Translocation of rhoA Associated with Ca\(^{2+}\) Sensitization of Smooth Muscle*

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We determined the relationship between the localization of rhoA and Ca\(^{2+}\) sensitization of force in smooth muscle. In \(\alpha\)-toxin-permeabilized rabbit portal vein at \(pCa\) 6.5, the particulate hydrophobic fraction of rhoA (10 ± 1.6% of the total) was significantly increased by phenylephrine to 18 ± 5.5% at 5 min, by AlF\(_4\)\(^–\) to 26 ± 8.4% at 20 min, and dose-dependently up to 62 ± 9.5% by guanosine 5′-O-(3-thiotriphosphate) (GTP\(_\gamma\)S; 0.3–50 μM). Translocation of rhoA was selective (Rac1 and Cdc42 were not translocated) and was quantitatively correlated (up to −50%; \(r = 0.91, p < 0.05\)) with Ca\(^{2+}\) sensitization; high GTP\(_\gamma\)S concentrations (10 μM) further increased translocation without increasing force. The initial recruitment of rhoA to the membrane paralleled the time course of contraction, but sensitization could be reversed without a decrease in particulate rhoA. High [Ca\(^{2+}\)]\(_i\) (pCa 4.5) also increased particulate rhoA to 31 ± 5.8%. Membrane-associated rhoA in unstimulated portal vein was a good substrate for in vitro ADP-riboylation, whereas the large amount translocated by GTP\(_\gamma\)S was not. We conclude that 1) translocation of rhoA plays a causal role in Ca\(^{2+}\) sensitization, and 2) membrane-bound rhoA can exist in two or more states.

**Materials and Methods**

Isometric Tension Measurement—Small strips (200 μm wide and 3 mm long) of rabbit portal vein and ileum longitudinal smooth muscle were dissected, and isometric tension was measured as published (15–17). Separation of Particulate and Cytosolic Fractions—A minimum of 10 small (200 μm wide and 3 mm long) strips of rabbit portal vein or ileum longitudinal smooth muscle were used to provide sufficient protein for reliable separation of cytosolic and particulate fractions. Stimulated and control 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 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin) and centrifuged at 100,000 \(\times\) g for 30 min at 4 °C (Optima™ TLX ultracentrifuge, TLA 120.1 rotor, Beckman Instruments), 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 or only 2% Triton X-114. The latter buffer was used to avoid the increase in the cloudy point of Triton X-114 by a second detergent. The extract was centrifuged at 800 \(\times\) g for 10 min. The supernatant was collected and is referred to as the particulate fraction, and the pellet was collected and is referred to as the detergent-insoluble particulate fraction. Cytosolic, particulate, and detergent-insoluble particulate fraction proteins were separated by SDS-polyacrylamide gel electrophoresis. Only the cytosolic and particulate p21\(^{\text{rhoA}}\) proteins are shown in most of the figures, as no immunoblot-detectable p21\(^{\text{rhoA}}\) was found in the detergent-insoluble particulate fraction. The absence of p21\(^{\text{rhoA}}\) in the detergent-insoluble particulate fraction verified the completion of the extraction of membrane p21\(^{\text{rhoA}}\) proteins and completion of homogenization. Prompt termination of the reaction in homogenization buffer was verified by the absence of translocation of p21\(^{\text{rhoA}}\) when control strips were homogenized in GTP\(_\gamma\)S (50 μM)-containing homogenization buffer.

Phase Separation by Triton X-114—Precondensed Triton X-114 stock solution was added to tissue homogenates or cytosolic fractions to a final concentration of ~2%, and proteins were extracted by incubation for 30 min on ice with occasional mixing (18). The mixture was centrifuged at 10,000 \(\times\) g for 10 min at 4 °C; the pellet was solubilized in sample buffer; and proteins were separated by SDS-polyacrylamide gel electrophoresis to determine cellular proteins insoluble in nonionic detergent. The supernatant was collected in a fresh tube and warmed to 37 °C in a water bath until the solution became cloudy (for ~5 min). Phase separation was achieved by centrifuging the solution in a tabletop centrifuge for 10 min at 800 \(\times\) g at room temperature. The upper aqueous phase contains soluble proteins, and the lower, detergent-enriched phase contains proteins bearing hydrophobic domains.

