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Down-Regulation of G-Protein-mediated Ca^{2+} Sensitization in Smooth Muscle

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Prolonged treatment with guanosine 5′-[γ-thio]triphosphate (GTPγS; 5–16 h, 50 μM) of smooth muscle permeabilized with *Staphylococcus aureus* α-toxin down-regulated (abolished) the acute Ca^{2+} sensitization of force by GTPγS, AlF_4^-, phenylephrine, and endothelin, but not the response to phorbol dibutyrate or a phosphatase inhibitor, tautomycin. Down-regulation also abolished the GTPγS-induced increase in myosin light chain phosphorylation at constant [Ca^{2+}] and was associated with extensive translocation of $p21^{rhoA}$ to the particulate fraction, prevented its immunoprecipitation, and inhibited its ADP ribosylation without affecting the immunodetectable content of G-proteins ($p21^{rhoA}$, $p21^{ras}$, $G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β) or protein kinase C (types α, β_1, β_2, δ, ϵ, η, θ, and ζ). We conclude that the loss of GTPγS- and agonist-induced Ca^{2+} sensitization through prolonged treatment with GTPγS is not due to a decrease in the total content of either trimeric ($G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β) or monomeric ($p21^{rhoA}$ and $p21^{ras}$) G-protein or protein kinase C but may be related to a structural change of $p21^{rhoA}$ and/or to down-regulation of its (yet to be identified) effector.

INTRODUCTION

Activation of G-protein-coupled excitatory receptors initiates at least two pathways of pharmacomechanical coupling (Somlyo and Somlyo, 1968) that can increase the force developed by smooth muscle independently of changes in membrane potential. The first, phosphatidylinositol hydrolysis, produces inositol 1,4,5-trisphosphate that releases Ca^{2+} from the sarcoplasmic reticulum; the ensuing activation of myosin light chain kinase by $[Ca^{2+}]_4$-calmodulin increases phosphorylation of the regulatory myosin light chain (MLC_{20})[1] and, consequently, initiates contraction (for review, Hartshorne, 1987; Somlyo and Somlyo, 1994). The second pathway increases MLC_{20} phosphorylation by inhibiting its dephosphorylation by smooth muscle myosin phosphatase (SMPP-1 M; Somlyo *et al.*, 1989; Kitazawa *et al.*, 1991a; Somlyo and Somlyo, 1994). SMPP-1 M is a trimeric enzyme associated with myosin filaments (Alessi *et al.*, 1992; Shirazi *et al.*, 1994; Shimizu *et al.*, 1994; Haystead *et al.*, 1995) and, therefore, its inhibition by a surface membrane-bound receptor–G-protein complex has to be signaled to the contractile apparatus by a second messenger(s). The G-proteins and effectors transmitting this signal have yet to be definitively identified, but possibilities suggested include protein kinase C (PKC) activated by trimeric G-proteins through diacylglycerols released by phospholipases (for review, Walsh *et al.*, 1994), a $p21^{rhoA}$ signaling cascade (Hirata *et al.*, 1992; Satoh *et al.*, 1993; Mukai *et al.*, 1994; Fujita *et al.*, 1995; Itagaki *et al.*, 1995; Leung *et al.*, 1995; Gong *et al.*, 1996; Kimura *et al.*, 1996; Matsui *et al.*, 1996), an unidentified phosphatase-associated kinase (Trinkle-Mulcahy *et al.*, 1995; Ichikawa *et al.*, 1996; Jensen *et al.*, 1996, Gailly, *et al.*, 1997) and/or arachidonic acid released by phospholipase A_2 and/or phospholipase D (Gong *et al.*, 1992, 1996). These possibilities, of course,

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[1] Abbreviations used: C_3, *Clostridium botulinum* exoenzyme C_3; GTPγS, guanosine 5′-[γ-thio]triphosphate; MLC_{20}, 20-kDa myosin light chain; PDBu, phorbol-12,13-dibutyrate; PIP_2, L-α-phosphatidylinositol 4,5-diphosphate; PKC, protein kinase C; SMPP-1 M, regulatory subunit of smooth muscle myosin phosphatase.

are not mutually exclusive but may constitute cross-talking and even redundant mechanisms.

The purpose of the present study was to determine the functional effects of chronic treatment of permeabilized smooth muscle with guanosine 5′-[γ-thio]-triphosphate (GTPγS) on Ca^{2+} sensitization and on the cellular content of G-proteins and PKCs implicated in the Ca^{2+}-sensitizing effect of agonists and GTPγS. Down-regulation of conventional and novel PKC isoforms by chronic exposure to phorbol esters does not affect GTPγS-induced Ca^{2+} sensitization (Hori *et al.*, 1993; Jensen *et al.*, 1996), indicating that these isoforms are not required for G-protein–coupled Ca^{2+} sensitization. We now show that, conversely, chronic treatment with GTPγS abolishes the Ca^{2+} sensitization of force and MLC_{20} phosphorylation by agonists, GTPγS and AlF_4^-, without affecting Ca^{2+} sensitization by phorbol ester; such down-regulation with GTPγS is associated with a change in the reactivity of $p21^{rhoA}$, without a detectable decrease in the content of trimeric ($G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β) or monomeric ($p21^{rhoA}$ and $p21^{ras}$) G-proteins implicated in G-protein-coupled Ca^{2+} sensitization.

