Role of G proteins in agonist-induced Ca\(^{2+}\) sensitization of tracheal smooth muscle

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Croxton, Thomas L., Boris Lande, and Carol A. Hirshman. Role of G proteins in agonist-induced Ca\(^{2+}\) sensitization of tracheal smooth muscle. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L748–L755, 1998.—Increased sensitivity to intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) is an important mechanism for agonist-induced contraction of airway smooth muscle, but the signal transduction pathways involved are uncertain. We studied Ca\(^{2+}\) sensitization with acetylcholine (ACh) and endothelin (ET)-1 in porcine tracheal smooth muscle by measuring contractions at a constant [Ca\(^{2+}\)] in strips permeabilized with α-toxin or β-escin. The peptide inhibitor G protein antagonist 2A (GP Ant-2A), which has selectivity for Gq over Gi, inhibited contractile responses to ET-1, ACh, and guanosine 5′-O-(3-thiotriphosphate) (GTP\(\gamma\)S), but the proportional inhibition of ACh responses was less than that of ET-1. Pretreatment with pertussis toxin reduced ACh contractions but had no effect on those of ET-1 or GTP\(\gamma\)S. Clostridium botulinum C3 exoenzyme, which inactivates Rho family monomeric G proteins, caused similar reductions in contractile responses to ACh, ET-1, and GTP\(\gamma\)S. Farnesyltransferase inhibition, which inhibits Ras proteins, reduced responses to ET-1. We conclude that the heterotrimeric G proteins G\(q\) and G\(i\) both contribute to Ca\(^{2+}\) sensitization by ACh, whereas ET-1 responses involve G\(i\) but not G\(q\). Both G\(q\) and G\(i\) pathways likely involve Rho family small G proteins. A Ras-mediated pathway also contributes to Ca\(^{2+}\) sensitization by ET-1 in airway smooth muscle.

Acetylcholine; airway smooth muscle contraction; endothelin; β-escin; heterotrimeric G proteins G\(q\) and G\(i\); monomeric G proteins Ras and Rho; staphylococcal α-toxin

A rise in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) has long been recognized as the normal trigger for smooth muscle contraction. However, it is now widely appreciated that many contractile agonists act both to increase [Ca\(^{2+}\)], and to enhance the effectiveness of Ca\(^{2+}\) for inducing contraction (1, 13, 16, 24, 30, 31, 38). The latter phenomenon can be readily demonstrated in membrane-permeabilized smooth muscle strips as a leftward shift of the force-[Ca\(^{2+}\)] curve or as an agonist-induced contraction at a constant [Ca\(^{2+}\)]. The signal transduction pathways involved in the regulation of Ca\(^{2+}\) sensitivity appear to be quite complex and smooth muscle type specific. In most smooth muscle prepa-

tations, agonist-induced enhancement of Ca\(^{2+}\) sensitivity or potentiation of Ca\(^{2+}\)-induced contractions involves a G protein-mediated cascade that results in inhibition of myosin light chain phosphatase (22, 24, 28). This leads to an increase in the level of myosin light chain phosphorylation, with increased numbers of attached cross bridges and increased force. Tyrosine kinases, protein kinase C, and regulatory thin filaments such as calponin are also involved (5, 19, 37).

Many lines of evidence support the involvement of members of the Ras superfamily of monomeric G proteins (e.g., Rho A and H-Ras) in the signaling pathways mediating agonist-induced potentiation of Ca\(^{2+}\)-induced contraction. First, agonist effects can be mimicked by the poorly hydrolyzable GTP analog guanosine 5′-O-(3-thiotriphosphate) (GTP\(\gamma\)S) (12, 18) and inhibited by guanosine 5′-O-(2-thiodiphosphate) (GDP\(\beta\)S) (12, 33). Second, potentiation of Ca\(^{2+}\)-induced contraction by agonists is associated with increases in myosin phosphorylation and inhibition of myosin light chain phosphatases (22, 24). Third, agonist-induced potentiation of Ca\(^{2+}\)-induced contraction is inhibited by Clostridium botulinum C3 exoenzyme or epidermal cell differentiation inhibitor (12, 16, 18, 20), specific inhibitors of Rho proteins. Fourth, GTP\(\gamma\)S inhibition of myosin light chain phosphatase is attenuated by C3 exoenzyme (28). Fifth, Rho-associated kinase, which is activated by GTP-RhoA, inactivates myosin light chain phosphatase by phosphorylating its myosin binding unit (21). Sixth, constitutively active mutants of RhoA (16, 30), of H-Ras (33), and of the mitogen-activated protein kinase extracellular signal-regulated kinase 2, which is downstream to Ras (14), all potentiate Ca\(^{2+}\)-induced contraction in smooth muscle preparations. However, the relative importance of particular G proteins in the Ca\(^{2+}\)-sensitizing effects of specific agonists on specific smooth muscle types is not well characterized.