Western Blots—After transfer to polyvinylidene difluoride membrane, the membranes were blocked with 5% nonfat 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 (ECL, Amersham Corp.) 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) using a Mini-protein II multiscanner apparatus (Bio-Rad). Preliminary experiments established that the amount of protein loaded was within the range of linearity of the assays. The percent of particulate p21\textsuperscript{rhoA} was calculated according to particulate p21\textsuperscript{rhoA})(particulate + cytosolic p21\textsuperscript{rhoA}).

Monoclonal anti-p21\textsuperscript{rhoA} antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) generated against amino acids 120–150 of human p21\textsuperscript{rhoA} was used at a 1:2500 dilution. Polyclonal anti-G\textsubscript{alpha}q, anti-Rac1, and anti-Cdc42 antibodies (Santa Cruz Biotechnology, Inc.) generated against amino acids 341–359 common to G\textsubscript{alpha}q and G\textsubscript{alpha}11, amino acids 178–191 of Rac1, and amino acids 166–182 of Cdc42 were used at 1:5000, 1:500, and 1:1000 dilutions, respectively.

ADP-ribosylation of Cytosolic and Particulate p21\textsuperscript{rhoA}—The detergent concentration and the volumes of cytosolic and particulate fractions were adjusted to identical values, and the following reagents were added: 200 \mu M GTP, 10 mM dithiothreitol, 2 mM thymidine, and 1 \mu g/ml Clostridium botulinum exoenzyme C3. After initiation of ADP-ribosylation by addition of [\textsuperscript{32}P]NAD (final concentration of 50 \mu Ci/ml), the mixture (total volume of 100 \mu l) was incubated for 30 min at 30 °C. The reaction was stopped by trichloroacetic acid (24%, 250 \mu l) and deoxycholate (2%, 6 \mu l), and the final volume was adjusted to 1 ml with water. After centrifugation (5000 \times g, 10 min), the supernatant was carefully removed, and the pellet was resuspended in 20 \mu l of 2 x sample buffer. 10 \mu l of 1 x Tris base was added to neutralize the pH. Samples were heated at 85 °C for 5 min, and the proteins were separated by SDS-polyacrylamide gel electrophoresis. ADP-ribosylation of p21\textsuperscript{rhoA} in \beta-esscin-permeabilized strips was carried out as described previously (11).

Details of the solutions used for study of permeabilized strips were described previously (15–17). The pretreatment with A23187 and the presence of 10 mM EGTA assured (15–17) that the changes in force and MLC\textsubscript{20} phosphorylation observed under these conditions were not due to changes in [Ca\textsuperscript{2+}]. \alpha-Toxin was purchased from List Biological Laboratories Inc. (Campbell, CA). GTP\textsubscript{S} was from Boehringer (Mannheim, Germany). ADP-ribosyltransferase C3, taumycin, and A23187 were from Calbiochem. [\textsuperscript{32}P]NAD (30 Ci/mmole) was from DuPont NEN. Statistical comparisons were made using Student’s t test; all values are given as mean ± S.E.

