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Role of guanine nucleotide-binding proteins—ras-family or trimeric proteins or both—in Ca^{2+} sensitization of smooth muscle

(rho protein/ADP-ribosylation/pertussis toxin/epidermal cell differentiation inhibitor)

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ABSTRACT **The purpose of this study was to identify guanine nucleotide-binding proteins (G proteins) involved in the agonist- and guanosine 5′-[γ-thio]triphosphate (GTP[γ-S])-induced increase in the Ca^{2+} sensitivity of 20-kDa myosin light chain (MLC_{20}) phosphorylation and contraction in smooth muscle. A constitutively active, recombinant val14p21^{rhoA}·GTP expressed in the baculovirus/Sf9 system, but not the protein expressed without posttranslational modification in *Escherichia coli*, induced at constant Ca^{2+} (pCa 6.4) a slow contraction associated with increased MLC_{20} phosphorylation from 19.8% to 29.5% ($P < 0.05$) in smooth muscle permeabilized with β-escin. The effect of val14p21^{rhoA}·GTP was inhibited by ADP-ribosylation of the protein and was absent in smooth muscle extensively permeabilized with Triton X-100. ADP-ribosylation of endogenous p21^{rho} with epidermal cell differentiation inhibitor (EDIN) inhibited Ca^{2+} sensitization induced by GTP [in rabbit mesenteric artery (RMA) and rabbit ileum smooth muscles], by carbachol (in rabbit ileum), and by endothelin (in RMA), but not by phenylephrine (in RMA), and only slowed the rate without reducing the amplitude of contractions induced in RMA by 1 μM GTP[γ-S] at constant Ca^{2+} concentrations. AlF_4^--induced Ca^{2+} sensitization was inhibited by both guanosine 5′-[β-thio]diphosphate (GDP[β-S]) and by EDIN. EDIN also inhibited, to a lesser extent, contractions induced by Ca^{2+} alone (pCa 6.4) in both RMA and rabbit ileum. ADP-ribosylation of trimeric G proteins with pertussis toxin did not inhibit Ca^{2+} sensitization. We conclude that p21^{rho} may play a role in physiological Ca^{2+} sensitization as a cofactor with other messengers, rather than as a sole direct inhibitor of smooth muscle MLC_{20} phosphatase.**

Contraction and relaxation of smooth muscle are primarily regulated by, respectively, phosphorylation and dephosphorylation of the 20-kDa regulatory light chain of myosin (MLC_{20}; reviewed in ref. 1). The protein phosphatase that dephosphorylates MLC_{20} can be inhibited by a guanine nucleotide-binding protein (G protein)-coupled mechanism (2–4), resulting in higher levels of MLC_{20} phosphorylation and contraction at a given submaximal intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. This process of Ca^{2+} sensitization can be inhibited by guanosine 5′-[β-thio]diphosphate (GDP[β-S]) (5–8), but the G protein(s) involved and the transduction pathways between plasma membrane-bound receptors and the myosin filament-bound protein phosphatase (9–11) have not been definitively identified. Both a constitutively active ras (12) and p21^{rhoA} activated with guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) (13, ‖) have been reported to Ca^{2+}-sensitize smooth muscle, whereas the Ca^{2+}-sensitizing effect of fluoroaluminates (14) implicated trimeric G protein(s) (ref. 15; reviewed in ref. 4).

The purpose of the present study was: (*i*) to determine whether p21^{rho} was involved in Ca^{2+} sensitization by using a constitutively active recombinant protein, the effects of which would not be confounded by the release of free GTP[γ-S] (16); (*ii*) to establish whether such effects were accompanied by an increase in MLC_{20} phosphorylation; (*iii*) to evaluate whether the effects, if any, of ADP-ribosylation of endogenous p21^{rho} by a highly purified bacterial ADP-ribosyltransferase, epidermal cell differentiation inhibitor (EDIN; refs. 17 and 18), were tissue- or agonist-specific or both; (*iv*) whether the Ca^{2+}-sensitizing activity of p21^{rho} is dependent on posttranslational modification; and (*v*) to determine, with what turned out to be somewhat surprising results, the effects of ADP-ribosylation of p21^{rho} on the Ca^{2+}-sensitizing activity of AlF_4^-.

MATERIALS AND METHODS

Preparation of val14p21^{rhoA}·GTP. Two liters of Sf9 insect cells were grown to a cell density of 1.5×10^6 cells per ml in TC100 medium supplemented with 10% (vol/vol) fetal calf serum, 100 μg of streptomycin per ml, and 60 μg of penicillin per ml. Recombinant *val14 rhoA* was subcloned from *E. coli* (19) into baculovirus (Invitrogen). The cells were infected for 62 hr at a multiplicity of infection of 5:1 with baculovirus containing the recombinant *val14 rhoA*, harvested, and stored at −80°C.

