechanism (Somlyo *et al.*, 1989) that operates largely through inhibition of SMPP-1 M, a trimeric, type 1 protein phosphatase that contains a regulatory/targeting subunit that enhances its catalytic activity toward MLC_{20} (Alessi *et al.*, 1992; Shimizu *et al.*, 1994; Shirazi *et al.*, 1994; Gailly *et al.*, 1996). Inhibition of SMPP-1 M at submaximal levels of Ca_4-calmodulin increases the level of MLC_{20} phosphorylation, resulting in force

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[1] Abbreviations used: C3, *Clostridium botulinum* exoenzyme C3; GEF, guanine nucleotide exchange factor; MLC_{20}, the 20-kDa light chains of myosin; PE, phenylephrine; PVDF, polyvinylidene difluoride; rhoGDI, rho guanine-nucleotide dissociation inhibitor; SMPP-1 M, smooth muscle myosin phosphatase 1 M.

development independently of a change in $[Ca^{2+}]_i$ ("Ca^{2+}-sensitization"; Kitazawa *et al.*, 1991). The complete sequence and components of the signal-transduction cascade between activation of a plasma membrane-bound receptor, inhibition of the cytosolic enzyme (SMPP-1 M), and phosphorylation of its substrate (MLC_{20}) have not been identified; however, several studies have implicated RhoA in this process (see DISCUSSION). Ca^{2+}-sensitization of smooth muscle (Gong *et al.*, 1996, and references therein), as well as other effects of RhoA, including stress-fiber formation (Ridley and Hall, 1992), exocytosis (Mariot *et al.*, 1996), lymphocyte aggregation (Tominaga *et al.*, 1993), and phospholipase D activity (Malcolm *et al.*, 1996), is inhibited by ADP ribosylation of RhoA with the *Clostridium botulinum* exoenzyme C3 (C3; Chardin *et al.*, 1989) at residue Asn 41 (Sekine *et al.*, 1989) or the staphylococcal exoenzyme EDIN (Sugai *et al.*, 1992).

Enzymes that ADP-ribosylate RhoA, until recently, had to be introduced by permeabilization with detergents, except in the case of some cultured cells. Such treatment, however, can cause relocalization of RhoA to the particulate fraction (our unpublished observations), complicating the interpretation of results. A recently developed chimeric toxin (DC3B) consists of C3 and the (noncatalytic) B fragment of diphtheria toxin; the latter allows the introduction of active C3 into intact cells that contain diphtheria toxin receptors (Aullo *et al.*, 1993; Boquet *et al.*, 1995). The fortunate presence of such receptors enabled us to determine the effects of ADP-ribosylation of RhoA in intact rabbit vascular smooth muscle on its cellular localization and Ca^{2+}-sensitizing activity. We also obtained information about the in vivo mechanism of RhoA inhibition by C3 and further evidence of separate pathways of, respectively, phorbol ester- and G-protein-coupled Ca^{2+}-sensitization.

MATERIALS AND METHODS

Construction of Chimeric Toxin

The preparation of the chimeric toxin DC3B and its properties have been published (Aullo *et al.*, 1993; Boquet *et al.*, 1995).

Preparation of Smooth Muscle and Treatment with DC3B

Small strips (200 μm wide, 3 mm long) of rabbit portal vein were dissected and placed in HEPES-buffered salt solution with DC3B (10^{-6}M) for 2 h at 4°C (pH 7.3) to allow DC3B to bind to diphtheria toxin receptor without endocytosis taking place (Aullo *et al.*, 1993). Control tissues without the chimeric toxin, but with inactive B-fragment of the diphtheria toxin, were carried through the same protocol as used for DC3B. To aid internalization, the tissues were then washed twice with HEPES-buffered salt solution (containing 10 mM NH_4Cl and adjusted at pH 4.9 with 10% acetic acid, at 37°C), and incubated in this buffer with or without DC3B for 30 min at 37°C (pH 4.9). The buffer was changed to serum-free DMEM + F12 at a 1:1 ratio, 50 μg/ml penicillin and 50 IU/ml streptomycin, L-glutamine, 200 mg/l, and insulin, 2.85 mg/l, and the tissues were incubated in organ culture (Lesh *et al.*, 1995) with DC3B (2×10^{-7} M) at 37°C in 5% CO_2 for 24 h or 48 h. After incubation, the tissues were placed in HEPES-buffered salt solution at room temperature before use.

Isometric Tension Measurement

Isometric tension was measured in intact or *Staphylococcus aureus* α-toxin-permeabilized smooth muscle as described previously (Kitazawa *et al.*, 1989; Kobayashi *et al.*, 1989, 1991), and force was expressed as percent of the maximal Ca^{2+}-induced contraction obtained in permeabilized tissues at the end of the experiment.

Separation of Particulate and Cytosolic Fractions

A minimum of 10 small (200 μm wide and 3 mm long) control or DC3B-treated, resting or GTPγS-stimulated strips of rabbit portal vein smooth muscle were used to provide sufficient protein for reliable separation of cytosolic and particulate fractions. Strips were homogenized in ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM AEBSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin) with glass microhomogenizers, centrifuged at 100,000 $\times$ *g* for 30 min at 4°C (Beckman, Fullerton, CA; Optima TLX Ultracentrifuge, TLA 120.1 rotor), and the supernatant was collected as the cytosolic fraction. Pellets were resuspended and membrane proteins were extracted by incubation for 30 min in homogenization buffer containing 1% Triton X-100 and 1% sodium cholate. The extract was centrifuged at 800 $\times$ *g* for 10 min, and the supernatant was collected as the detergent-soluble particulate fraction and the pellet was resuspended in 1$\times$ Laemmli sample buffer as the detergent-insoluble particulate fraction. Cytosolic, detergent-soluble particulate and detergent-insoluble particulate fraction proteins were separated by SDS-PAGE. Only the cytosolic and detergent-soluble particulate RhoA are shown in the illustrations, as no detectable RhoA was found in the detergent-insoluble particulate fraction. The absence of RhoA in the detergent-insoluble particulate fraction verified the complete extraction of membrane-associated RhoA. Prompt termination of translocation by the ice-cold homogenization buffer was verified by the absence of translocation of RhoA when the control strips were homogenized in homogenization buffer containing GTPγS (50 μM).