MATERIALS AND METHODS

Isometric Tension Measurement and Down-Regulation Protocol

Portal veins were removed from 2- to 3-kg male rabbits, anesthetized with halothane, and killed by rapid exsanguination through the carotid artery, as approved by the Animal Research Committee of the University of Virginia (Charlottesville, VA). The adventitia was carefully removed and small strips (200-μm wide and 3-mm long) were dissected and stretched to 1.3 times resting length. Isometric tension was measured with a force transducer (AE801; SensoNor, Horten, Norway) in a well on a "bubble" plate at 22°C. After the steady responses to high $[K^+]$ were observed, the strips were incubated in normal relaxing solution (Ca^{2+}-free, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid) for about 5 min and permeabilized with 17.5 μg/ml α-toxin (List Biological Laboratories, Campbell, CA) for 60 min. To deplete the sarcoplasmic reticulum of calcium, all permeabilized strips were treated with A23187 (10 μM; Calbiochem, La Jolla, CA) for 10 min in relaxing solution (Kitazawa *et al.*, 1989; Kobayashi *et al.*, 1991). Strips permeabilized with α-toxin were incubated with pCa 6.7 (as control) or in the same solution containing GTPγS (50 μM, from 5 h to 16 h). After incubation, the strips were washed in Ca^{2+}-free solution and the responses to G-protein activators (GTPγS, AlF_4^-), agonists (phenylephrine, endothelin), a PKC activator [phorbol-12,13-dibutyrate (PDBu)], and a phosphatase inhibitor (tautomycin) were determined and compared with the responses of control strips. The pretreatment with A23187 and presence of 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid in the experimental solution assured that, as previously verified (Kitazawa *et al.*, 1989; Kobayashi *et al.*, 1989, 1991), the changes in force and MLC_{20} phosphorylation observed under these conditions were not due to changes in $[Ca^{2+}]$.

MLC_{20} Phosphorylation

Phosphorylation of MLC_{20} was measured with two-dimensional gel electrophoresis on minigels transferred to polyvinylidene difluoride (PVDF) membranes and stained with colloidal gold (Kitazawa *et al.*, 1991a).

Western Blots

Total tissue homogenates (including membrane and cytosol fraction) were obtained by homogenizing strips in a small glass homogenizer in sample buffer [62.5 mM Tris-HCl, pH 7.0, 1% SDS, 15% glycerol, 15 mM dithiothreitol (DTT)], and 0.004% bromphenol blue]. The homogenates were heated at 85°C for 5 min and centrifuged for 10 min at 14,000 rpm, and the supernatants were loaded for gel electrophoresis. To immunoblot $p21^{rhoA}$, $p21^{ras}$, $G_{\alpha i3}$, $G_{\alpha q/11}$, and G_β, we used 15% SDS-PAGE gels and the proteins were transferred to a PVDF membrane for 1 h at 100 V at 4°C. Homogenates for Western blots of PKC isoforms were electrophoresed on 10% SDS-PAGE gels and transferred overnight at 30 V at 4°C. Equal protein loading among samples was verified by Coomassie blue staining of the protein remaining in the gel after transfer. After transfer, the PVDF 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 primary antibody for 3 h and secondary antibody for 1 h at room temperature. Blots were detected with enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantitated by densitometry using a Bio-Rad GS-670 imaging densitometer. Optimal primary antibody concentration was determined by antibody titration (1:100, 1:500, 1:1000, and 1:5000 dilutions) using Mini-protein II multiscreen apparatus (Bio-Rad, Hercules, CA). Preliminary experiments established that the amount of protein loaded and the enhanced chemiluminescence developing condition were within the range of linearity of the assays. With defined exposure times, the density of the Western blot signals as a function of the amount of tissue loaded (one, two, or five strips) was linear ($r^2 \geq 0.998$).

Epitope, source, and dilution of antibodies used in the present study are listed in Table 1.

Separation of Particulate and Cytosolic Fractions

Treated strips were homogenized immediately in ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA, 250 mM sucrose, 1 mM DTT, 1 mM [4-(2-aminoethyl)benzenesulfonyyl fluoride], 20 μg/ml leupeptin, 20 μg/ml aprotinin), centrifuged at 100,000 × *g* for 30 min at 4°C (Beckman, Optima TLX Ultracentrifuge, TLA 120.1 rotor; Beckman, Fullerton, CA), and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in homogenization buffer containing 1% Triton X-100 and 1% sodium cholate, incubated for 1 h at 4°C to extract membrane proteins, and centrifuged at 800 × *g* for 10 min, and the supernatant was collected as the detergent-soluble particulate fraction and the pellet was collected as the detergent-insoluble particulate fraction. Proteins of cytosolic, detergent-soluble, and detergent-insoluble particulate fractions were separated by SDS-PAGE. A minimum of 10 small (200-μm wide and 3-mm long) rabbit portal vein strips were used to provide sufficient protein for reliable separation of cytosolic, detergent-soluble, and detergent-insoluble particulate fractions. Rapid termination of the reaction in homogenization buffer was indicated by the absence of translocation of $p21^{rhoA}$ or PKC isoforms when control strips were homogenized in homogenization buffer containing GTPγS (50 μM).