Airway smooth muscle expresses M\(_2\) and M\(_3\) muscarinic receptors, with greater than 80% being of the M\(_2\) subtype (7, 9, 11, 32). Both endothelin (ET\(_A\)) and ET\(_B\) receptors are present in airway smooth muscle (15). Muscarinic M\(_3\) and ET receptors couple to phospholi-

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with pentobarbital sodium (30 mg/kg iv), and killed by exsanguination through the femoral arteries. The tracheae were removed and placed in cold physiological salt solution for transport to the laboratory. Tissues were stored for up to 48 h at 4°C in similar solutions bubbled with 95% O2-5% CO2. Pairs of tracheal smooth muscle strips 0.2–0.3 mm in width and ~12 mm in length were dissected from the posterior aspect of the upper half of the trachea with a binocular microscope and tied at either end with 6-0 silk suture for later attachment to a chamber and force transducer (Grass FT-03).

Experimental chambers and solutions. Pairs of smooth muscle strips were mounted vertically and studied simultaneously in identical 800-µl chambers made from 1⁄4-dram glass vials. The chamber solution was stirred continuously with a magnetic bar. [Ca2+] was changed by flushing the chambers with ~4 ml of solution added through polyethylene tubing to the bottom of the chamber. Excess solution was removed by suction at the top. Solutions contained (in mM) 130 potassium propionate, 20 monohydrate (hydroxymethyl)aminoethylphosphonic acid; (2,2-dimethyl-1-oxopropoxy)-methyleneester, 7.0 MgCl2, 4.6 Na2ATP, 2.0 EGTA, 2.0 phosphocreatine, and 1.0 dithiothreitol plus 8 U/ml of creatine phosphokinase, 10-6 M leupeptin, and sufficient CaCl2 to provide the desired concentration of free Ca2+. Calmodulin was not added. Solutions of 10-9 and 3 x 10-4 M free Ca2+ were prepared and carefully adjusted to pH 7.10. Solutions of intermediate [Ca2+] were made by mixing these solutions in appropriate proportions. All experiments were performed at room temperature.

Smooth muscle strip permeabilization. Permeabilization with staphylococcal α-toxin was performed before the strips were mounted in the chamber by 50–60 min of incubation with 830 µM of α-toxin in 300 µl of relaxing solution ([Ca2+] = 10-9 M, composition as described in Experimental chambers and solutions). Permeabilization with β-escin (60 µM) was performed in the experimental chambers while isometric force in the presence of 10-5 M Ca2+ was measured. The β-escin concentration was increased to 80 µM in some experiments as needed to increase the rate of increase in force. The β-escin was washed out with relaxing solution when force had nearly plateaued (after ~45 min).

Drug treatments. Smooth muscle strips used in experiments involving pertussis toxin or farnesyltransferase inhibitor FPT III were incubated before α-toxin permeabilization in the presence or absence of the drug for 18–20 h in 500 µl of medium in a 24-well plate at 31°C in a humidified atmosphere of 5% CO2 in air. Preliminary results indicated better preservation of contractile responses in tissues pretreated at this reduced temperature. The culture medium was Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 250 ng/ml of amphotericin B, and 100 U/ml of nystatin. Pertussis toxin was prepared as a 500 µg/ml stock solution in water and was added at a final concentration of 20 µg/ml. FPT III was used at a final concentration of 5 x 10-4 M. Smooth muscle strips used for studies of G protein antagonist 2A (GP Ant-2A) were permeabilized with β-escin to allow entry of the peptide antagonist. GP Ant-2A (5 x 10-5 M) was added during permeabilization, to the relaxing solution used to wash out β-escin, and to the 3.6 x 10-7 M Ca2+ solution used during the experimental period. Incubation with C3 exoenzyme (2.5 µg/ml) was similar to that with GP Ant-2A except that 3 x 10-5 M β-NAD was added to both treated and control tissues during the treatment and C3 exoenzyme was washed out before the experimental period.