Western Blots

After proteins were transferred to polyvinylidene difluoride (PVDF) membranes (100 V, 1 h), the membranes were blocked with 5% fat-free dry milk in phosphate buffered saline containing 0.05% Tween-20 for 1 h and then incubated with monoclonal anti-RhoA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, generated to amino acids 120–150 of human RhoA at 1:2,500 dilution) for 3 h at room temperature. After washing, the membranes were incubated with secondary (antimouse; Goldmark, Inc., 1:65,000) antibody for 1 h at room temperature. Proteins were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantitated by densitometry using a Bio-Rad GS-670 imaging densitometer (Bio-Rad, Richmond, CA). The percent of particulate RhoA (membrane-associated RhoA) was calculated according to particulate RhoA/(particulate + cytosolic) RhoA.

For Western blots for actin, monoclonal anti-α smooth muscle actin antibody was used at 1:5,000 dilution followed by the secondary antibody (antimouse).

Immunoprecipitation

Samples treated and prepared as above were precleared with Protein A-agarose (1 h, room temperature) to prevent nonspecific binding of proteins in the immunoprecipitated complex. Precleared homogenates were incubated with either anti-RhoA monoclonal antibody conjugated to agarose beads (10 μg) or anti-rho guanine-

nucleotide dissociation inhibitor (rhoGDI) polyclonal antibody (1 μg) overnight at 4°C, rotating. rhoGDI immunoprecipitates were then incubated with Protein A-agarose for 1 h at room temperature. Immune complexes were centrifuged and the supernatants collected and saved for analysis. The precipitates were washed three times in ice-cold phosphate-buffered saline and resuspended in Laemmli sample buffer. Antibodies and Protein A-agarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

ADP Ribosylation of RhoA with ^{32}P-NAD

To determine the extent of ADP ribosylation of RhoA by DC3B, tissues were subjected to further, in vitro, ADP ribosylation by C3. After 24 or 48 h incubation with DC3B, three strips were homogenized in homogenization buffer (total volume, 100 μl) to determine the *subsequent* C3-catalyzed ADP-ribosylatability of RhoA in the tissue. For determination of ADP ribosylation in the cytosolic and particulate fractions, the volumes and detergent concentrations of the cytosolic and particulate fractions were preadjusted to identical values (0.1% Triton X-100, total volume 200 μl). The following reagents were added: 200 μM GTP, 10 mM dithiothreitol, 2 mM thymidine, 4×10^{-8} M C3. After initiation of ADP ribosylation by addition of ^{32}P-NAD (50 μCi/ml, Dupont NEN, Boston, MA), the mixture was incubated for 30 min at 30°C. The reaction was stopped by addition of 24% trichloroacetic acid (250 μl) and 2% deoxycholate (6 μl), and the final volume was adjusted to 1 ml with water. After centrifugation (5,000 × *g*, 10 min), the supernatant was removed and the pellet was resuspended in 2× sample buffer, and 1 M Tris-Base was added to neutralize the pH. Samples were heated at 85°C for 5 min, and the proteins were separated by SDS-PAGE and transferred to PVDF membrane. Autoradiographs and Western blots were obtained from the same PVDF membrane.

RhoA complexed with rhoGDI is not readily ADP ribosylated (Bourmeyster *et al.*, 1992). Therefore, to explore the possibility of a residual non-ADP-ribosylated pool of RhoA complexed with rhoGDI (see RESULTS), ADP ribosylation with ^{32}P-NAD was also performed in the presence of 0.05% SDS to dissociate the complex (Williamson *et al.*, 1990; Just *et al.*, 1993).

Details of the solutions used for study of permeabilized strips have been published (Kitazawa *et al.*, 1989; Kobayashi *et al.*, 1989, 1991). α-Toxin was purchased from List Biochemicals (Campbell, CA), GTPγS from Boehringer Mannheim (Boehringer Mannheim, Mannheim, Germany), C3 (Upstate Biotechnology, Lake Placid, NY), A23187 from Calbiochem (La Jolla, CA), and ^{32}P-NAD (30 Ci/mmol) from Dupont NEN. A point-mutated, catalytically inactive diphtheria toxin (CRM 197) used as a control was a generous gift from Dr. John R. Murphy, Boston University Medical Center Hospital (Boston, MA). Statistical comparisons were made using analysis of variance and paired *t* test; all values are given as mean ± SEM.

RESULTS

DC3B ADP-Ribosylates RhoA in Intact Smooth Muscle

Treatment of intact rabbit portal vein smooth muscle with DC3B (10^{-6} M) for 24 or 48 h decreased the subsequent C3-catalyzed ADP ribosylation of RhoA with ^{32}P-NAD in whole homogenate at 24 h (control as 100%) to 67% ± 29.1% (n = 3) and at 48 h to 15% ± 6.1%, (n = 6, $p < 0.0001$). In view of the much more extensive ADP ribosylation after 48-h treatment with DC3B compared with 24-h treatment, all the subsequent results reported were obtained with the 48-h protocol.

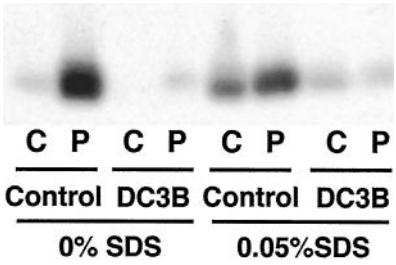

Figure 1. ADP ribosylation of RhoA in intact portal vein smooth muscle by DC3B. The effect of SDS (0.05%) on subsequent C3-catalyzed ADP ribosylation of RhoA in cytosolic (C) and membrane (P) fraction. Representative of three experiments.