Immunoprecipitation of $p21^{rhoA}$

Treated samples were homogenized in ice-cold homogenization buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM AEBSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin). Membrane proteins were extracted in the above homogenization buffer on ice for 30 min and centrifuged at 300 × *g* for 5 min to remove nuclei and cell fragments. The supernatant was collected and used for immunoprecipitation. Samples were precleared with protein A (1 h, room temperature) to

Table 1. Epitope, source and dilution of antibodies used in the present study

Antibody	Epitope	Dilution	Supplier
$p21^{rhoA}$ (p)	119–132	1:5000	Santa Cruz
$p21^{rhoA}$ (m)	120–150	1:2500 for Western blot; 1:100 for immunoprecipitation	Santa Cruz
$p21^{ras}$ (m)	Entire 21 kDa	1:500	Transduction Laboratories, (Lexington, KY)
$G_{\alpha q/11}$ (p)	341–359	1:5000	Santa Cruz
$G_{\alpha i3}$ (p)	345–354	1:5000	Santa Cruz
G_{β} (p)	1–14	1:5000	Santa Cruz
Phosphatase (m)	M110	1:200,000	Gift from Dr. David J. Hartshorne (Tucson, AZ)
$PKC_{\beta 1}$ (p)	656–671	1:1000	Santa Cruz
$PKC_{\beta 2}$ (p)	657–673	1:1000	Santa Cruz
PKC_{η} (p)	669–683	1:100	Santa Cruz
PKC_{θ} (p)	656–671	1:100	Santa Cruz
PKC_{ε} (m)	1–175	1:500	Transduction Laboratories (Lexington, KY)
PKC_{α} (m)		1:5000	Gift from Dr. Peter Parker (Protein Phosphorylation, London, UK)

p, polyclonal; m, monoclonal.

prevent nonspecific binding of proteins and then incubated with anti-$p21^{rhoA}$ monoclonal antibody conjugated to agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and rotated overnight at 4°C. Immune complexes were centrifuged and the supernatants and pellets were collected and saved for analysis. The precipitates were washed and resuspended in Laemmli sample buffer.

ADP Ribosylation of $p21^{rhoA}$

The ADP ribosylation reaction was carried out in the whole tissue homogenate of control and GTPγS-down-regulated strips by incubation for 30 min at 30°C with a solution of 1 μg/ml *Clostridium botulinum* exoenzyme C_3 (C_3) transferase, 50 μCi/ml [^{32}P]NAD, 2 mM thymidine, 10 mM DTT, and 200 μM GTP in a total volume of 100 μl. The reaction was stopped by 24% trichloroacetic acid (250 μl) and 2% deoxycholate (6 μl), and the final volume was adjusted to 1 ml with water. After centrifugation (5000 × *g*, 10 min), the supernatant was carefully removed, and the pellet was resuspended in 20 μl of 2× sample buffer. Ten microliters of 1 M Tris base were added to neutralize the pH. Samples were heated at 85°C for 5 min, and the proteins were separated by SDS-PAGE. Autoradiographs and Western blots were obtained from the same PVDF membrane.

[^{32}P]GTP Overlay Assay

[^{32}P]GTP overlay assay was carried out according to McGrath *et al.* (1984). Proteins of control and GTPγS-down-regulated tissue were separated in a large (14 cm × 24 cm) 15% polyacrylamide gel to achieve better resolution (range, 20–30 kDa), and the gel area of interest was excised and transferred to PVDF membrane. The PVDF membrane was rinsed for two 5-min periods in Tris-buffered saline (containing 0.05% Tween 20). Nonspecific nucleotide binding was blocked by incubating the membrane in ATP overlay buffer (50 mM Tris-HCl, pH 7.6, 10 μM $MgCl_2$, 0.3% Tween 20, 100 mM DTT, 100 μM ATP) for 10 min. [^{32}P]GTP (10 mCi/ml stock; 2 μCi/ml, final concentration) was added to ATP overlay buffer and incubated for 60 min. After extensive washing, the PVDF membrane was dried, and an autoradiograph was obtained.

Details of the solutions used on permeabilized strips were as described (Kitazawa *et al.*, 1989; Kobayashi *et al.*, 1989, 1991). α-Toxin was purchased from List Biological Laboratories, GTPγS was obtained from Boehringer Mannheim (Indianapolis, IN). PDBu, ADP-ribosyltransferase C3, tautomycin, and A23187 were purchased from Calbiochem; [^{32}P]NAD (30 Ci/mmol) and [^{32}P]GTP (10 mCi/mmol) were obtained from Dupont NEN (Boston, MA); and PIP_2 (L-α-phosphatidylinositol 4,5-diphosphate) was purchased from Sigma (St. Louis, MO).

Statistics

Statistical comparisons were made with Student's *t* test, unless noted otherwise.