For protein purification, carried out at 4°C, 10 g of frozen cells were resuspended in 70 ml of 20 mM Hepes, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/20 μM GTP (buffer A), disrupted in a glass homogenizer, and centrifuged at 640 × *g* for 15 min, and the supernatant was decanted. The residue was twice resuspended in 70 ml of buffer A and recentrifuged. The combined supernatants containing the cell membrane fragments were centrifuged at 186,000 × *g* for 90 min, and the pellet was resuspended in 50 ml of buffer A containing 2% octyl glucoside, homogenized, and gently shaken for 45 min. The solution was centrifuged at 142,000 × *g* for 90 min, and the supernatant was filtered through a 0.45-μm Minisart cellulose acetate filter (Sartorius). The solution (≈0.4 mg of protein per ml) was loaded into a 10-ml MonoS column equili-

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Abbreviations: EDIN, epidermal cell differentiation inhibitor; GTP[γ-S], guanosine 5′-[γ-thio]triphosphate; PE, phenylephrine; MLC_{20}, 20-kDa myosin light chain; PT, pertussis toxin; G protein, guanine nucleotide-binding protein; GDP[β-S], guanosine 5′-[β-thio]diphosphate; RMA, rabbit mesenteric artery; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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brated with 20 mM Hepes, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/20 μM GTP/1% octyl glucoside (buffer B) and was eluted with a linear gradient from 0 to 0.7 M NaCl in buffer B over 1 hr at a flow rate of 1 ml/min; the eluate was collected in 1-min fractions. Fractions were analyzed by SDS/polyacrylamide gel electrophoresis and [^{3}H]GDP binding assays (20). Those containing val14p21^{rhoA} (≈20 ml) were pooled and dialyzed overnight against 20 mM Tris, pH 8.0/5 mM $MgCl_2$/1 mM dithiothreitol/0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 20 μM GTP (buffer C). The solution (≈0.4 mg of protein per ml) was loaded into a 1-ml MonoQ column equilibrated with buffer C and was eluted with a linear gradient of 0–1 M NaCl over 45 min at a flow rate of 1 ml/min; the eluate was collected in 1-min fractions. SDS/polyacrylamide gel electrophoresis showed that the majority of the p21^{rhoA} was eluted in a single fraction that was desalted in a PD 10 column equilibrated with 20 mM Hepes, pH 7.5/5 mM $MgCl_2$; 0.5-ml fractions were collected, and the protein peak was detected by absorbance at 280 nm. Samples were rapidly frozen and stored at −80°C.

The purified sample was analyzed by SDS/gel electrophoresis with Coomassie blue staining and also by immunoblot (Western blot) analysis with anti-rho antibody. The nucleotide content and identity of the nucleotide bound to val14p21^{rhoA} was determined by HPLC. This procedure also gave the concentration of nucleotide (and hence protein, assuming stoichiometric binding) by comparison of the areas of the peak with standards of known concentration.

The concentration of CHAPS in the final protein solution was determined by the following method based on cholesterol measurement (21): sixty microliters of 6 mM $FeCl_3$ in glacial acetic acid was mixed with 30 μl of sample, and 60 μl of concentrated H_2SO_4 was added; the mixture was incubated at 100°C for 3 min and briefly centrifuged. The concentration of CHAPS was calculated from a standard curve of CHAPS concentration against the absorbance at 600 nm.

The protein concentration was determined (*i*) from the absorbance at 280 nm by using a theoretical extinction coefficient (25.9×10^3 M^{-1}) calculated from the aromatic amino acid content (22) and taking into account the absorption of guanine nucleotide and (*ii*) from the nucleotide concentration of the complex as measured by HPLC. Both methods gave values in close agreement. The Bradford assay with bovine serum albumin as the standard gave values ≈20% higher. The concentrations of the complex (containing >95% GTP and <5% GDP) used in our study are based on nucleotide concentration, since this is the active species. There was no detectable (<0.001%) CHAPS in the final protein solutions.

Force Measurement. Helical strips (100 μm wide × 4 mm long) were cut from the second or third branch of male rabbit (4 to 6 lb) mesenteric artery (RMA; o.d. = 200 to 300 μm). The longitudinal muscle layer was peeled off from rabbit ileum and cut into 200-μm-wide and 4-mm-long strips. Isometric tension was measured at 24°C with a force transducer (AE 801; SensoNor, Horten, Norway) in a well on a "bubble" plate (23). Muscles were stretched to 1.3 × rest length. After normal responses to depolarization with 154 mM K^+ and to 100 μM phenylephrine (PE) were observed, the strips were permeabilized by incubation with 50 μM β-escin (Sigma) for 15 min at 24°C and treated with 10 μM A23187 to deplete Ca^{2+} stores (24). Details of the solutions used and methods for measurement of MLC_{20} phosphorylation have been published (8, 24, 25). Calmodulin (0.1 μM) was added to all intracellular solutions.