Cytosolic RhoA, presumably complexed with rhoGDI, is a poor substrate for ADP ribosylation by C3 in smooth muscle (Gong *et al.*, 1997a). Because SDS has been reported to increase ADP ribosylation of Rho proteins through dissociation of the complex (Just *et al.*, 1993), we used it to determine whether there was a population of RhoA inaccessible to C3 treatment (Figure 1). This, indeed, was the case in control tissues (after 48 h incubation) in which cytosolic RhoA was a poor substrate for C3-catalyzed ADP ribosylation, and SDS (0.05%) markedly increased the extent of ADP ribosylation expressed as the densitometric ratio of ^{32}P-autoradiographic signal/actin content by more than 10-fold (from 0.11 ± 0.05 [n = 6] to 1.35 ± 0.60 [n = 6]). In contrast, in DC3B-treated tissues (48-h incubation), treatment with SDS had no significant effect on subsequent C3-catalyzed ADP ribosylation with ^{32}P-NAD of cytosolic RhoA (0.14 ± 0.08 [n = 6] vs. 0.28 ± 0.15 [n = 6]), indicating that RhoA was already ADP ribosylated, and there was very little remaining non-ADP-ribosylated cytosolic RhoA that could be unmasked by SDS. In unstimulated smooth muscle, the small fraction of RhoA that is membrane associated is a better substrate than the large amount of cytosolic RhoA (Gong *et al.*, 1997a), and the extent of ADP ribosylation of the membrane-associated fraction was not affected by SDS in either control or DC3B-treated tissues. DC3B inhibited ($p < 0.05$) the subsequent ADP ribosylation of membrane-associated RhoA (Figure 1). Western blots of RhoA, normalized to Western blots for actin for each lane using the same PVDF membrane, indicated that DC3B had no effect on the amount of total cellular RhoA content (number of experiments for each group was the same as shown above).

In summary, these results showed that treatment of intact organ-cultured smooth muscle for 48 h with DC3B resulted in extensive ADP ribosylation of RhoA in both cytosolic and membrane fractions, including the component complexed with rhoGDI.

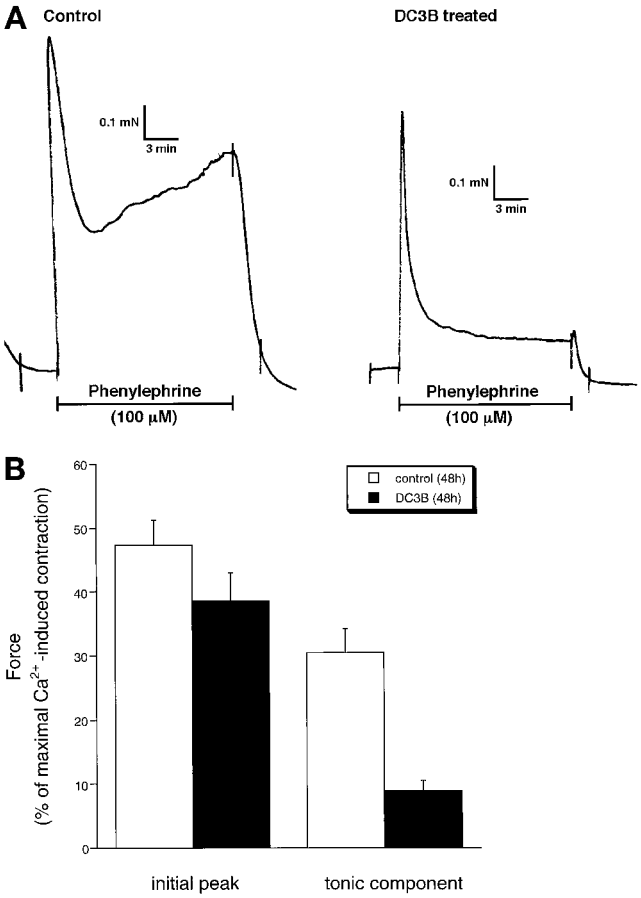

Figure 2. Phenylephrine-induced contraction in intact portal vein smooth muscle. (A) Isometric tension was measured after treatment with or without DC3B for 48 h. Note that the contractile response of untreated smooth muscle was biphasic, consisting of a phasic transient followed by a tonic phase. DC3B treatment inhibited the tonic phase of contraction with little effect on the initial, transient phase. (B) Summary of the effect of DC3B on PE-induced contraction in intact portal vein smooth muscle. DC3B significantly inhibited the tonic phase of contraction. Amplitudes of contraction in each phase were normalized to maximal Ca^{2+}-induced contraction as 100% after permeabilization with α-toxin. The maximal Ca^{2+}-induced contractions were: control, 1.6 ± 0.21 mN (n = 26); DC3B = 1.1 ± 0.17 mN (n = 30). The difference between these values was not statistically significant (p = 0.08).

ADP Ribosylation of RhoA by DC3B Decreases the Tonic Component of Contraction Induced by Phenylephrine in Intact Portal Vein Smooth Muscle

Phenylephrine (PE; 100 μM)-induced contractions are biphasic in intact portal vein smooth muscle, consisting of an initial transient, followed by a slow, tonic phase that reaches a plateau (Figure 2A). Incubation with DC3B for 48 h significantly ($p < 0.0001$) inhibited (Figure 2B) the tonic phase of contraction (control 31% ± 3.6% [n = 22], DC3B 9% ± 1.5% [n = 25]). There was a trend toward a slight decrease in the initial transient phase of contraction in DC3B-treated muscles (Figure 2B), but this was not statistically significant ($p > 0.05$; control 47% ± 4.0% [n = 22], DC3B 39% ± 4.4% [n = 25]).

Incubation with DC3B (48 h) significantly inhibited high K^+-induced contractions (initial peak; control 48% ± 3.9% [n = 23], DC3B 28% ± 3.8% [n = 24], p = 0.0006 vs. control). Diphtheria toxin has been reported to increase the permeability of plasma membrane to monovalent cations (such as K^+; Sandvig and Olsnes, 1988), but CRM 197 that contains the intact B-fragment had no effect on either the PE- or high K^+-induced contraction (n = 5 for both control and the treated group). The effects of DC3B on K^+-contractions were not further explored.

ADP Ribosylation of RhoA by DC3B Inhibits Ca^{2+} Sensitization Induced by Phenylephrine, Endothelin, and GTPγS, but Not That by Phorbol Ester

After incubation, the muscle strips were permeabilized with α-toxin (see MATERIALS AND METHODS) to determine the effect of DC3B on Ca^{2+} sensitization of contraction by agonists or GTPγS (Gong *et al.*, 1996). DC3B (48 h) also inhibited (Figure 3) phenylephrine (PE) (100 μM) plus GTP (10 μM)-induced Ca^{2+} sensitization at pCa 6.5 (control 21% ± 2.0% [n = 22], DC3B 5% ± 0.8% [n = 25], $p < 0.0001$) and significantly inhibited total Ca^{2+} sensitization (GTP + PE + GTPγS; control 59% ± 2.2%; [n = 22], DC3B 32% ± 2.5% [n = 25], $p < 0.0001$).