Experimental protocols. In all cases, drug effects were evaluated in pairs of smooth muscle strips that were preincubated, permeabilized, and studied in parallel by identical methods, except for the presence or absence of the stated drug. Preliminary experiments indicated that baseline forces of ~2 mN were optimal for active force generation in these tissues. Hence, after permeabilization with either α-toxin or β-escin, tissues were bathed with relaxing solution (10-5 M free Ca2+) and were stretched until a stable baseline force of ~2 mN was obtained. This and all solutions used subsequently contained 10-6 M calcium ionophore A-23187 to deplete intracellular Ca2+ stores. After a 20-min exposure to A-23187, the [Ca2+] was increased to a value that typically increased force by ~5% of the maximum Ca2+ response (10-7 and 3.6 x 10-7 M for experiments employing α-toxin and β-escin, respectively). In experiments involving pertussis toxin, GP Ant-2A or FPT III, cumulative additions of drugs were then made at 10- to 15-min intervals in the following order: GTP, ACh, atropine, ET-1, and GTP>Y>S. All drugs were then washed out with 3 x 10-4 M free Ca2+ solution, and the difference between the maximum force obtained in this solution and the force obtained in the relaxing solution before the protocol was used to normalize the drug-induced changes in force. It was assumed that smooth muscle strips treated in a given manner exhibiting essentially maximum force response. We chose to study the [Ca2+] required for 50% maximum active force (EC50) in each tissue. Mean log(EC50) values were compared by paired t-tests. Unless stated otherwise, drug-induced changes in isometric force at a constant [Ca2+] were normalized by the change in force induced by high [Ca2+] in that strip. The GTP>Y>S response was measured relative to the value obtained before addition of GTP. Effects of pretreatments on force responses were analyzed by paired two-tailed t-tests, with n = number of animals and P < 0.05 considered significant.

RESULTS

As has been previously shown in tracheal smooth muscle strips from other species (1, 29), ACh produced a leftward shift of the [Ca2+]i-force curve of porcine tracheal smooth muscle (Fig. 1). Log(EC50) values for Ca2+ were −6.71 ± 0.09 and −6.42 ± 0.10 log(M) in the presence and absence of 10-5 M ACh, respectively (P = 0.01; n = 4). A free [Ca2+] of 10-4 M produced an essentially maximum force response. We chose to study the pathways that contribute to this sensitizing effect by measuring agonist-induced contractions at a constant [Ca2+] in permeabilized smooth muscle strips. Figure 2A shows a typical protocol in which ACh produced contraction of α-toxin-permeabilized strips at 10-7 M free Ca2+ in the presence of 5 x 10-6 M GTP. Atropine reversed the ACh response, and subsequent additions of ET-1 and GTP>Y>S to the same tissues produced similar contractions.

Figure 2B shows results from an experiment similar to that of Fig. 2A but with a strip permeabilized with
In general, active forces recorded in β-escin-permeabilized strips were smaller than those obtained with α-toxin-permeabilized strips of similar size (Fig. 3A). In 21 control strips permeabilized with β-escin, the active force induced by Ca^{2+} (3.24 ± 0.25 mN) tended to be less than that recorded in 18 α-toxin-permeabilized strips studied in the absence of drugs (5.39 ± 0.75 mN; P = 0.054). Contractile responses to 10^{-4} M GTP \gamma S were significantly less in 13 β-escin-permeabilized strips than in 18 α-toxin-permeabilized strips (P = 0.0001). Responses to 10^{-7} M ET-1 were also significantly different in these same tissues (P = 0.0017). Responses to 10^{-4} M ACh were less in β-escin- than in α-toxin-permeabilized strips (0.29 ± 0.05 mN, n = 15 vs. 0.53 ± 0.28 mN, n = 5), but this difference was not significant (P = 0.79). Differences between β-escin and α-toxin remained significant when the responses to ET-1 and GTP \gamma S were normalized by the Ca^{2+}-induced force in the same strips (P = 0.0037 and 0.0001, respectively; Fig. 3B). However, when normalized by the contractile response to GTP \gamma S in the same strip, contractile responses to ET-1 were not significantly different in β-escin- and α-toxin-permeabilized strips (36 ± 4 vs. 48 ± 4%; P = 0.06; Fig. 3C).