ADP-Ribosylation of p21^{rho} with EDIN. Permeabilized strips were incubated for 40 min in 250 μl of relaxing solution also containing 12.5 μCi (1 Ci = 37 GBq) of [^{32}P]NAD (Amersham Life Science) or 100 μM NAD (when only tension was determined), 2 mM thymidine, 10 mM dithiothreitol, 200 μM GTP, 100 μg of leupeptin per ml, and 2 μg of aprotinin per ml, with or without 800 ng or 1 μg of EDIN per ml. Relaxing solution contained 74 mM potassium methanesulfonate, 30 mM Pipes, 2 mM magnesium methanesulfonate, 4.5 mM MgATP, 5.1 mM ATP (total), 10 mM creatine phosphate, and 1 mM K_2EGTA. Next, the strips were washed twice for 2.5 min in relaxing solution and then were stimulated with submaximal Ca^{2+} (pCa 6.4) followed by 10 μM GTP and one of the following agonists: 100 μM carbachol, 1 μM endothelin, 100 μM PE, or 50 μM GTP[γ-S]; 5 μM calmodulin was also added to verify transmembrane penetration of a protein with a molecular mass comparable to that of p21^{rho}. Three such treated strips were pooled for each autoradiographic measurement of ADP-ribosylation. Homogenates of three other intact strips of identical size were also incubated under identical conditions. Reactions were stopped by adding sample buffer (62.5 mM Tris·HCl, pH 7.0/1% SDS/15% glycerol/15 mM dithiothreitol/0.004% bromphenol blue) to the strips that were homogenized, boiled for 2 min, centrifuged for 10 min at 16,000 × *g* run in 15% SDS/polyacrylamide gel, and electrotransferred to poly(vinylidene difluoride) (PVDF) membrane for autoradiographs and Western blots with anti-p21^{rhoA} antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Equal protein loading among samples was verified by blotting the same membrane with anti-actin antibody and by Coomassie blue staining of the protein remaining in the gel after transfer. Western blotting was detected with enhanced chemiluminescence (ECL).

EDIN was purified to homogeneity, with only one band, corresponding to ADP-ribosylating activity, detected in silver-stained SDS/PAGE gels (17, 18).

ADP-Ribosylation of Trimeric G Protein(s) with Pertussis Toxin (PT). Strips permeabilized with *Staphylococcus aureus* α-toxin were incubated with 21 μg of PT (preactivated for 1 hr at 37°C with 50 mM dithiothreitol per ml, 15 μCi of [^{32}P]NAD, 10 mM thymidine, and 10 mM dithiothreitol for 4 hr to ADP-ribosylate endogenous α subunits of inhibitory G proteins ($G_i\alpha$) and regulatory G proteins ($G_0\alpha$). After incubation, the responses to submaximal Ca^{2+} (pCa 6.4), 10 μM GTP, and 100 μM PE were determined, and the strips were homogenized as above.

RESULTS

Slow Dose-Dependent Ca^{2+} Sensitization of Permeabilized RMA Is Caused by val14p21^{rhoA}·GTP Expressed and Post-translationally Modified in the Baculovirus/Sf9 System, but Not By Unmodified Protein Expressed in *E. coli*. Recombinant val14p21^{rhoA}·GTP (3 μM) expressed in a baculovirus system caused a very slow, dose-dependent increase in tension in β-escin-permeabilized RMA at constant, submaximal free [Ca^{2+}] (pCa 6.4, containing 10 mM EGTA) (Fig. 1 *Upper*). The delay and $t_{1/2}$ of force elicited by supramaximal (6 μM) val14p21^{rhoA}·GTP were 1.9 ± 0.22 min (n = 6) and 19 ± 0.58 min (n = 6), respectively. In comparison, the delay (0.55 ± 0.044 min; n = 8) and $t_{1/2}$ (2.6 ± 0.36 min; n = 5) of force development in response to GTP[γ-S] were much faster, as were the responses to the agonists PE (Fig. 1) and endothelin (data not shown). The amplitude [44 ± 7.8% (n = 6) of maximal Ca^{2+}-induced contraction] of contractile response to val14p21^{rhoA} was smaller, although not statistically significant, than the contraction induced by 50 μM GTP[γ-S] [57 ± 7.8% (n = 5) of maximal Ca^{2+}-induced contraction]. GTP plus PE, added after the contraction induced by 6 μM val14p21^{rhoA}·GTP had reached steady state (Fig. 1 *Upper*), caused further contraction (17 ± 3.4%; n = 5), raising the possibility that PE can activate an additional Ca^{2+}-sensitizing pathway not mediated by p21^{rhoA}. ADP-ribosylation of val14p21^{rhoA} *in vitro* markedly inhibited its Ca^{2+}-sensitizing effect (Fig. 1 *Lower*), and the recombinant val14p21^{rhoA}·GTP (3.0–12 μM) expressed in *E. coli* and therefore