Endothelin (10^{-7} M) plus GTP (10 μM)-induced Ca^{2+} sensitization at pCa6.5 was also inhibited by DC3B (control 20% ± 1.7% [n = 5], DC3B 6% ± 2.9% [n = 5], p = 0.0007; Figure 3).

To determine whether inactivation of RhoA affects Ca^{2+} sensitization induced by conventional and novel protein kinase C(s) (Jensen *et al.*, 1996; Gailly *et al.*, 1997; Gong *et al.*, 1997b), phorbol-12,13-dibutyrate (PDBu; 1 μM) was applied at pCa 6.5. PDBu (1 μM) caused Ca^{2+}-sensitization, increasing force at constant [Ca^{2+}] (control 34% ± 3.6%, [n = 4]), and this was not inhibited by DC3B (29% ± 3.0% [n = 4]; Figure 3).

Treatment with the inactive diphtheria toxin construct, CRM 197 (the same concentration as DC3B) for 48 h had no effect on PE (100 μM) plus GTP-(10 μM) or GTPγS-induced Ca^{2+} sensitization at pCa6.5 (n = 5 for both control and the treated group).

DC3B had no significant effect on the pCa–tension relationship of smooth muscles in which G-proteins were not activated (Figure 4).

The Effect of ADP Ribosylation of RhoA by DC3B on Its GTPγS-induced Translocation and Association with a Putative Effector

The purpose of the following experiments was to establish whether the inhibitory effects of ADP ribosylation involved inhibition of the GTPγS-induced trans-

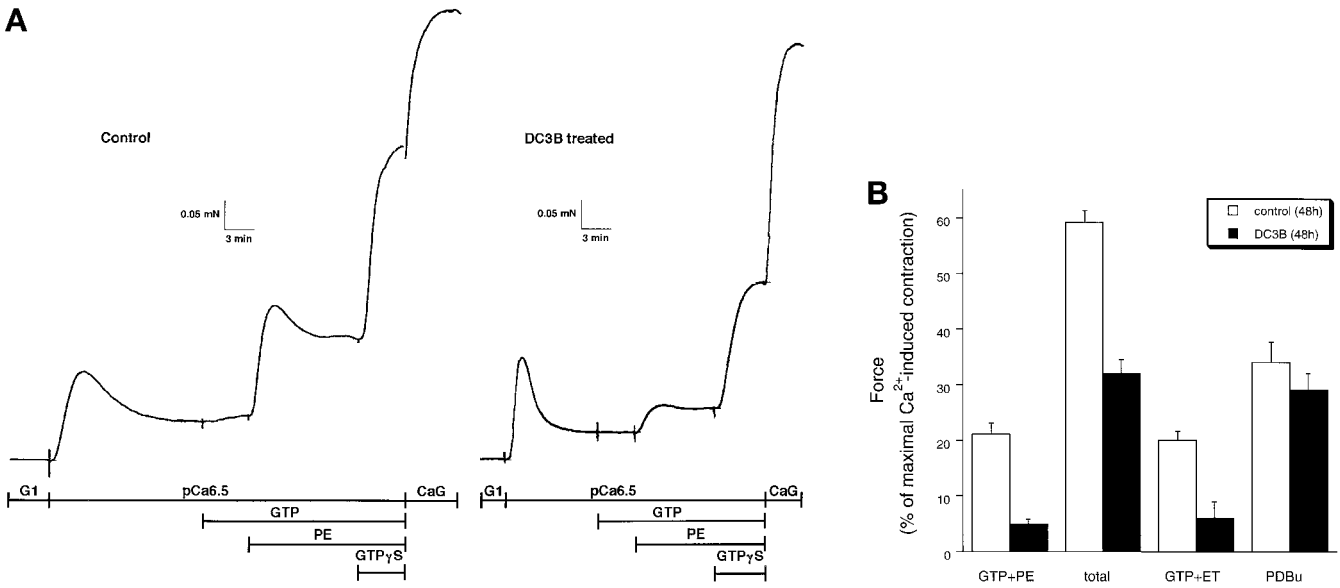

Figure 3. ADP ribosylation of RhoA by DC3B inhibits Ca^{2+} sensitization by phenylephrine, endothelin, and GTPγS, but not by phorbol ester. (A) Rabbit portal vein strips incubated for 48 h with or without DC3B were permeabilized with α-toxin (see MATERIALS AND METHODS). Isometric tension was measured at constant [Ca^{2+}] (pCa 6.5), followed by stimulation with phenylephrine (100 μM) plus GTP (10 μM) or endothelin (100 nM) plus GTP (10 μM). GTPγS (50 μM) was applied on the plateau phase of PE plus GTP-induced contraction. Traces shown are representative of phenylephrine plus GTP-induced Ca^{2+} sensitization, followed by stimulation with GTPγS. G1; calcium-free solution containing 1 mM EGTA, CaG; pCa 4.5, 10 mM EGTA buffered. (B) Summary of the effect of DC3B on the phenylephrine-, endothelin- and GTPγS-induced, and lack of effect on phorbol ester-induced, Ca^{2+} sensitization. PE, phenylephrine (100 μM); ET, endothelin (100 nM); PDBu, phorbol-12,13-dibutyrate (1 μM). Phenylephrine-, endothelin-, and total (PE + GTP + GTPγS)-induced Ca^{2+}-sensitization were significantly inhibited by DC3B (48 h).

location of RhoA from the cytosol to the membrane (Gong *et al.*, 1997a,b). After 48 h incubation, the amount of RhoA in the membrane (% memb) of α-toxin–permeabilized smooth muscle was 16% ± 3.3% (of total RhoA; n = 8) in control and 16% ± 3.5% (n = 7) in the DC3B-treated group (Figure 5), indicating that DC3B had no significant effect on the basal levels of membrane-associated RhoA.

The GTPγS (50 μM)-induced translocation of RhoA in the control group (% memb, 46% ± 6.9%, n = 8) was completely inhibited by DC3B treatment (% memb, 8% ± 1.8%, n = 10) (Figure 5). CRM 197, used as a control, had no effect on GTPγS (50 μM)-induced RhoA translocation (n = 4 for control and n = 6 for the treated group; our unpublished results).