RESULTS

Chronic (5–16 h) Treatment with GTPγS (50 μM) of α-Toxin-permeabilized Rabbit Portal Vein Abolishes Ca^{2+} Sensitization Induced by Agonists (Phenylephrine and Endothelin) and G-Protein Activators (GTPγS and AlF_4^-)

During prolonged (≥5 h) exposure to GTPγS (50 μM), the large initial contraction induced by GTPγS at constant [Ca^{2+}] gradually relaxed to baseline. Addition of fresh GTPγS to these strips induced no significant contraction (0.4 ± 0.41% of the maximal Ca^{2+}-induced contraction, n = 10) compared with 39 ± 3.8% (n = 5, $p < 0.001$) in paired controls incubated with pCa 6.7 for the same length of time in the absence of GTPγS (Figures 1 and 2). The Ca^{2+} sensitizing effect of AlF_4^- (Kawase and van Breemen, 1992; Gong *et al.*, 1996) was also significantly inhibited by prolonged treatment with GTPγS,

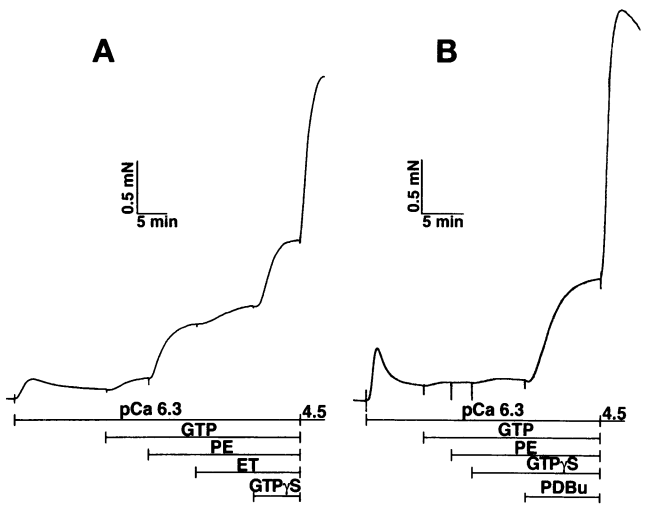

Figure 1. Inhibition of contractile responses at constant $[Ca^{2+}]$ to α-adrenergic agonist (100 μM phenylephrine plus 10 μM GTP, PE + GTP) and GTPγS (50 μM) but not to PDBu (1 μM), by chronic (6-h) treatment with GTPγS (50 μM) in α-toxin-permeabilized rabbit portal vein. Force traces from timed control (A) and chronic GTPγS (50 μM, 6 h)-treated (B) strips are shown. After washing, a submaximal response to pCa 6.3 was obtained, and the response to freshly added agonists (GTP + PE + Endothelin ET in control and GTP + PE in GTPγS-treated strips) and GTPγS (50 μM) were determined. Note that the contractile Ca^{2+}-sensitizing response to PE + GTP and GTPγS is abolished, whereas the response to PDBu is preserved after prolonged treatment with GTPγS.

from control 40 ± 3.1% (n = 4) to 4.3 ± 1.6% (n = 4, $p < 0.001$) (Figures 1 and 2).

The Ca^{2+} sensitization of force by agonists was also abolished by prolonged GTPγS treatment. No

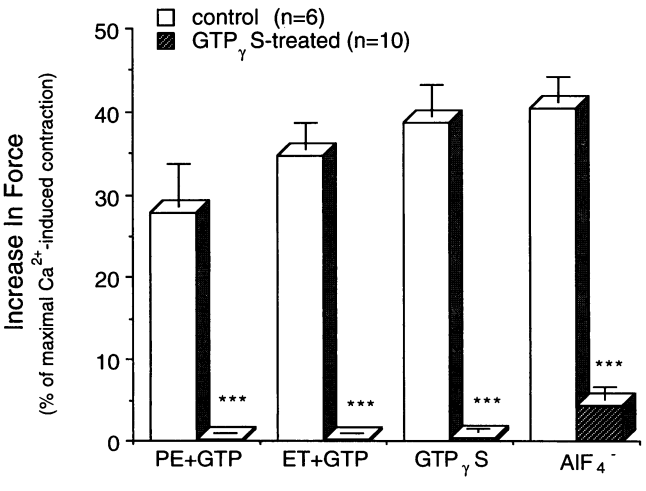

Figure 2. Abolition of agonist (100 μM phenylephrine or 100 nM endothelin) and G-protein activator (50 μM GTPγS and AlF_4^-)-induced Ca^{2+} sensitization of force by chronic incubation (6 h) of α-toxin-permeabilized rabbit portal vein with GTPγS (50 μM). The protocol is similar to the protocol in Figure 1. ***, $p < 0.001$

contraction was induced by a combination of phenylephrine and GTP (0%, n = 10) or of endothelin and GTP (0%, n = 10) in GTPγS-treated strips, whereas in control strips a combination of phenylephrine and GTP caused 28 ± 5.4% (n = 6, $p < 0.001$) and of endothelin and GTP caused 35 ± 3.3% (n = 4, $p < 0.001$) of the maximal Ca^{2+}-induced contraction (Figures 1 and 2).