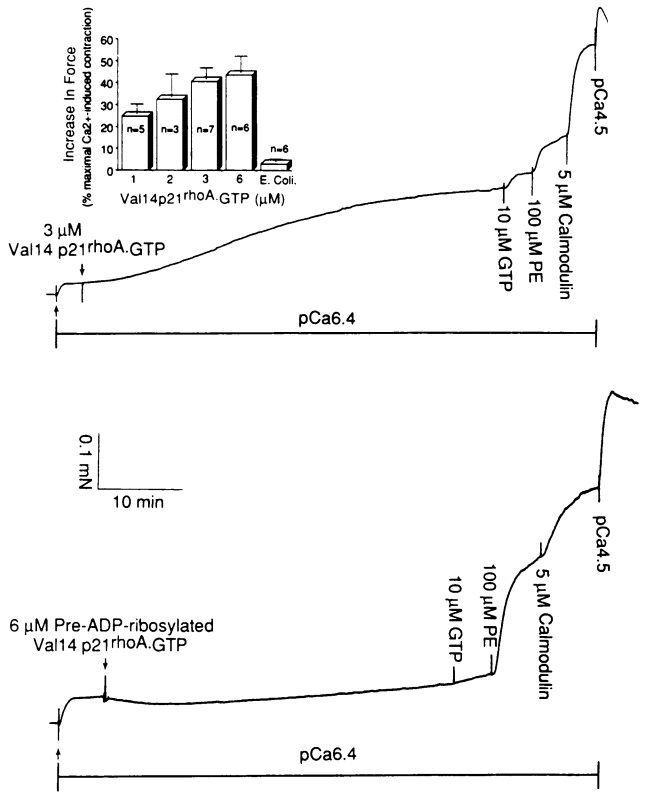

FIG. 1. Ca^{2+}-sensitizing effect of val14p21^{rhoA}·GTP expressed in baculovirus/Sf9 system on RMA. RMA strip permeabilized with β-escin in relaxing solution (1 mM EGTA) was submaximally contracted by transferring it (at arrow head) into buffered (with 10 mM EGTA) pCa 6.4 solution. (*Upper*) val14p21^{rhoA}·GTP (expressed in the baculovirus/Sf9 system) added at the arrow caused slow but significant force development, and subsequent addition of 10 μM GTP and 100 μM PE caused further and more rapid contraction. The fast and large contraction induced by 5 μM calmodulin indicates free diffusion of a 17-kDa protein in the β-escin-permeabilized system. (*Inset*) Summary of the dose-dependent Ca^{2+}-sensitizing effect of val14p21^{rhoA}·GTP expressed in the baculovirus/Sf9 system and the lack of effect of val14p21^{rhoA}·GTP expressed in *E. coli*. (*Lower*) ADP-ribosylation of val14p21^{rhoA}·GTP by EDIN *in vitro* inhibited its Ca^{2+}-sensitizing effect. val14p21^{rhoA}·GTP expressed in the baculovirus/Sf9 system was ADP-ribosylated by incubating it with 800 ng of EDIN per ml/2 mM thymidine/10 mM dithiothreitol/200 μM GTP for 40 min at 24°C in Ca^{2+}-free relaxing solution (containing 30 mM Pipes, 1 mM EGTA, 2 mM Mg^{2+}, 4.5 mM MgATP, and 0.5 mM ATP). The buffer was exchanged from the reaction mixture to pCa6.4 intracellular solution by using Microcon 10 and then was added to β-escin-permeabilized RMA strips. (The data are representative of three paired experiments.)

not geranylgeranylated had no significant effect [2.9 ± 1.3% (n = 6) on the maximal Ca^{2+}-induced contraction, 6 μM; Fig. 1 *Inset*].

Val14p21^{rhoA}·GTP Increases MLC_{20} Phosphorylation But Does Not Directly Inhibit Smooth Muscle Myosin Phosphatase. Contractions induced at pCa 6.4 by val14p21^{rhoA}·GTP (6 μM) expressed in baculovirus/Sf9 system were associated with an increase in MLC_{20} phosphorylation from 19.8 ± 2.48% (n = 3) to 29.5 ± 1.89% (n = 5; $P < 0.05$), suggesting that p21^{rhoA} operated through the same mechanism as agonists and GTP[γ-S], which cause Ca^{2+}-sensitization by inhibiting MLC_{20} phosphatase (2, 3).

Vigorous permeabilization with Triton X-100 abolishes the Ca^{2+}-sensitizing effect of GTP[γ-S] because of loss of a diffusible cofactor and/or uncoupling of a membrane-bound G protein from its effector, while retaining the "Ca^{2+}-sensitizing" effect of the protein phosphatase inhibitor microcystin (26). In Triton X-100-treated preparations, val14p21^{rhoA}·GTP (up to 6 μM) had no effect (Fig. 2), indicating that it is not a direct myosin phosphatase inhibitor.

ADP-Ribosylation of Endogenous p21^{rho} and Its Effect on Agonist (Carbachol, Endothelin, and PE)- and GTP[γ-S]-Induced Ca^{2+} Sensitization and on Ca^{2+}-Induced Contraction. The role of endogenous p21^{rho} in Ca^{2+}-sensitization was tested by determining whether ADP-ribosylation of endogenous p21^{rho} by EDIN, a *Staphylococcus aureus* enzyme that specifically ADP-ribosylates p21^{rho} (18), inhibits agonist- and GTP[γ-S]-induced Ca^{2+} sensitization.

ADP-ribosylation by EDIN of endogenous p21^{rho} in RMA strips permeabilized with β-escin was verified in the same strips in which tension was also recorded (see below). Autoradiography showed reproducibly a band at about 21 kDa (n = 6) (Fig. 3*A*) and sometimes a second faint band that migrates just above 21 kDa. Western blots of the same membrane with anti-p21^{rhoA} polyclonal antibody showed a band at about 21 kDa that overlayed the band on the autoradiograph. Following ADP-ribosylation of strips by nonradioactive NAD, there was no subsequent ADP-ribosylation of homogenates detectable with [^{32}P]NAD (data not shown), indicating that all of the p21^{rhoA} available to EDIN was ADP-ribosylated.