Activation with GTPγS abolished the immunoprecipitability of RhoA with the RhoA antibody, even in the presence of a detergent, Nonidet-P40 (our unpublished observation). Therefore, we wanted to determine the effect of GTPγS on the immunoprecipitability of membrane-associated RhoA that had been ADP ribosylated with DC3B. In DC3B-treated tissues, GTPγS not only failed to translocate RhoA, but even in its presence the small amount of RhoA in the membrane remained immunoprecipitable (our unpublished results).

The Ca^{2+}-sensitizing phorbol ester (see DISCUSSION), PDBu (1 μM for 20 min), had no effect on

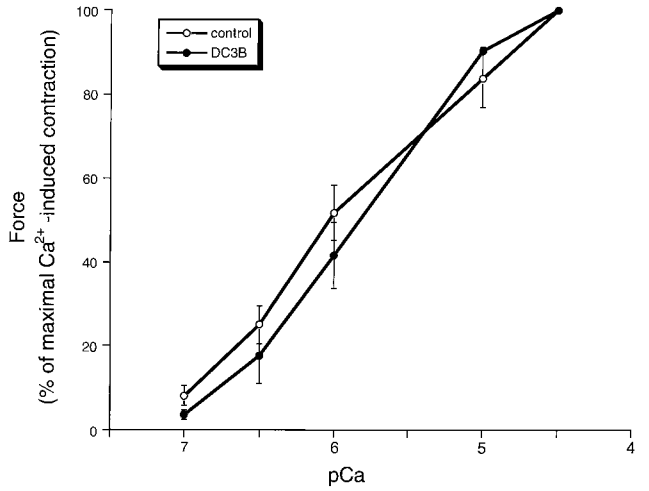

Figure 4. pCa–tension relationship is not affected by DC3B treatment. Rabbit portal vein strips incubated for 48 h with or without DC3B were permeabilized with α-toxin (see MATERIALS AND METHODS). Isometric tension was measured at each pCa and normalized to the maximal Ca^{2+}-induced contraction (pCa 4.5) of each strip as 100%.

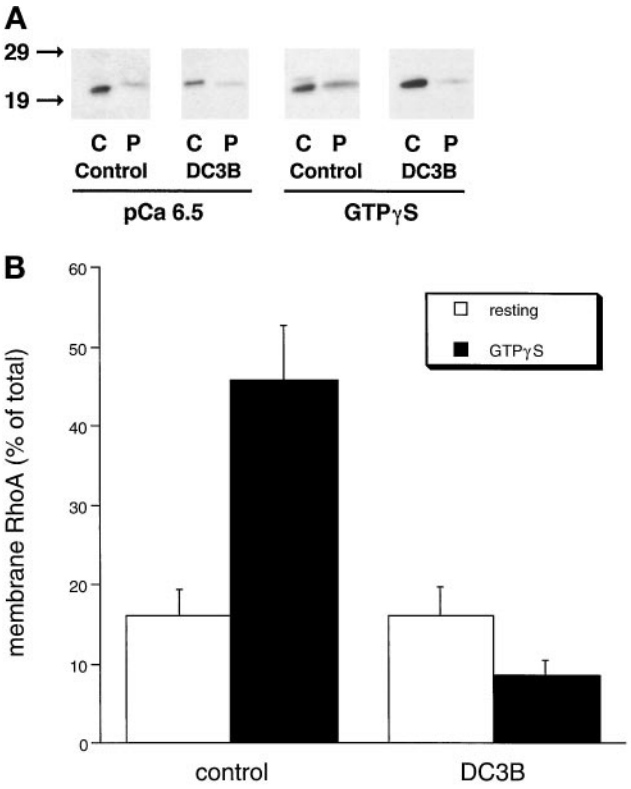

Figure 5. ADP ribosylation of RhoA by DC3B (48 h) inhibits GTPγS (50 μM)-induced RhoA translocation from the cytosolic to the membrane fraction. (A) After incubation with or without DC3B (48 h), α-toxin–permeabilized tissues were stimulated with GTPγS (50 μM) for 20 min and homogenized and fractionated (see MATERIALS AND METHODS); translocation of RhoA to the membrane fraction was inhibited by DC3B treatment (48 h). Representative Western blots of RhoA. (B) Summary of the effect of DC3B on the translocation of RhoA by GTPγS (50 μM) from cytosolic to the membrane fraction. DC3B significantly inhibited translocation of RhoA.

translocation of RhoA (resting at pCa 6.5, 18.0% ± 3%, n = 6; PDBu, 12.5% ± 3%, n = 6).

ADP Ribosylation of Cytosolic RhoA by DC3B Does Not Interfere with Complexation with rhoGDI

To further elucidate the effects of ADP ribosylation in intact smooth muscle, we studied its effects on the complexation of RhoA with rhoGDI. Cytosolic RhoA was not immunoprecipitable with the antibody to RhoA, but in control tissues (Figure 6), treatment of the cytosolic extract with 0.05% SDS rendered RhoA immunoprecipitable. In tissues treated with DC3B (Figure 6), in which RhoA was ADP-ribosylated, cytosolic RhoA was similarly nonimmunoprecipitable in the absence of SDS, but immunoprecipitated in its presence. Membrane-associated RhoA in both the control and DC3B-treated muscle could be immunoprecipitated (our unpublished results).

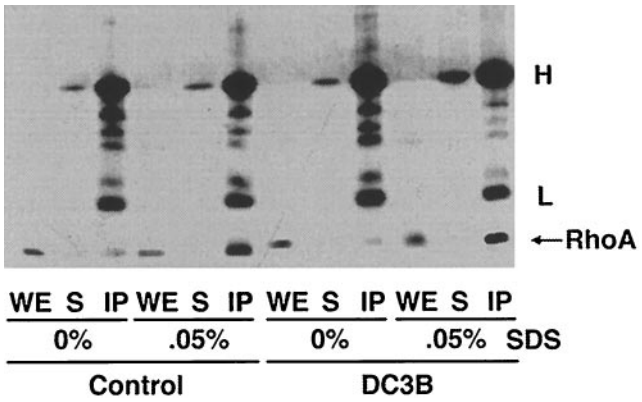

Figure 6. Recovery of the immunoprecipitability of cytosolic RhoA by detergent treatment. Cytosolic fractions of control (untreated) and DC3B (48 h) treated tissues were incubated without or with 0.05% SDS for 30 min on ice and immunoprecipitated with anti-RhoA-agarose conjugated monoclonal antibody overnight at 4°C. Samples were electrophoresed and transferred to PVDF membranes. Membranes were blotted with anti-RhoA monoclonal antibody and visualized by enhanced chemiluminescence. WE, whole extract (before immunoprecipitation); S, supernatant (after immunoprecipitation); IP, immunoprecipitate. Note that the immunoprecipitating antibody heavy (H) and light (L) chains were recognized by the blotting antibody.