Maximal Ca^{2+}-induced contractions (about 150% of the initial high K^+-induced contraction in intact strips) were comparable after prolonged incubation in the presence or in the absence of GTPγS.

Prolonged Treatment with GTPγS (50 μM) Abolishes GTPγS-induced Increase in MLC_{20} Phosphorylation

Acute exposure to GTPγS (50 μM, 15 min) at pCa 6.3 increased MLC_{20} phosphorylation to 47 ± 1.7% (n = 4, $p < 0.01$) from control 33 ± 1.7% (n = 4, in pCa 6.3) in time-matched strips (incubated in pCa 6.7 without GTPγS for 15 h), whereas in strips chronically treated with GTPγS, there was no significant increase in MLC_{20} phosphorylation: 30 ± 2.5% (n = 4, $p > 0.5$) compared with 26 ± 2.3% (n = 4).

Prolonged Treatment with GTPγS Has No Significant Effect on Contraction Induced by Either Phorbol Ester (PDBu) or the Phosphatase Inhibitor Tautomycin

PKC has been implicated in GTPγS-induced Ca^{2+} sensitization in part because phorbol esters (activators of PKC, e.g., PDBu) induce Ca^{2+} sensitization in smooth muscle (Chatterjee and Tejada, 1986; Jensen *et al.*, 1996, and references therein; for review, Walsh *et al.*, 1994). The Ca^{2+} sensitizing effect of PDBu was not inhibited by chronic (5–6.5 h) exposure of smooth muscle to GTPγS (50 μM): the contraction induced by PDBu (1 μM) at pCa 6.3 was 26 ± 2.1% (n = 4) in control and 21 ± 3.0% (n = 10, $p > 0.05$, Figures 1 and 3) in GTPγS-treated strips.

Tautomycin, a phosphatase inhibitor, causes smooth muscle contraction through direct inhibition of MLC_{20} phosphatase, thus permitting unopposed MLC_{20} phosphorylation by a myosin light chain kinase (Gong *et al.*, 1992). The contraction induced by tautomycin (1 μM) at pCa 6.3 was not significantly different in control (86 ± 6.3%, n = 3) compared with chronically GTPγS-treated smooth muscle (88 ± 3.7%, n = 3; $p > 0.05$).

pCa Tension Curve Is Not Shifted by Chronic Treatment with GTPγS: Ca^{2+} Sensitivity Is Similar to that of Controls

To determine whether the loss of G-protein–coupled Ca^{2+} sensitization after long-time incubation with GTPγS was due to a residual increase in Ca^{2+} sensi-

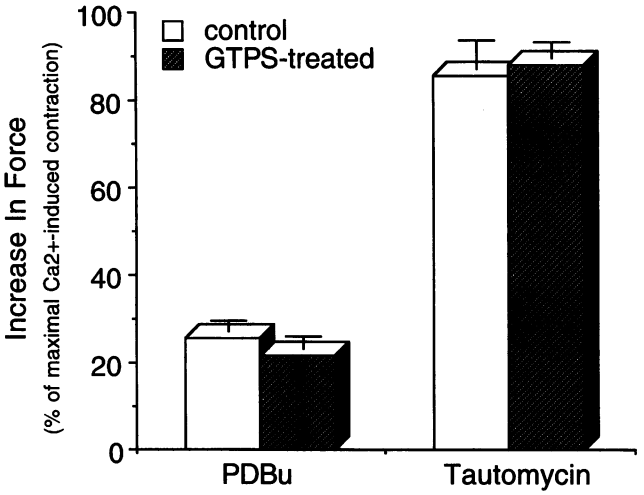

Figure 3. Absence of significant effect on PDBu- (1 μM) and tautomycin- (1 μM) induced contraction by chronic incubation with GTPγS (50 μM, 6 h) in α-toxin-permeabilized rabbit portal vein. The same protocol as in Figure 1 was used. ***, $p < 0.001$

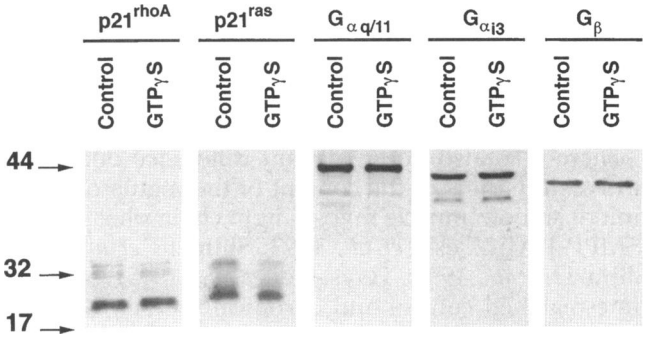

Figure 4. Western blots of $p21^{rhoA}$, $p21^{ras}$, $G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β in homogenates of timed control and chronically GTPγS-treated portal vein smooth muscle. Western blots were obtained as described in MATERIALS AND METHODS and are representative of three to six experiments.

tivity resulting from chronic exposure, pCa tension curves were compared between control and chronically (15 h) GTPγS-treated strips. There was no significant difference between control (EC_{50} = pCa 5.97 ± 0.02, n = 4) and chronically GTPγS-treated (EC_{50} = pCa 6.01 ± 0.004, n = 3) strips, although the Ca^{2+} sensitivity of both control and GTPγS-treated strips was slightly shifted to the right after prolonged (15-h) incubation compared with immediately after permeabilization (EC_{50} = pCa 6.24 ± 0.117, n = 6).