We also attempted, unsuccessfully, to further quantitate the degree of ADP-ribosylation *in situ* by measuring ADP-ribosylation in homogenates, in which EDIN is expected to have free access to and ADP-ribosylate all of the endogenous p21^{rhoA}. However, *in vitro* treatment with EDIN and [^{32}P]NAD after homogenization in relaxing solution of intact strips of identical size to those used in the previous experiments reproducibly (n = 6) yielded much fainter autoradiographic bands than did similar treatment of β-escin-permeabilized strips. Equal protein loading was verified by anti-actin staining of the same membrane or Coomassie blue staining of the gel after transfer (Fig. 3*B*). The level of ADP-ribosylation of the homogenate could not be increased by increasing either the incubation time (from 40 min to 2 hr) or the incubation temperature (from 24°C to 37°C). These results are consistent with reports of low stoichiometry of *in vitro* ADP-ribosylation of p21^{rho} (0.4 to 0.6 mol of ADP-ribose per mol of protein; refs. 27 and 28) and suggest that perhaps a cofactor that facilitated ADP-ribosylation *in situ* was removed from, or an inhibitor of ADP-ribosylation was bound to, p21^{rho} during homogenization (29–31).

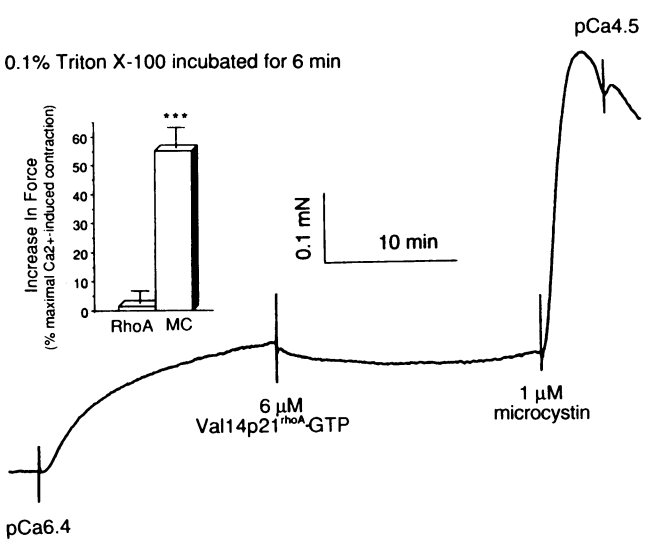

FIG. 2. Lack of effect of val14p21^{rhoA}·GTP expressed in baculovirus/Sf9 system on RMA permeabilized with Triton X-100. Neither 50 μM GTP[γ-S] nor val14p21^{rhoA}·GTP caused Ca^{2+} sensitization in RMA strips permeabilized with 0.1% Triton X-100 for 6 min at 24°C, in which the phosphatase inhibitor, microcystin (MC), caused large "Ca^{2+} sensitization." The data are representative of six experiments.

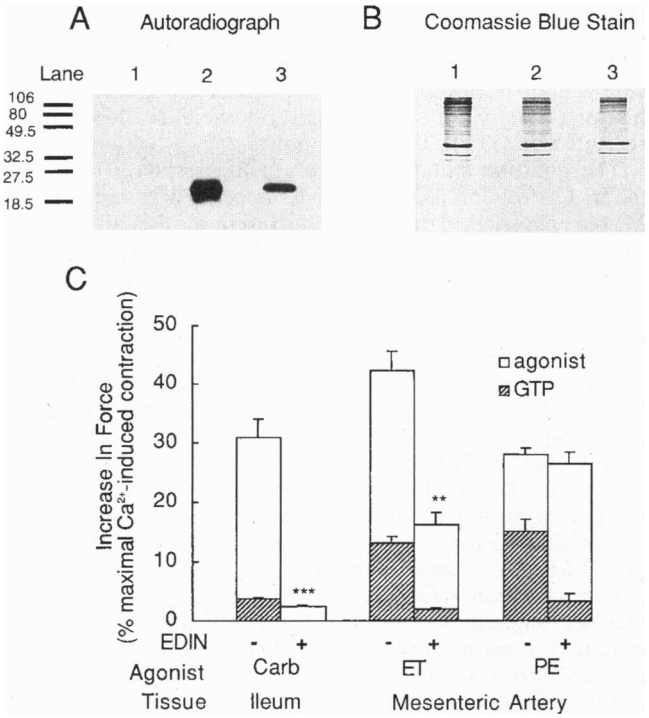

FIG. 3. The effect of ADP-ribosylation of endogenous $p21^{rho}$ by EDIN on GTP- and agonist-induced Ca^{2+} sensitization in β-escin-permeabilized rabbit smooth muscle. Endogenous $p21^{rho}$ in RMA and rat ileum longitudinal smooth muscle was ADP-ribosylated by incubation with 1 μg of EDIN per ml and other reagents (for details, see *Material and Methods*) for 40 min at 24°C. After the samples were washed, the responses to pCa 6.4, 10 μM GTP, and agonist were determined. (*A*) ADP-ribosylation of a 21-kDa protein by EDIN. ADP-ribosylation was carried out in β-escin-permeabilized strips of RMA or homogenates of identical size strips by incubation with 1 μg of EDIN per ml, 12.5 μCi of [^{35}P]NAD, and other reagents (for details, see *Materials and Methods*) for 40 min at 24°C. The reaction was stopped by adding sample buffer, and the proteins were separated with SDS/PAGE and transferred to polyvinylidene difluoride membrane. (*A*) Autoradiograph. Lanes: 1, three β-escin-permeabilized strips processed without EDIN during incubation; 2, ADP-ribosylation of three β-escin-permeabilized strips with EDIN; 3, ADP-ribosylation by EDIN of the homogenate of three strips. (*B*) Coomassie blue stain of the gel after transfer showing even protein loading among the three lanes. (*C*) Functional effects of EDIN. The 10 μM GTP-induced Ca^{2+} sensitization was significantly ($P < 0.001$) inhibited in both smooth muscles by EDIN, as were the effects of 100 μM carbachol (Carb) in ileum and of 1 μM endothelin (ET) in mesenteric artery. In contrast, EDIN did not inhibit the effect of 100 μM PE.