Immunoprecipitation using an anti-rhoGDI antibody followed by Western blotting with RhoA antibody showed that cytosolic RhoA was in a complex that coimmunoprecipitated with rhoGDI in both control and DC3B-treated tissues (Figure 7). In DC3B-treated tissues cytosolic RhoA was also coimmunoprecipitated with rhoGDI even after exposure to GTPγS. Treatment of cytosolic extracts with 0.05% SDS dissociated the complex, and RhoA was no longer coimmunoprecipitated with rhoGDI, indicating that ADP ribosylation of RhoA by DC3B did not prevent its reassociation with rhoGDI. Membrane-associated RhoA was not coimmunoprecipitated with rhoGDI in either control or DC3B-treated tissues, and no rhoGDI was detectable in the membrane fraction.

DISCUSSION

ADP Ribosylation of RhoA by DC3B, the Effect of rhoGDI, and the Inferred Shape of the RhoA–rhoGDI Complex

Treatment of intact smooth muscle with DC3B ADP-ribosylated endogenous RhoA without affecting the pCa–tension relationship of unstimulated smooth muscle (Figure 4). Therefore, the biological effects of this chimeric toxin on intact (nonpermeabilized) smooth muscle, like the effects of C3 or EDIN on permeabilized preparations (Gong *et al.*, 1996), can be ascribed to inhibition of RhoA-mediated mechanisms. The time course of ADP ribosylation of endogenous

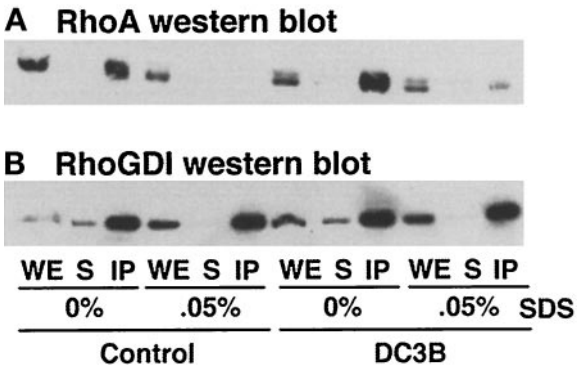

Figure 7. Cytosolic RhoA coimmunoprecipitates with rhoGDI in the absence of detergent. Cytosolic fractions of control (untreated) and DC3B-treated tissues were incubated without or with 0.05% SDS for 30 min on ice and immunoprecipitated with anti-rhoGDI polyclonal antibody overnight at 4°C and subsequently incubated with protein A-agarose for 1 h at room temperature. Samples were electrophoresed and transferred to PVDF membranes. Membranes were blotted with anti-RhoA monoclonal antibody (panel A) or antirhoGDI polyclonal antibody (panel B) and visualized by enhanced chemiluminescence. WE, whole extract (before immunoprecipitation); S, supernatant (after immunoprecipitation); IP, immunoprecipitate. In the absence, but not in the presence, of 0.05% SDS, cytosolic RhoA coimmunoprecipitates with rhoGDI.

RhoA was slow: at 24 h only about 30% of RhoA was ADP ribosylated. This may have been due to slow cellular uptake of DC3B, but most likely it reflects shielding of the ADP-ribosylation site (Asn 41) by rhoGDI in the cytosolic, RhoA–rhoGDI complex. According to this interpretation, ADP ribosylation of RhoA is rate limited by the slow, spontaneous equilibrium dissociation of the RhoA–rhoGDI complex that makes Asn 41 accessible to intracellular C3 during the 48-h incubation and is followed by reassociation of ADP-ribosylated RhoA with rhoGDI (Figure 7). Dissociation of the RhoA–rhoGDI complex with SDS (Figure 1) revealed that the RhoA in the heterodimer had been ADP ribosylated by DC3B. Cytosolic RhoA complexed with rhoGDI is not readily accessible to C3 (Just *et al.*, 1993; Gong *et al.*, 1996; present study), and our finding that prolonged treatment of DC3B ADP-ribosylates RhoA that reassociates with rhoGDI is consistent with a previous study that showed that RhoA ADP ribosylated in vitro can bind to rhoGDI (Hancock and Hall, 1993). In the present study, newly formed RhoA may also have been ADP ribosylated before it complexed with rhoGDI.

The crystal structure of RhoA (Wei *et al.*, 1997), combined with the results of ADP ribosylation and immunoprecipitation (present study) and the structure of rhoGDI published after our studies were completed (Gosser *et al.*, 1997; Keep *et al.*, 1997), allows us to deduce an approximate model of the RhoA–rhoGDI complex. The longest dimensions of the two proteins are comparable and significantly longer than their shorter dimensions (Gosser *et al.*, 1997; Keep *et al.*, 1997; Wei *et al.*, 1997). Thus, rhoGDI can interact with both "ends" of RhoA only if the long axes of the two proteins in the heterodimer are aligned in parallel. That such alignment occurs is suggested by the binding of the prenylated C terminus of RhoA in a C-terminal hydrophobic cavity of rhoGDI (Gosser *et al.*, 1997; Keep *et al.*, 1997) and our finding that complexation of rhoGDI with RhoA prevents immunoprecipitation of the latter with an antibody generated to the insert helix that is at the end of the RhoA structure opposite to that containing the C terminus (Wei *et al.*, 1997). An antibody to portions of rhoGDI (residues 178–198) can immunoprecipitate the heterodimer (Figure 7), indicating that the bottom sheet of the rhoGDI "β-sandwich" that contains these residues (Gosser *et al.*, 1997; Keep *et al.*, 1997) is solvent exposed, and either a β-sheet edge or the outer surface of the upper half of the β-sandwich of rhoGDI contacts RhoA. A structure that would account for both the nucleotide-inhibitory activity of rhoGDI and its ability to occlude the RhoA insert from immunoprecipitation is one in which a solvent-exposed surface of rhoGDI contacts the face of RhoA containing the nucleotide-binding pocket and shields, with its mobile N terminus, the insert helix of RhoA.