There Are No Immunodetectable Decreases in G-Proteins ($p21^{rhoA}$, $p21^{ras}$, $G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β) or PKC (α, β_1, β_2, δ, ϵ, η, θ, and ζ) in GTPγS (50 μM, up to 15 h)-Treated Strips

We estimated G-protein levels with Western blots to determine whether the abolition of GTPγS-induced sensitization of MLC_{20} phosphorylation and force by the prolonged treatment with GTPγS could be correlated with a decrease in the level of a specific G-protein. The levels of $p21^{rhoA}$, $p21^{ras}$, $G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β were determined because of their potential importance in Ca^{2+} sensitization in smooth muscle (for review, Somlyo and Somlyo, 1994; Gong *et al.*, 1996). The immunodetectable levels of $p21^{rhoA}$, $p21^{ras}$, $G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β in chronically GTPγS-treated tissue were, respectively, 102 ± 5.4% (n = 6, $p > 0.05$), 134 ± 31.9% (n = 4, $p > 0.05$), 94% ± 3.1% (n = 4, $p > 0.05$), 98% ± 9.5% (n = 3, $p > 0.05$), and 99 ± 13.4% (n = 3, $p > 0.05$) of controls. Figure 4 shows Western blots representative of three to six experiments.

To rule in or out the possibility that only a specific (e.g., membrane) pool of G-protein(s) was decreased by chronic GTPγS treatment, the detergent-soluble and detergent-insoluble particulate fractions and the cytosolic fractions were separated and their G-protein contents were determined. Most of the $p21^{ras}$, $G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β was detected in the detergent-soluble particulate fraction, and there was no significant difference in the G-protein content of either the cytosolic or the detergent-soluble particulate fractions of control and chronically GTPγS-treated strips. The distribution of $p21^{rhoA}$ is described below.

The levels of Gαs and Gαq can be reduced by prolonged treatment of cultured cells, including vascular smooth muscle (Kai *et al.*, 1996), with agonists (for review, Milligan *et al.*, 1995). Therefore, we wished to determine whether the absence of down-regulation of $G_{\alpha q/11}$ content by GTPγS in our study was due to a requirement for concurrent activation of receptors and G-proteins (Milligan *et al.*, 1995). However, prolonged (20-h) treatment of intact rabbit portal vein strips (not α-toxin permeabilized) with multiple agonists (100 μM phenylephrine and 100 nM endothelin) did not cause a significant decrease in G-protein ($p21^{rhoA}$, $p21^{ras}$, $G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β) levels, although it did inhibit subsequent responses to agonists (our unpublished results).

Exogenous PIP_2 (50 μM, dissolved in dimethyl sulfoxide), added to determine whether the down-regulation of G-protein-induced sensitization of force and MLC_{20} phosphorylation was due to depletion of endogenous substrate PIP_2 as the result of prolonged activation of phospholipase C, did not induce contraction (n = 3) nor did it rescue GTPγS-induced Ca^{2+} sensitization (our unpublished results).

PKC has been suggested to be involved in Ca^{2+} sensitization in smooth muscle, and the PKC α, β_1, β_2, θ, and ϵ isoforms are down-regulated by chronic activation with phorbol esters or bryostatin (Jensen *et al.*, 1996). Therefore, we determined whether down-regulation of any of the PKC isoforms could account for the abolition of agonist- and GTPγS-induced Ca^{2+} sensi-

tization by chronic GTPγS treatment. However, Western blots of PKC isoforms (α, β_1, β_2, δ, ε, η, θ, and ζ) showed no significant difference in the PKC contents of, respectively, timed controls and GTPγS-treated tissue (three to five experiments).

There was also no significant difference detectable on Western blots of the content of the regulatory subunit of smooth muscle myosin light chain phosphatase (SMPP-1 M; Alessi *et al.*, 1992; Shimizu *et al.*, 1994; Shirazi *et al.*, 1994; Haystead *et al.*, 1995) between time-matched control and chronically GTPγS-treated tissue (our unpublished results).

p21^{rhoA} Remains in the Detergent-Soluble Particulate Fraction and Is No Longer Immunoprecipitable, and Its ADP Ribosylation by C_3 Is Decreased in Chronically GTPγS-treated Smooth Muscle

Because prolonged treatment with GTPγS did not decrease the total cellular content of p21^{rhoA}, a monomeric GTP-binding protein that may play an important role in Ca^{2+} sensitization in smooth muscle (Hirata *et al.*, 1992; Satoh *et al.*, 1993; Fujita *et al.*, 1995; Itagaki *et al.*, 1995; Gong *et al.*, 1996), we examined some of its other properties that may have been affected so as to abolish its Ca^{2+}-sensitizing effects.

p21^{rhoA} translocates from the cytosol to the particulate fraction of smooth muscle during GTPγS-induced sensitization of force and MLC_{20} phosphorylation (Gong, Fujihara, Somlyo, and Somlyo, unpublished results). After treatment for 15 h with GTPγS (50 μM), when the GTPγS-induced sensitization of force and MLC_{20} phosphorylation was totally down-regulated, p21^{rhoA} remained in the particulate fraction. Approximately 57 ± 14.7% (n = 4, $p < 0.001$) of total p21^{rhoA} was detected in the particulate fraction compared with only 8 ± 1.5% (n = 19) in controls (Figure 5).