In β-escin-permeabilized rabbit ileum longitudinal smooth muscle, ADP-ribosylation of endogenous $p21^{rho}$ by EDIN significantly inhibited Ca^{2+}-sensitization by 10 μM GTP and 100 μM carbachol from, respectively, 3.8 ± 0.1% to 0 ($n = 3$ each; $P < 0.001$) and from 27.2 ± 2.9% to 2.4 ± 0.3% ($n = 3$ each; $P < 0.01$) (Fig. 3*C*).

In the ileum, EDIN did not block 50 μM GTP[γ-S]-induced contractions but only reduced their amplitude [from 49.2 ± 2.4% ($n = 6$) to 33.9 ± 2.9% ($n = 5$; $P < 0.01$)] and slowed the rate of force development [$t_{1/2}$ from 3.0 ± 0.6 min ($n = 5$) to 7.9 ± 0.69 ($n = 5$; $P < 0.01$)].

In β-escin-permeabilized RMA, experiments were conducted to determine whether the inhibitory effect of EDIN on carbachol-induced contraction was receptor or tissue specific or both. Ca^{2+} sensitization induced by 1 μM endothelin (32) was inhibited by EDIN (Fig. 3*C*) from 29.1 ± 3.2% to 14.2 ± 2.0% ($P < 0.05$; $n = 3$) of the maximal Ca^{2+}-induced contraction. EDIN did not inhibit PE-induced Ca^{2+} sensitization, although it inhibited in the same strips the GTP-induced Ca^{2+} sensitization from 15.1 ± 2.1% ($n = 13$) to 3.4 ± 1.3% ($n = 11$; $P < 0.00$) (Fig. 3*C*).

In RMA, EDIN did not reduce the amplitude [44.1 ± 4.3% ($n = 5$) versus 49.2 ± 6.1% ($n = 3$; $P > 0.05$)], although it decreased the rate [increased $t_{1/2}$ from 2.6 ± 0.36 min ($n = 5$) to 9.6 ± 1.9 min ($n = 3$; $P < 0.01$] of force development induced by 1 μM GTP[γ-S] at pCa 6.4.

Surprisingly, EDIN also inhibited submaximal Ca^{2+}-induced contractions, shifting the EC_{50} from pCa 5.65 to pCa 5.24 ($n = 5$ each; $P < 0.05$). On the other hand, GDP[β-S] had no significant effect on the pCa tension curve (data not shown).

Inhibition of AlF_4^--Induced Ca^{2+} Sensitization by EDIN. In view of the report that AlF_4^- activates the heterotrimeric G proteins but not the monomeric ras superfamily of G proteins (15), we determined whether EDIN affects AlF_4^--induced Ca^{2+} sensitization in β-escin-permeabilized rabbit ileum.

The combination of 10 μM $AlCl_3$ and 3 mM NaF caused Ca^{2+}-sensitization, increasing force at pCa 6.3 by 25 ± 1.5% of the maximal Ca^{2+}-induced contraction ($n = 4$); 3 mM GDP[β-S] relaxed the AlF_4^--induced contraction by 41.8 ± 5.3% ($n = 4$). Contrary to expectation, ADP ribosylation with EDIN reduced the amplitude of Ca^{2+} sensitization induced by AlF_4^- [from 25 ± 1.5% ($n = 4$) to 14.8 ± 2.2% ($n = 4$; $P < 0.01$) of the maximal Ca^{2+}-induced contraction] and prolonged the $t_{1/2}$ of contraction [from 1.7 ± 0.14 ($n = 4$) to 6.2 ± 0.48 ($n = 4$; $P < 0.001$) min.

In heavily saponin-permeabilized (150 μg/ml for 15 min at 24°C) strips, Ca^{2+}-sensitization could not be induced by either 300 μM GTP[γ-S] or by AlF_4^- (10 μM $AlCl_3$/3 mM NaF), whereas the phosphatase inhibitor microcystin-LR at 1 μM caused the usual large contraction (26, 33). These results (see also refs. 14 and 34) indicate that the effect of AlF_4^- is mediated through G protein(s) rather than direct inhibition of protein phosphatase or interaction with actomyosin ATPase.

Lack of Effect of PT-induced ADP-Ribosylation on PE-, Carbachol-, and GTP[γ-S]-Induced Ca^{2+} Sensitization in *S. aureus* α-Toxin-Permeabilized RMA and Ileum. The Ca^{2+}-sensitizing effect of AlF_4^- and the fact that Ca^{2+}-sensitizing agonists coupled to trimeric G proteins also activate the phosphatidylinositol cascade suggest that heterotrimeric G protein(s) may be involved in Ca^{2+} sensitization (4).