RESULTS

\textit{p21\textsuperscript{rhoA} Is Translocated by Agonists, GTP\textsubscript{S}, and Aluminum Fluoride from the Cytosol to the Particulate Fraction Concomitantly with Ca\textsuperscript{2+} Sensitization of Rabbit Portal Vein Smooth Muscle—Phenylephrine (PE; 100 \mu M) plus GTP (10 \mu M) increased force from 13 ± 1.1% (n = 18) to 41 ± 5.4% (n = 6, p < 0.001) of the maximal Ca\textsuperscript{2+}-induced contraction in \alpha-toxin-permeabilized rabbit portal vein strips at constant free Ca\textsuperscript{2+} (pCa 6.5). Such contractions are the result of increased MLC\textsubscript{20} phosphorylation (2, 4). Force development was accompanied by translocation of p21\textsuperscript{rhoA} induced by GTP\textsubscript{S} (0.3–50 \mu M). A linear fit of the data gives the correlation coefficient r = 0.9995 (p < 0.001).

FIG. 1. Translocation of p21\textsuperscript{rhoA} correlated with Ca\textsuperscript{2+} sensitization of force by PE, AlF\textsubscript{4}–, and GTP\textsubscript{S}. \alpha-Toxin-permeabilized rabbit portal vein strips were incubated with pCa 6.5 solution (control) or were stimulated with PE (100 \mu M) plus GTP (10 \mu M) for 5 min, with AlF\textsubscript{4}– (10 mM NaF + 30 \mu M AlCl\textsubscript{3}) for 20 min, or with various concentrations of GTP\textsubscript{S}. Stimulated and control tissues were homogenized in ice-cold homogenization buffer and fractionated by centrifugation at 100,000 \times g, and p21\textsuperscript{rhoA} in the particulate fraction was quantitated on Western blots (see "Materials and Methods"). Force is normalized to the maximal Ca\textsuperscript{2+}-induced contraction (100%). In A, the solid line represents an exponential fit to the data with the correlation coefficient r = 0.91 (p < 0.05, n = 4–23). ●, control; ○, 0.3 \mu M GTP\textsubscript{S}; □, PE + GTP; □, 1 \mu M GTP\textsubscript{S} ×, AlF\textsubscript{4}–; ▲, 10 \mu M GTP\textsubscript{S}; ●, 50 \mu M GTP\textsubscript{S}. In B is shown the dose-dependent p21\textsuperscript{rhoA} translocation induced by GTP\textsubscript{S} (0.3–50 \mu M). A linear fit of the data gives the correlation coefficient r = 0.9995 (p < 0.001).
Selective Translocation of p21rhoA, but not Rac1 and Cdc42, induced by GTPyS (50 μM, 20 min). Results are representative of five to six experiments showing that p21rhoA, but not Rac1 and Cdc42, is translocated from the cytosol (C) to the particulate (P) fraction; note that only Rac1 is present in the detergent-insoluble (DI) particulate fraction.

From 13 ± 1.1% (n = 18) to 53 ± 3.1% (n = 4) of the maximal Ca2+-induced contraction and particulate p21rhoA from the control value of 10 ± 1.6% (n = 23) to 26 ± 8.4% (n = 4, p < 0.01) (Fig. 1A).

As shown in Fig. 1A, the translocation of p21rhoA up to 50%, was quantitatively correlated (r = 0.91, p < 0.05) with Ca2+-sensitization of force induced by agonist (PE plus GTP), AlF4−, and various concentrations of GTPyS. Higher concentrations (≥10 μM) of GTPyS caused further translocation of p21rhoA without further increase in force, indicating a “ceiling effect.”

To ascertain whether the observed translocation is α-adrenergic receptor-specific, we also determined the effect of a muscarinic agonist on the localization of p21rhoA in permeabilized ileum smooth muscles. Surprisingly, a high percentage (61 ± 6.8%, n = 14) of p21rhoA was located in the particulate fraction of unstimulated ileum. This high basal level of particulate p21rhoA was not due to Ca2+ (submaximal, pCa 6.5) because 59 ± 1.4% (n = 2) of p21rhoA was particulate even at cytoplasmic [Ca2+] < pCa 8 (no free Ca2+ added, 10 mM EGTA present). In contrast, inclusion of the muscarinic antagonist atropine (10 μM) during and following dissociation decreased particulate p21rhoA to 28 ± 11.6% (n = 6, p < 0.05). This significant decrease in particulate p21rhoA by a highly specific muscarinic antagonist indicates that acetylcholine released from nerve endings in the richly innervated ileum causes translocation of p21rhoA to the particulate fraction and that such translocation is not limited to the action of α-adrenergic agonists.