In the absence of chronic GTPγS treatment, ADP ribosylation of p21^{rhoA} is inhibited when it is activated and translocated to the particulate fraction by acute GTPγS treatment (Gong, Fujihara, Somlyo, and Somlyo, unpublished results). ADP-ribosylation of p21^{rho} by C_3 (carried out in tissue homogenate; see MATERIALS AND METHODS) was also reduced in chronically GTPγS-down-regulated strips (Figure 6) to 51 ± 8.3% (n = 8, $p < 0.05$ using the Wilcoxon test) of timed controls, with the autoradiographic signals of ADP ribosylation of p21^{rho} normalized to their respective densities on Western blots.

A monoclonal antibody generated against p21^{rhoA} immunoprecipitated p21^{rhoA} from resting α-toxin-permeabilized rabbit portal vein tissue. When α-toxin-permeabilized strips were incubated with GTPγS (50 μM, 15 h), p21^{rhoA} was no longer immunoprecipitable by the above antibody (Figure 7A). In contrast, chronic treatment with GTPγS had no effect on the immunoprecipitability of $G_{\alpha/q11}$ (Figure 7B).

GTP binding by low molecular weight G-proteins in chronically GTPγS-treated tissue was determined by

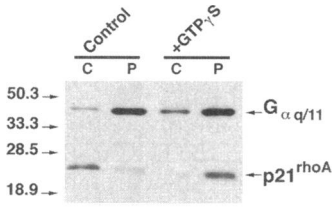

Figure 5. p21^{rhoA} is largely in the particulate fraction in chronically GTPγS-treated (50 μM, 15 h) rabbit portal vein. α-Toxin-permeabilized rabbit portal vein strips were incubated in pCa 6.7 (as control) or plus GTPγS (50 μM) for 15 h, homogenized, and fractionated as described in MATERIALS AND METHODS. C, cytosol; p, pellet. Data are representative of four experiments. $G_{\alpha q/11}$ showed no significant translocation after a 15-h treatment with GTPγS.

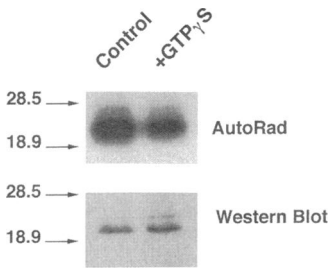

Figure 6. Decreased ADP ribosylation of p21^{rhoA} in GTPγS-treated (50 μM, 15 h) rabbit portal vein. Whole homogenates of time-matched control and chronic GTPγS-treated rabbit portal vein (three strips per group) were incubated for 30 min at 30°C in the presence of 1 μg/ml C_3, 10 mM DTT, 2 mM thymidine, 200 μM GTP, and 50 μCi/ml [^{32}P]NAD. The reaction was stopped with trichloroacetic acid and proteins were separated by SDS-PAGE. The autoradiograph and Western blot were obtained from the same PVDF membrane. This blot is representative of eight experiments.

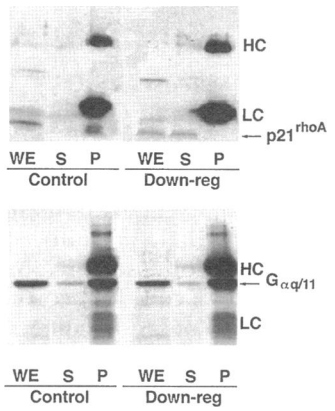

Figure 7. Chronic incubation (15 h) of α-toxin-permeabilized rabbit portal vein with GTPγS (50 μM) interferes with the immunoprecipitation of p21^{rhoA} but not $G_{\alpha/q11}$. Samples were immunoprecipitated with either anti-p21^{rhoA} (1:100 dilution, A) or $G_{\alpha/q11}$ (1:100 dilution, B) overnight at 4°C, electrophoresed, and transferred to PVDF membranes. Membranes were incubated with anti-p21^{rhoA} (A) or $G_{\alpha/q11}$ (B) and visualized with enhanced chemiluminescence. WE, whole extract (before immunoprecipitation); S, supernatant (after immunoprecipitation); P, precipitate. Note: Since the same species antibodies were used for immunoblotting and for immunoprecipitation (RhoA, monoclonal, $G_{\alpha/q11}$, polyclonal), the IgGs of immunoprecipitating antibodies were recognized by the blotting antibodies. That is, the mouse IgGs of the monoclonal immunoprecipitating RhoA antibody were recognized by the secondary anti-mouse antibody used in conjunction with the monoclonal anti-RhoA blotting antibody. Similarly, the rabbit IgGs of the polyclonal immunoprecipitating $G_{\alpha/q11}$ antibody were recognized by the secondary anti-rabbit antibody used in conjunction with the polyclonal anti-$G_{\alpha/q11}$ blotting antibody.