In *S. aureus* α-toxin-permeabilized (7) muscles, ADP-ribosylation of a 44-kDa protein ($G_i\alpha$ and/or G_0) with PT (21 μg/ml for 4 hr; ref. 35) verified by autoradiography (Fig. 4) did not inhibit Ca^{2+} sensitization by 10 μM GTP, 100 μM PE, or 300 μM GTP[γ-S] that was 24 ± 4.4% ($n = 5$), 18 ± 3.5% ($n = 5$), and 12 ± 2.8% ($n = 5$) in control strips and 33 ± 1.2% ($n = 8$; $P < 0.05$), 22 ± 2.2% ($n = 8$; $P > 0.05$), and 13 ± 1.2%

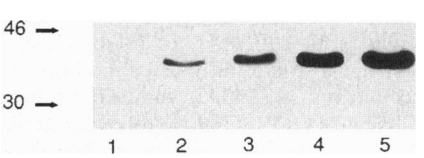

FIG. 4. ADP-ribosylation with PT of a G protein in *S. aureus* α-toxin-permeabilized RMA. Autoradiographs showing different levels of ADP-ribosylation reached by various PT concentrations or various PT incubation times at 30°C. Lanes: 1–4, muscle strips treated with PT at 10.5 μg/ml for 2-hr incubation (lane 1), at 21 μg/ml for 2-hr incubation (lane 2), at 10.5 μg/ml for 4-hr incubation lane 3, and at 21 μg/ml for 4-hr incubation (lane 4); 5, tissue homogenate treated with PT at 1 μg/ml for 30-min incubation. Note that incubation with PT at 21 μg/ml for 4 hr, used to determine the effects on Ca^{2+} sensitization, induced similar ADP-ribosylation levels in strips as in homogenates in which, presumably, PT had free access to all G protein(s) and complete ADP-ribosylation of $G_i\alpha$ and/or $G_0\alpha$ had been achieved. The arrows indicate the position of molecular markers ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). Only one band, at about 43 kDa, was labeled in the autoradiograms.

($n = 8$, $P > 0.05$) in PT-treated mesenteric artery strips. In ileum, Ca^{2+} sensitization induced by 10 μM GTP, 100 μM carbachol, and 300 μM GTP[γ-S] was, respectively, 11.1 $\pm$ 1.6% ($n = 6$), 45.5 $\pm$ 3.2% ($n = 6$), and 19.0 $\pm$ 2.2% ($n = 6$) in control strips and 9.2 $\pm$ 1.5% ($n = 6$; $P > 0.05$), 46.1 $\pm$ 3.5% ($n = 6$; $P > 0.05$) and 15.3 $\pm$ 2.0% ($n = 6$; $P > 0.05$) in PT-treated strips. The somewhat greater Ca^{2+} sensitization induced by GTP in the PT protocol was due to prolonged (4 hr) incubation. The apparently greater percentage of Ca^{2+} sensitization by the agonists than by GTP[γ-S] in these and other experiments reflects experimental protocols in which GTP[γ-S] was added "on top" of agonist-induced Ca^{2+} sensitization, and its magnitude reflected the limitation of the total (agonist-induced plus GTP[γ-S]-induced) Ca^{2+} sensitization attainable by this mechanism.

DISCUSSION

Our results not only indicate that posttranslationally modified monomeric G proteins can modulate the Ca^{2+} sensitivity of MLC_{20} phosphorylation and contraction but also point to the complexity of the mechanisms involved, as shown by the slow kinetics of val14p21^{rhoA}·GTP action, its absence in extensively permeabilized smooth muscle, and the unexpected inhibition of the Ca^{2+}-sensitizing action of AlF_4^- by ADP-ribosylation of endogenous p21^{rho}.

The participation of p21^{rho} in Ca^{2+} sensitization of smooth muscle had been suggested by its abundance in aortic smooth muscle (28), the ability of a wild-type recombinant p21^{rho} activated with GTP[γ-S] to cause contraction at constant Ca^{2+}, and by the apparent block of this effect by ADP-ribosylation of endogenous p21^{rho} (13). We used a mutated val14p21^{rhoA} that, because of its very low GTPase activity, is constitutively active in its GTP-bound form (36), eliminating effects due to release of free GTP[γ-S] from wild-type p21^{rhoA}. Constitutively active val14p21^{rhoA} caused contraction at constant [Ca^{2+}] and, although such an effect can also be produced by H-ras p21 (12), by detergents (0.3% Na cholate) (M.C.G., S.K., A.V.S., and A.P.S., unpublished observations), and even by some batches of bovine serum albumin (M.C.G., S.K., A.V.S., and A.P.S., unpublished observations), the inhibition of the Ca^{2+}-sensitizing effect of val14p21^{rho} by ADP-ribosylation *in vitro* supports its specificity of action, as do the inhibitory effects of ADP-ribosylation of endogenous p21^{rhoA} on Ca^{2+} sensitization by GTP, carbachol, and endothelin (Fig. 3). However, the contractile responses to val14p21^{rhoA}·GTP were slow, developed after a long delay, and were abolished by heavy permeabilization of smooth muscle with Triton X-100. The much slower rate of contraction induced by val14p21^{rhoA}·GTP than by Ca^{2+}-sensitizing agonists, GTP, and GTP[γ-S] (see Fig. 1) could not be ascribed to the slower diffusion of the 21-kDa protein or to deficiency of GDP-release-stimulating factor (GDS) because addition of calmodulin, a protein of similar molecular mass (17 kDa), induced a brisk contraction (Fig. 1) and because GDS is not required for activation of the constitutively active GTP-bound protein. The slow contractile response to and the lack of effect of val14p21^{rhoA}·GTP on smooth muscles permeabilized with Triton X-100 (present study) suggest that p21^{rho} is an upstream messenger of Ca^{2+} sensitization, rather than a direct inhibitor of MLC_{20} dephosphorylation. ADP-ribosylation of endogenous p21^{rho} by EDIN inhibited endothelin-induced, but not PE-induced, Ca^{2+} sensitization in the same (RMA) smooth muscle, suggesting that the participation of p21^{rhoA} in such cascades may be, at least quantitatively, agonist dependent. When endogenous p21^{rho} in smooth muscle strips was ADP-ribosylated in the presence of nonradioactive NAD, subsequent attempts to ADP-ribosylate homogenates with [^{32}P]-NAD showed no further ADP-ribosylation, indicating that all of the available substrate had been ADP-ribosylated. Unfortunately, homogenates of intact tissue were less extensively ADP-ribosylated than strips (see *Results*), precluding normalization to the homogenate value as 100%. Therefore, we cannot exclude the possibility that some endogenous p21^{rho}, perhaps protected by GDP exchange inhibitor (GDI) (31), escaped ADP-ribosylation and could be recruited by GTP[γ-S] or an agonist.