Selective Translocation of p21rhoA, but Not Rac1 and Cdc42, by GTPyS—To determine the specificity of translocation of p21rhoA by GTPyS, we also determined the distribution of two other Rho family proteins, Rac1 and Cdc42, under conditions identical to those used for determining the partitioning of p21rhoA. GTPyS (50 μM, 20 min) (Fig. 2) had no significant effect on the amount of either Rac1 or Cdc42 present in the particulate fraction: 46 ± 2.9% (n = 5) of Rac1 and 14 ± 4.1% (n = 5) of Cdc42 in pCa 6.5 solution and 47 ± 4.2% (n = 6, p > 0.005) of Rac1 and 19 ± 4.8% (n = 6, p > 0.05) of Cdc42 after stimulation with GTPyS. Unlike p21rhoA and Cdc42, which were not detected in the detergent-insoluble particulate fraction, 23 ± 7.0% (n = 5) of Rac1 was in the detergent-insoluble particulate fraction, and this fraction was also not changed by GTPyS (25 ± 5.7%, n = 6, p > 0.05).

Relaxation of Phenylephrine-induced Ca2+ Sensitization Is Unaccompanied by the Return of p21rhoA from the Particulate to the Cytosolic Fraction—To determine whether the return of p21rhoA from the particulate to the cytosolic fraction is required for the reversal of Ca2+ sensitization, the distribution of p21rhoA after “washout” of the agonist was determined. After increasing the steady-state pCa 6.5-induced contraction by PE plus GTP from 7 ± 2.2% (n = 3) to 32 ± 1.2% (n = 3, p < 0.001), the muscles were transferred into relaxing solution (no added Ca2+, PE, or GTP and containing 1 mM EGTA) and exchanged three times for a total of 25 min. At this time, the pCa 6.5-induced contraction was not significantly different from before exposure to PE plus GTP (9 ± 0.6%, n = 3), indicating that the muscles were no longer Ca2+-sensitized, but 21 ± 4.6% (n = 9) of p21rhoA remained in the particulate fraction; this was not significantly different from that found in the presence of PE (18 ± 5.5%, n = 9, 5 min, p > 0.05). Even when strips were washed in 10 mM EGTA-containing solution for 60 min, the translocated p21rhoA remained in the particulate fraction (data not shown).

Time Courses of GTPyS-induced Contraction and Translocation of p21rhoA and Gαq/11—The time courses of GTPyS-induced potentiation of force and translocation of p21rhoA were determined to evaluate whether they were kinetically consistent with the potential role of p21rhoA as a mediator of agonist-induced Ca2+ sensitization. As shown in Fig. 3 (A and B), within 1 min following addition of GTPyS to permeabilized portal vein smooth muscle at pCa 6.5, force reached 21 ± 4.2% (n = 10) of the maximal GTPyS-induced contraction; this was accompanied by translocation of p21rhoA to the particulate fraction (increasing from the control value of 10 ± 1.6% (n = 23) to 32 ± 9.7% (n = 6, p < 0.0001)). Thus, within the time resolution of this study, the kinetics of translocation of p21rhoA were consistent with its role in GTPyS-induced Ca2+ sensitization of force. However, the later time course of GTPyS-induced p21rhoA...
translocation was slower than that of force development; contraction peaked at 5 min, at which time ~51 ± 4% (n = 6) of p21\textsuperscript{rhoA} was in the particulate fraction, whereas p21\textsuperscript{rhoA} continued to translocate, reaching its peak of 62 ± 9.5% (n = 4) at 20 min, consistent with the ceiling effect in the translocation-force relationship (Fig. 1A).