[^{32}P]GTP overlay assay. Three major bands between molecular masses 20 and 30 kDa were present in autoradiographs, with the middle band identified as p21^{rhoA} with a Western blot. There was no significant difference in autoradiographically detectable GTP binding in any of these three bands between time-matched control and chronically GTPγS-treated tissues (n = 3).

DISCUSSION

Our results show that the receptor-activated G-protein-coupled mechanism that Ca^{2+} sensitizes MLC_{20} phosphorylation and contraction (Somlyo *et al.*, 1989; Kitazawa *et al.* 1991b) can be down-regulated at the postreceptor level by prolonged exposure to GTPγS. Such down-regulation eliminates the acute Ca^{2+}-sensitizing effect of not only GTPγS but also AlF_4^-, an α-adrenergic agonist (phenylephrine), and endothelin, indicating that these excitatory agents utilize the same postreceptor pathway(s). The persistence of the Ca^{2+} sensitizing effect of GTPγS in preparations down-regulated with PDBu already indicated that the Ca^{2+} sensitization by GTPγS does not require conventional or novel PKCs (Hori *et al.*, 1993; Jensen *et al.*, 1996). Accordingly, unlike the marked reduction in PKCs induced by phorbol esters (Hori *et al.*, 1993; Jensen *et al.*, 1996), down-regulation with GTPγS caused no loss of immunodetectable PKCs or inhibition of PDBu-induced Ca^{2+} sensitization (present study). These and earlier results are consistent with the conclusion that Ca^{2+} sensitization by the two kinds of agents (phorbol esters and G-protein activators) proceeds through relatively separate upstream pathways with conventional and novel PKCs playing only a minor role in G-protein-coupled Ca^{2+} sensitization (Hori *et al.*, 1993; Jensen *et al.*, 1996; present study).

The abolition of the Ca^{2+}-sensitizing effect of GTPγS on MLC_{20} phosphorylation and force by chronic exposure to GTPγS was not associated with any detectable decrease in either trimeric ($G_{\alpha q/11}$, $G_{\alpha i3}$, and G_β) or monomeric (p21^{ras} and p21^{rhoA}) G-proteins. Furthermore, even extensive stimulation of two highly effective G-protein-coupled receptors (α-adrenergic and endothelin) failed to cause detectable down-regulation of either class of G-proteins, although in cultured cells down-regulation of trimeric G-proteins has been observed and related to stimulation and down-regulation of receptors (Milligan *et al.*, 1995). The differences between these and our results may be due to more rapid turnover of G-proteins in cultured cells. In any event, the abolition of the Ca^{2+}-sensitizing effect of GTPγS by chronic exposure to this nucleotide cannot be ascribed to a detectable loss of the G-proteins or to depletion of PIP_2, because the response to GTPγS could not be restored by exogenous PIP_2 (present study) and the depletion of PIP_2 is transient (Kai *et al.*, 1996). Similarly, down-regulation of the G-protein-coupled response was not due to loss of smooth muscle myosin light chain phosphatase, because the effects of PDBu and of the conventional phosphatase inhibitor (tautomycin) persisted (Figure 3) and levels of SMPP-1 M were not decreased in the GTPγS-down-regulated preparations.

Some of the properties of p21^{rhoA}, a ras family monomeric G-protein implicated in Ca^{2+} sensitization of smooth muscle (Hirata *et al.*, 1992; Fujita *et al.*, 1995; Itagaki *et al.*, 1995; Gong *et al.*, 1996), were affected by down-regulation by GTPγS and may be related to the loss of its Ca^{2+}-sensitizing effect. The disappearance of the normally present immunoprecipitability of p21^{rhoA} in the preparations chronically treated with GTPγS (Figure 7) may indicate a conformational change or association with a protein affecting residues 119–132 (the parent epitope) that prevents p21^{rhoA} from interacting productively with its effector(s). The reduced availability of p21^{rhoA} for C_3-catalyzed ADP ribosylation of Asn^{41} in down-regulated tissue is also compatible with such modification. Acute exposure of rabbit portal vein to GTPγS induced within 1 min significant translocation of p21^{rhoA} to the membrane; within 20 min (the only time point tested) translocated protein also exhibited reduced immunoprecipitability and availability for ADP ribosylation (our unpublished observations). However, the time course of down-regulation of G-protein-mediated Ca^{2+} sensitization cannot be determined from the observations alone, because it can be affected by recruitment of available cytosolic p21^{rhoA} from its *rho*GDI complex and by the, yet unknown, inactivation kinetics of the relevant downstream effectors. As an hypothesis, we suggest that, in analogy with certain PKC isoforms, both activation and subsequent down-regulation of the *rhoA* pathway progress through translocation of p21^{rhoA} to the membrane. However, we cannot exclude the possibility that the functional down-regulation occurs downstream, within the effector pathway between the G-protein and myosin phosphatase. In either case, we conclude that G-protein–coupled Ca^{2+} sensitization can be down-regulated independently of the phorbol ester-induced process and that down-regulation by GTPγS can take place directly at the level of the G-protein or downstream and need not involve receptor activation.

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