With the use of the protocol illustrated in Fig. 2, we studied the effects of the \( G_q \)-selective inhibitor GP Ant-2A on agonist-induced contractions at a constant [Ca^{2+}] in β-escin-permeabilized strips. As shown in Fig. 4, contractions induced by ACH and ET-1 were significantly reduced in the presence of GP Ant-2A (P = 0.007 and P = 0.008, respectively; n = 6). The proportional decrease in the ACH response (~28%) was approximately one-half that of the ET-1 response (~46%). GP Ant-2A attenuated the contractile response to GTP \gamma S by ~47% (P = 0.006). The force response to high [Ca^{2+}] was slightly different between treated and control tissues (2.41 ± 0.26 vs. 2.80 ± 0.23 mN; P = 0.025; n = 6).

To further investigate the role of large G proteins in the potentiation of Ca^{2+}-induced contractions by ET-1 and ACh, we performed similar studies in strips pre-
treated with pertussis toxin to inactivate G. Strips were permeabilized with α-toxin and studied as shown in Fig. 2. Contractile responses to ACh were diminished in pertussis toxin-treated strips, whereas responses to ET-1 and to GTPγS were unaffected. A summary of data from 11 experiments with smooth muscle strips from 9 animals is given in Fig. 5. An ~28% decrease in ACh-induced force in pertussis toxin-treated strips was significant (P < 0.027; n = 9), whereas responses to other stimuli did not differ between treated and control tissues (P = 0.65 and 0.69, respectively, for ET-1 and GTPγS). Maximum Ca2+-induced increases in force did not differ between treated and control strips (6.02 ± 1.02 vs. 6.37 ± 1.16 mN; P = 0.55; n = 9).

To evaluate the role of Rho in agonist enhancement of Ca2+-induced contractions, we studied β-escin-permeabilized tracheal smooth muscle strips after pretreatment with C3 exoenzyme, which inactivates that family of small G proteins by ADP ribosylation (2). C3 exoenzyme decreased the contractile responses at a constant [Ca2+] produced by ACh, ET-1, and GTPγS (P = 0.029, 0.009, and <0.0001, respectively; n = 8, 6, and 7, respectively; Fig. 6). The proportional decrease in contraction produced by C3 exoenzyme was similar for all three stimuli. High [Ca2+]−induced force was not significantly different in C3 exoenzyme-treated strips and control strips (3.17 ± 0.34 vs. 3.46 ± 0.45 mN; P = 0.21; n = 8 for strips contracted with ACh and 3.40 ± 0.42 vs. 3.50 ± 0.58 mN; P = 0.71; n = 6 for strips contracted with ET-1).

A summary of data obtained with smooth muscle strips preincubated in the presence or absence of the farnesyltransferase inhibitor FPT III is shown in Fig. 7. Inhibition of Ras with this drug caused a significant reduction in ET-1 contraction at a constant Ca2+ (18.6 ± 2.1 vs. 25.7 ± 3.4%; P = 0.02; n = 7) but had no significant effect on contractions induced by ACh (P = 0.33; n = 5) or GTPγS (P = 0.43; n = 7). High

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**Fig. 4.** Effects of 5 x 10^-5 M G protein antagonist 2A (GP Ant-2A) on responses of β-escin-permeabilized tracheal smooth muscle strips to 10^-5 M ACh, 10^-7 M ET-1, and 10^-4 M GTPγS at constant 3.6 x 10^-7 M Ca2+. *Significant difference from control condition, P < 0.05.

**Fig. 5.** Effects of pretreatment with pertussis toxin (20 µg/ml for 18–20 h at 31°C) on responses of α-toxin-permeabilized tracheal smooth muscle strips to 10^-5 M ACh, 10^-7 M ET-1, and 10^-4 M GTPγS at constant 10^-7 M Ca2+. *Significant difference from control condition, P < 0.05.
[Ca\textsuperscript{2+}]\textsuperscript{-induced forces did not differ between FPT III-treated and untreated strips (4.51 ± 0.78 vs. 4.72 ± 0.66 mN; \( P = 0.66; n = 7 \)).