We also determined the time course of translocation of G\textsubscript{aq/11}, the heterotrimeric G-protein implicated in the activation of phospholipase C, a major contributor to pharmacomechanical coupling in smooth muscle (reviewed in Ref. 4). Under control conditions (pCa 6.5), 86 ± 1.7% (n = 25) of the total G\textsubscript{aq/11} was in the particulate fraction, and this was reduced by GTP\textsubscript{S} to 60 ± 12% (n = 6, p < 0.01) at 1 min and 70 ± 8.8% (n = 6, p < 0.05) at 5 min (Fig. 3, A and B). In contrast to p21\textsuperscript{rhoA}, the translocation of G\textsubscript{aq/11} was transient; by 60 min, the previously translocated protein had returned to the particulate fraction (Fig. 3, A and B).

**Particulate p21\textsuperscript{rhoA} Is Hydrophobic**—The hydrophobic domain of cytosolic, geranylgeranylated p21\textsuperscript{rhoA} is masked by bound Rho-GDI, and activated p21\textsuperscript{rhoA} is thought to bind to the cell membrane through the unmasked hydrophobic geranylgeranyl group exposed by the release of Rho-GDI (22). Because the particulate fraction obtained through centrifugation may contain both hydrophobic (membrane) and nonhydrophobic (e.g. cytoskeletal) components, we determined by phase separation with Triton X-114 whether the p21\textsuperscript{rhoA} translocated to the particulate fraction of GTP\textsubscript{S} in vitro was hydrophilic, as indicated by partitioning into Triton X-114. Indeed, GTP\textsubscript{S} (50 \muM, 30 min) increased the fraction of p21\textsuperscript{rhoA} partitioned into the detergent phase when whole homogenates were treated with Triton X-114. p21\textsuperscript{rhoA} in the Triton X-114 phase increased from the control value of 22 ± 6.6% (n = 11) to 81 ± 18% (n = 6, p < 0.0001), indicating that most of the particulate p21\textsuperscript{rhoA} was associated with hydrophobic (presumably membrane) components.

To further evaluate whether cytosolic p21\textsuperscript{rhoA} (complexed with Rho-GDI) is hydrophilic, whereas particulate p21\textsuperscript{rhoA} is hydrophobic, the whole homogenate was first separated into cytosolic and particulate fractions, which were subsequently phase-separated with Triton X-114 (see “Materials and Methods”). As shown in Fig. 4, only 5 ± 1.8% (n = 9) of the cytosolic p21\textsuperscript{rhoA} partitioned into the Triton X-114 phase. In contrast, most of the particulate p21\textsuperscript{rhoA} partitioned into the Triton X-114 phase, with only 7 ± 2.5% (n = 8) partitioning into the aqueous phase. Again, there was a dramatic increase from 11 ± 8.6% (n = 3) to 63 ± 13.5% (n = 3, p < 0.05) in the amount of p21\textsuperscript{rhoA} in the Triton X-114-treated particulate fraction of GTP\textsubscript{S} (50 \muM, 20 min)-stimulated muscles. The small quantities of cytosolic p21\textsuperscript{rhoA} partitioning into the Triton X-114 phase and particulate p21\textsuperscript{rhoA} partitioning into the aqueous phase may result from “carryover” during the experimental procedure.

**Translocated p21\textsuperscript{rhoA} Is Not A Good Substrate for C3-catalyzed ADP-ribosylation**—The cytosolic and particulate fractions of control and GTP\textsubscript{S}-stimulated tissues were incubated with C3 and \textsuperscript{32}P\textsubscript{NAD} (see “Materials and Methods”) to determine if the translocated p21\textsuperscript{rhoA} was a good substrate for C3-catalyzed ADP-ribosylation. In unstimulated strips, the largely cytosolic p21\textsuperscript{rhoA} (Figs. 1, 2, and 5) was only minimally ADP-ribosylated; a very faint band was detected in the autoradiograph (AutoRad). The particulate p21\textsuperscript{rhoA} in the control strips was a good substrate for C3-catalyzed ADP-ribosylation, whereas the particulate p21\textsuperscript{rhoA} in the GTP\textsubscript{S}-stimulated strips was a poor substrate, although markedly increased (see Western blots). Results are representative of three independent experiments. C, cytosol; P, particulate.