The Effect of ADP Ribosylation on the Translocation of RhoA to the Membrane and the Mechanism of Inhibition of RhoA Action

Approximately 50–60% of RhoA is translocated by high (50 μM) concentrations of GTPγS to the membrane, and lesser amounts by lower concentrations and by AlF_4^- or by Ca^{2+}-sensitizing (e.g., muscarinic, α-adrenergic) agonists (Gong *et al.*, 1997a,b). We now show that ADP ribosylation of RhoA in intact smooth muscle with DC3B completely blocks the translocation of RhoA by GTPγS, while also inhibiting the Ca^{2+}-sensitizing effects of agonists and GTPγS (see below). Based on several lines of evidence obtained in smooth muscle and other cells (present study; Fleming *et al.*, 1996; Gong *et al.*, 1996, 1997a), the initiation of RhoA-mediated processes involves dissociation of the rhoGDI complex, guanine nucleotide exchange factor (GEF)-facilitated exchange of GTP for guanosine diphosphate, and association of Rho-GTP with a membrane-associated effector (Bokoch *et al.*, 1994). The precise sequence of these events is not known, although it has been suggested that, in neutrophils, the RhoA–rhoGDI complex is first translocated to the plasma membrane, where it encounters a GEF that facilitates nucleotide exchange and dissociation of the complex (Bokoch *et al.*, 1994). In vitro ADP ribosylation of constitutively active Val14-RhoA·GTP inhibits its effects on stress-fiber assembly in fibroblasts (Paterson *et al.*, 1990) and Ca^{2+} sensitization in smooth muscle