The question whether the contractile response to monomeric G proteins (12, 13, 37) is associated with increased MLC_{20} phosphorylation was answered in the affirmative: val14p21^{rhoA}·GTP significantly increased the phosphorylation of MLC_{20}. This finding and a recent study showing that in cultured cells ADP-ribosylation of endogenous rho protein partially reverses the inhibition of MLC_{20} dephosphorylation by GTP[γ-S] (38) are consistent with rho being at least one of the G proteins involved in the G-protein-coupled inhibition of MLC_{20} phosphatase first implicated in G-protein-mediated Ca^{2+} sensitization (2, 3).

Only the posttranslationally modified val14p21^{rhoA} expressed in the baculovirus/Sf9 system, but not unmodified protein expressed in *E. coli*, had a Ca^{2+}-sensitizing effect. Inasmuch as geranylgeranylation promotes the association of p21^{rho} with the plasma membrane (39, 40), both this finding and the abolition of Ca^{2+} sensitization in Triton X-100-treated muscles, suggest that the Ca^{2+}-sensitizing effect of p21^{rho} requires its association with an intact or only moderately, permeabilized plasma membrane. The very slow Ca^{2+}-sensitization by val14p21^{rhoA} (Fig. 1) may be due to the slow rate of this association, whereas a pre-formed endogenous p21^{rho}-effector complex could respond more rapidly to an agonist or GTP[γ-S].

The inhibition of the Ca^{2+}-sensitizing effect of AlF_4^- by ADP-ribosylation of endogenous p21^{rhoA} with EDIN was unexpected, because fluoroaluminates are not thought to activate monomeric G proteins (15). Similar effects on AlF_4^--induced Ca^{2+} sensitization have also been observed recently following ADP-ribosylation of guinea pig vas deferens with C3 exoenzyme (41). We can suggest two possible mechanisms responsible for this effect: (*i*) p21^{rho} may be a cofactor of a Ca^{2+}-sensitizing trimeric G protein, and EDIN inhibits the interaction between the (putative) trimeric G protein and p21^{rhoA}; and (*ii*) AlF_4^- may be able to interact with p21^{rhoA} complexed with another protein or proteins in the same manner in which association with a ribosome allows the otherwise forbidden interaction between elongation factor G and fluoroaluminates (42).

Contrary to a previous report that "the contraction induced by any concentration of Ca^{2+} in the absence of GTP[γ-S] was not affected by EDIN" (13), we found that ADP-ribosylation of these same (RMA) smooth muscles with EDIN inhibited the contractile response to submaximal [Ca^{2+}]. Another study, published after completion of our experiments (37), showed that ADP-ribosylation of endogenous p21^{rho} with C_3 enzyme also right-shifted the pCa–tension curve of β-escin-skinned guinea pig ileum; this rightward shift was eliminated by pretreatment of the muscles with GDP[β-S]. The inhibitory effect of C_3 (37) and EDIN (present study) on submaximal Ca^{2+}-induced tension may reflect the presence of a p21^{rho}-mediated baseline Ca^{2+} sensitization, although in our experiments GDP[β-S] itself did not affect the pCa–tension curve. Also, contrary to a report (13), we found that EDIN did not block the Ca^{2+}-sensitizing effect of GTP[γ-S] in RMA (see *Results*) but only slowed the rate of force development induced by GTP[γ-S]. The complete block of GTP[γ-S]-induced Ca^{2+}-sensitization reported by Takai and colleagues (13) may have been due to their more drastic method of permeabilization (with saponin) and the consequently more rapid "rundown" of the Ca^{2+}-sensitizing effect of GTP[γ-S] that can be abolished with heavy saponin treatment.

In conclusion, our results are consistent with an accessory, and possibly agonist-dependent, role of p21^{rhoA} in G-protein-

mediated Ca^{2+} sensitization of MLC_{20} phosphorylation and force in smooth muscle, and indicate that $p21^{rhoA}$ requires a diffusible cofactor or association with a relatively intact intact plasma membrane, or both, in order to cause Ca^{2+} sensitization by inhibiting smooth muscle MLC_{20} phosphatase.

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