**Effect of Ca\textsuperscript{2+} and of the Phosphatase Inhibitor Tautomycin on the Localization of p21\textsuperscript{rhoA}**—To determine whether Ca\textsuperscript{2+} alone can induce translocation of p21\textsuperscript{rhoA}, α-toxin-permeabilized rabbit portal vein strips were incubated in Ca\textsuperscript{2+}-free solution (no Ca\textsuperscript{2+} added and containing 10 mM EGTA) for 15 min, homogenized, and fractionated. The particulate fraction under this Ca\textsuperscript{2+}-free condition contained 9 ± 3.0% (n = 6) of the total p21\textsuperscript{rhoA} which is not significantly different from strips incubated in pH 6.5 solution: 10 ± 1.6% (n = 23, p > 0.05). However, increasing Ca\textsuperscript{2+} to very high levels (pCa 4.5, 15 min) increased the p21\textsuperscript{rhoA} content of the particulate fraction to 31 ± 5.8% (n = 10, p < 0.01). This translocation of p21\textsuperscript{rhoA} induced by pCa 4.5 was not due to the release of norepinephrine from nerve endings because inclusion of the α-adrenergic blocker.
prazosin (10 μM) in the solutions during permeabilization and thereafter had no effect on the Ca^{2+}-induced translocation of p21^{rhoA} to the particulate fraction (39 ± 6.9%, n = 8, p > 0.05).

To exclude the possibility that the translocation of p21^{rhoA} by Ca^{2+} was due to trapping of cytosolic p21^{rhoA} in the cytoskeletal components of the contracted tissue, cytosolic and particulate fractions of Ca^{2+}-stimulated tissues were phase-separated by Triton X-114. Similar to the GTP-γ-S-stimulated tissues, 89 ± 8.6% (n = 3) of p21^{rhoA} in the particulate fraction (29.0 ± 13.9% (n = 3) of the total p21^{rhoA}) was partitioned into the Triton X-114 phase, suggesting that high [Ca^{2+}] alone can translocate p21^{rhoA} to the membrane.

To determine whether p21^{rhoA} translocated by high [Ca^{2+}] returns to the cytosol after removal of Ca^{2+}, portal vein strips stimulated with pCa 4.5 for 15 min were washed five times in Ca^{2+}-free solution (no Ca^{2+} added and containing 10 mM EGTA) for a total of 60 min, and the distribution of p21^{rhoA} was determined. Even after this extensive wash, the same amount (38 ± 2%, n = 3) of translocated p21^{rhoA} remained in the particulate fraction.

The Ca^{2+} dependence of GTP-γ-S-induced translocation of p21^{rhoA} was also determined by adding GTP-γ-S to α-toxin-permeabilized smooth muscle in the presence and absence of Ca^{2+}. GTP-γ-S (50 μM, 60 min) caused similar p21^{rhoA} translocation in Ca^{2+}-free, 10 mM EGTA-containing solution as in pCa 6.5 solution: p21^{rhoA} in the particulate fraction was 65 ± 6.9% (n = 6) versus 62 ± 9.5% (n = 4, p > 0.05), respectively.

Tautomycin, a potent inhibitor of protein phosphatases 1 and 2A, (25) causes substantial MLC_{20} phosphorylation and smooth muscle contraction even in the absence of Ca^{2+} (26) by inhibiting the catalytic subunit of SMPP-1M. We also wished to localize p21^{rhoA} in tautomycin-stimulated tissues to determine whether phosphorylation of a protein that can be dephosphorylated by phosphatase 1 or 2A is involved in regulating translocation of p21^{rhoA}. No significant translocation of p21^{rhoA} was detected in tautomycin-stimulated tissue at pCa 6.5 (data not shown), although it caused 86 ± 6.3% (n = 3) of the maximal Ca^{2+}-induced contraction.