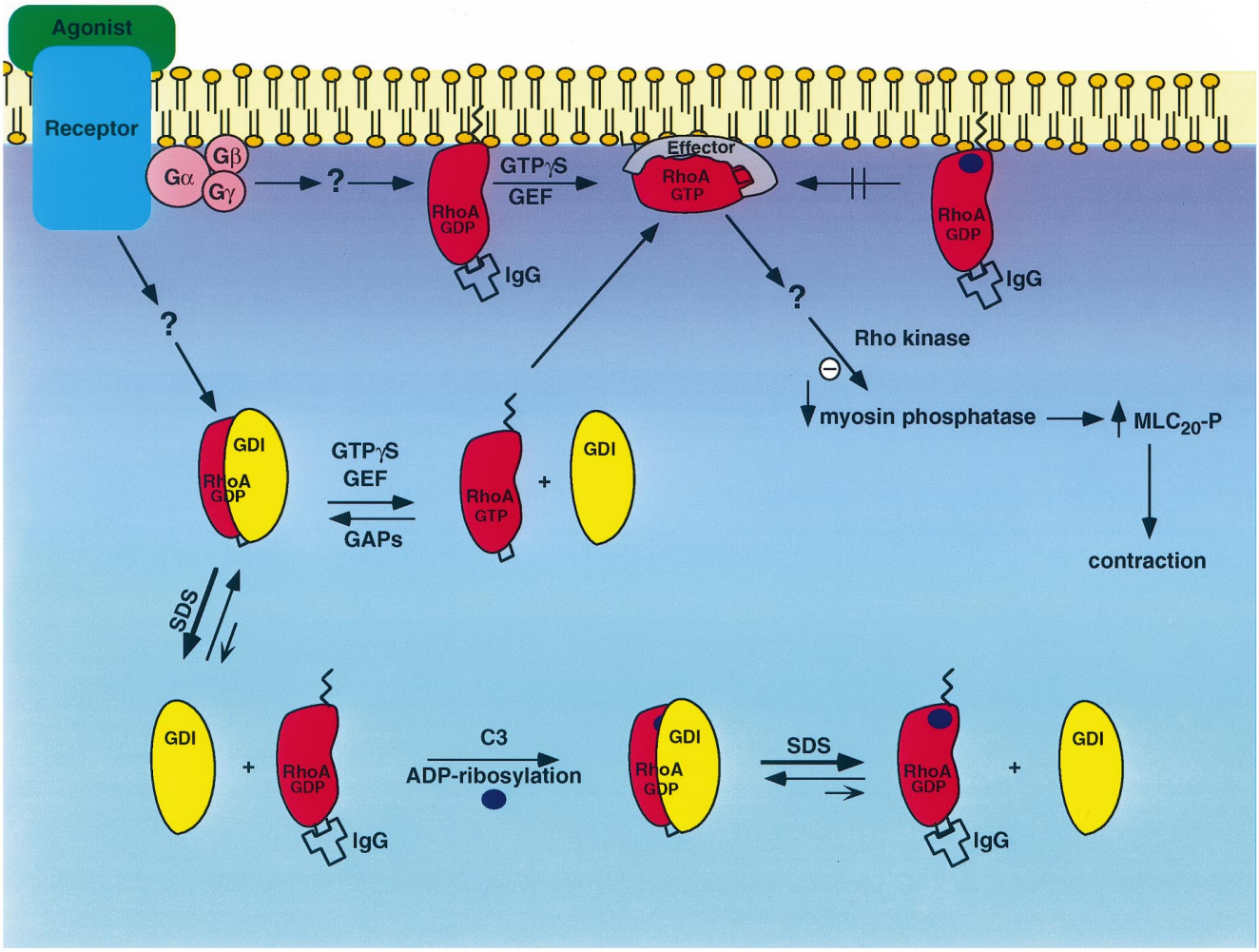

Figure 8. Steps of RhoA activation and of the inhibition of RhoA activity by ADP ribosylation of its Asn 41 residue. The alignment of RhoA and rhoGDI in the heterodimer formed by the two proteins, while schematic, is based on combining the results of ADP ribosylation and immunoprecipitation of RhoA with the crystal and nuclear magnetic resonance structures of the respective proteins (Gosser *et al.*, 1997; Keep *et al.*, 1997; Wei *et al.*, 1997). ADP ribosylation of RhoA is indicated by dark blue circles; the insert loop (Wei *et al.*, 1997), the region of the epitope to which the immunoprecipitating antibody was generated, is indicated by a square; and the zigzag line represents the flexible, prenylated C terminus of RhoA that is thought to be largely responsible for membrane binding. The main phenomena illustrated are: 1) in unstimulated smooth muscle RhoA is present mainly as a cytosolic complex with rhoGDI that can be neither immunoprecipitated nor ADP ribosylated, and a small fraction of inactive, membrane-associated RhoA that can be ADP ribosylated and immunoprecipitated. 2) Dissociation of the RhoA–rhoGDI complex, accelerated by SDS, renders it available for both immunoprecipitation and ADP ribosylation. 3) Activation of RhoA with GTPγS (or GTP plus an agonist) causes translocation of RhoA to the membrane and activates already membrane-associated, inactive RhoA, making both the ADP-ribosylation site (Asn 41) and the immunoprecipitating epitope unaccessible, due to the association of RhoA with a putative effector and/or other membrane structure. 4) Treatment with DC3B for 48 h results in ADP ribosylation of both the cytosolic rhoGDI-complex and membrane-associated RhoA, and prevents the association of RhoA with the membrane-bound effector, as indicated by its immunoprecipitability after exposure to GTPγS. The site of dissociation of the heterodimer, whether within the cytosol or during transient association with the membrane, is not known. Association of activated RhoA with its effector results in inhibition of smooth muscle myosin phosphatase, increased phosphorylation of the regulatory myosin light chain (MLC_{20}), and contraction. GDI, rhoGDI; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein.

(Gong *et al.*, 1996), whereas ADP ribosylation of RhoA does not interfere with nucleotide binding (Hancock and Hall, 1993), guanosine triphosphatase (GTPase) activity (Braun *et al.*, 1989), or interaction with GTPase-activating proteins (Paterson *et al.*, 1990). These and our present results suggest that the most likely in vivo mechanism of inhibition of RhoA-mediated effects by ADP ribosylation is interference with the association between RhoA and a membrane-bound effector. RhoA activated with GTPγS is translocated and forms a membrane-bound complex that cannot be immunoprecipitated with an antibody

raised against residues 120–150 (our unpublished results and Gong *et al*., 1997a,b). This translocation is prevented by ADP ribosylation with DC3B, and both cytosolic RhoA (dissociated from rhoGDI with SDS; Figure 7) and membrane-associated RhoA ADP-ribosylated with DC3B can be immunoprecipitated even in the presence of GTPγS. The immunoprecipitability of ADP-ribosylated membrane-associated RhoA suggests that inhibition of the activity of RhoA by its ADP ribosylation is not due to inhibition of translocation *per se*, but to the prevention of the association of RhoA with the membrane-bound effector that would normally result in occlusion of the RhoA insert helix (residues 124–136; Wei *et al.*, 1997), and that, in addition to insertion of the prenylated C terminus into the lipid bilayer, association of the RhoA helix with a protein target also directs the specificity of binding of activated RhoA to the membrane.

The Effects of ADP Ribosylation of RhoA on Contraction of Intact Smooth Muscle, and on Ca^{2+} Sensitization by Agonists and GTPγS, but Not by Phorbol Ester

The tonic phase of contraction induced by the α_1-adrenergic agent, phenylephrine, was markedly inhibited in intact smooth muscles treated with DC3B (Figure 2), whereas the initial transient was only slightly reduced. This finding, in conjunction with earlier results showing dissociation, in nonpermeabilized smooth muscle, between agonist-induced force development and $[Ca^{2+}]_i$ (Bradley and Morgan, 1987; Himpens *et al*., 1990; reviewed by Somlyo and Somlyo, 1994), indicates that RhoA-mediated Ca^{2+} sensitization can operate under physiological conditions. The inhibition of the tonic phase of muscarinic-induced contractions by toxin B of *Clostridium difficile* also led to this conclusion (Otto *et al.*, 1996); however, the effects of this toxin are less selective than that of C3 because it monoglucosylates and inhibits not only RhoA, but all members of the Rho subfamily (Aktories and Just, 1995). Toxins that inactivate RhoA inhibit Ca^{2+} sensitization of smooth muscle by a variety of agents (α-adrenergic, muscarinic, endothelin; Kokubu *et al.*, 1995; Gong *et al.*, 1996) that activate receptors that are also present on nonmuscle cells. Therefore, it is likely that a RhoA cascade similar to that operating in smooth muscle plays a signaling function in nonmuscle cell processes that involve nonmuscle myosin regulated by phosphorylation/dephosphorylation of MLC_{20} (Somlyo and Somlyo, 1994; Goeckeler and Wysolmerski, 1995; Burridge and Chrzanowska-Wodnicka, 1996). The downstream mechanisms mediating increased MLC_{20} phosphorylation have not yet been fully determined, with recent studies implicating inhibitory phosphorylation of SMPP-1 M by Rho kinase (Kimura *et al.*, 1996; Kureishi *et al.*, 1997) and/or other kinases (Amano *et al*., 1996), including atypical protein kinase Cs not activated by phorbol esters (Ichikawa *et al.*, 1996; Gailly *et al.*, 1997).

Phorbol ester-induced Ca^{2+} sensitization that is mediated by conventional and/or novel protein kinase C(s) was not inhibited by DC3B, in contrast to the inhibitory effect of ADP ribosylation on G-protein–coupled Ca^{2+} sensitization. This finding confirms that the two mechanisms are separate upstream, and the effect of phorbol ester is not mediated by RhoA (Jensen *et al.*, 1996; Gailly *et al.*, 1997), although activation of conventional and/or novel kinase Cs by phorbol esters can, like the G-protein–coupled mechanism, Ca^{2+} sensitize smooth muscle by increasing phosphorylation of MLC_{20} (Itoh *et al.*, 1994; Masuo *et al.*, 1994; Ikebe and Brozovich, 1996; Jensen *et al.*, 1996).

Finally, although DC3B caused extensive ADP ribosylation of endogenous RhoA and inhibited Ca^{2+} sensitization by GTPγS, neither of these effects was complete. We have previously shown that translocation of only 30% of total RhoA to the membrane fraction is sufficient for maximal Ca^{2+} sensitization with GTPγS (Gong *et al.*, 1997a). Therefore, it remains to be determined whether the GTPγS-mediated Ca^{2+} sensitization still remaining after DC3B treatment is due to activation of the residual, non-ADP-ribosylated RhoA or to some other Ca^{2+}-sensitizing mechanism (Gong *et al.*, 1992; Lee and Severson, 1994; Masuo *et al.*, 1994; Walsh *et al.*, 1994; Gailly *et al.*, 1997).

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