**DISCUSSION**

The relationships between the extent and time course of translocation of p21^{rhoA} to the particulate fraction (Figs. 1 and 3) and enhancement of force at constant [Ca^{2+}] are consistent with a causal role of p21^{rhoA} recruitment to the membrane in Ca^{2+}-sensitization. This conclusion is also supported by the hydrophobicity of particulate p21^{rhoA} (partitioning into Triton X-114; this study), the abolition of the Ca^{2+}-sensitizing effect of recombinant p21^{rhoA} by extensive permeabilization of smooth muscle with detergent, and the inactivity of nonprenylated p21^{rhoA} (11). Similarly, GTP-γ-S-induced activation of NADPH oxidase in neutrophils involves the dissociation of p21^{rhoA} from Rho-GDI and its translocation to the membrane by a mechanism that requires a heat- and trypsin-labile membrane component (27). The translocation was limited to p21^{rhoA} in our study: the localization of two other Rho family proteins, Rac1 and Cdc42, was not affected by GTP-γ-S under conditions causing extensive translocation of p21^{rhoA}.

The α-adrenergic agonist phenylephrine, GTP-γ-S, and AlF_{4} induced translocation of p21^{rhoA}, whereas the muscarinic agonist atropine inhibited the translocating effect of endogenous acetylcholine. The significant reduction by atropine of the large amount of particulate p21^{rhoA} in "unstimulated" ileum smooth muscle and the translocation of p21^{rhoA} by high [Ca^{2+}] indicate that preparatory conditions alone can affect its localization. Thus, caution is required in interpreting results of fractionation obtained in the presence of high [Ca^{2+}] and/or locally stored and released transmitters.
This would account for the more rapid rate of Ca$^{2+}$ sensitization achieved by agonists or GTP$\gamma$S than by exogenous p21$\text{rhoA}_{\text{GTP}}$ (11). Another state (or states) is much less available for ADP-ribosylation, as indicated by the large quantity of p21$\text{rhoA}_{\text{GTP}}$ that was translocated by GTP$\gamma$S to the membrane, but not ADP-ribosylated (Fig. 5). This may represent a combination of transitional states being activated through membrane association, as well as a third membrane-bound but inactivated state of p21$\text{rhoA}_{\text{GTP}}$ (31). However, we cannot completely rule out the possibility that a very small fraction of activated p21$\text{rhoA}_{\text{GTP}}$ that was not translocated is involved in Ca$^{2+}$ sensitization.

Tautomycin, a potent inhibitor of protein phosphatases 1 and 2, did not translocate p21$\text{rhoA}_{\text{GTP}}$, although it had a large "Ca$^{2+}$-sensitizing" effect on contraction (see also Ref. 26). Therefore, it is unlikely that phosphorylation of a site susceptible to dephosphorylation by these phosphatases plays a role in the translocation of p21$\text{rhoA}_{\text{GTP}}$. The possibility of a role of tyrosine phosphorylation in this process is currently under study.

Ca$^{2+}$-sensitizing mechanisms, whether mediated by protein kinase C (34, 35, 41) or other effectors, appear to converge to inhibit dephosphorylation of a cytosolic SMPP-1M substrate, MLC$_{20}$ (2). Smooth muscle myosin light chain phosphatase is inhibited by p21$\text{rhoA}_{\text{GTP}}$ (11). Another state (or states) is much less available for ADP-ribosylation. This would account for the more rapid rate of Ca$^{2+}$ sensitization achieved by agonists or GTP$\gamma$S than by exogenous p21$\text{rhoA}_{\text{GTP}}$.