**DISCUSSION**

Permeabilized smooth muscle preparations have been used extensively to characterize the cellular mechanisms that regulate Ca\textsuperscript{2+} sensitivity. One concern with the use of these methods is that the chemicals used to permeabilize the cell membrane may alter the function of relevant cellular pathways. Staphylococcal \( \alpha \)-toxin is thought to be less injurious to tissues than detergents such as \( \beta \)-escin, but our use of large-molecular-weight drugs (e.g., C3 exoenzyme) required that we perform certain experiments in \( \beta \)-escin-permeabilized strips. To compare these permeabilization methods, we evaluated contractile responses in control tissues permeabilized with \( \alpha \)-toxin or \( \beta \)-escin. Although all strips were of similar size, we generally obtained lower force responses to ACh, ET-1, GTP\textsubscript{S}, and high Ca\textsuperscript{2+} in those permeabilized with \( \beta \)-escin relative to those permeabilized with \( \alpha \)-toxin (Figs. 2 and 3A). The attenuation of high Ca\textsuperscript{2+} responses suggests loss of "distal" cellular components such as calmodulin, myosin light chain kinase, or actin in \( \beta \)-escin-permeabilized strips. Addition of calmodulin to the bathing solutions has been shown to reduce the decline of force over time in saponin-permeabilized smooth muscle strips (3).

Significant differences in ET-1 and GTP\textsubscript{S} responses persisted after normalization by the high Ca\textsuperscript{2+}-induced contraction (Fig. 3B). These data suggest an additional attenuation of pathways activated by G proteins, possibly due to diffusive loss of small G proteins. However, responses to ACh and ET-1 were similar in \( \alpha \)-toxin- and \( \beta \)-escin-permeabilized strips when normalized by the GTP\textsubscript{S} response. This indicates that the "upstream" portions of pathways activated by these contractile agonists (e.g., receptors and heterotrimeric G proteins) are not differentially affected by the method of permeabilization. Although quantitative differences do exist,
we conclude that both methods of permeabilization are useful for studies of agonist-induced Ca\textsuperscript{2+} sensitization. However, results obtained by different methods of permeabilization should probably not be compared numerically.

Muscarinic cholinergic receptors of types M\textsubscript{3} and M\textsubscript{2} are known to couple to the heterotrimeric (large) G proteins G\textsubscript{q} and G\textsubscript{i}, respectively, in airway smooth muscle (25). ET\textsubscript{A} and ET\textsubscript{B} receptors are expressed in porcine airway smooth muscle (15), and each of these receptor subtypes can couple to both G\textsubscript{i} and G\textsubscript{q} (34). G\textsubscript{q} activation causes contraction of airway smooth muscle through release of Ca\textsuperscript{2+} from intracellular stores and enhancement of Ca\textsuperscript{2+} entry, whereas G\textsubscript{i} activation antagonizes relaxation through inhibition of adenylyl cyclase (6, 9, 35). The roles of G\textsubscript{q} and G\textsubscript{i} in the enhancement of [Ca\textsuperscript{2+}]\textsubscript{i} sensitivity are not known. We initially addressed this issue in experiments with GP Ant-2A, an analog of substance P that has been shown to inhibit activation of large G proteins with selectivity for G\textsubscript{i} over G\textsubscript{q} (27). Preliminary experiments indicated that a higher concentration of GP Ant-2A was required to have effects in our smooth muscle strips than was reported to be effective in a vesicular preparation (27). We attribute this relative insensitivity to limitations of drug access but cannot rule out the possibility of nonspecific effects in our experiments. GP Ant-2A inhibited the contractile responses to ACh and to ET-1 in permeabilized smooth muscle strips studied at a constant [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 4), consistent with the hypothesis that activation of G\textsubscript{q} contributes to the potentiation of Ca\textsuperscript{2+}-induced contractions by these agonists. The effect of GP Ant-2A was more pronounced on ET-1-induced contractions than on ACh-induced contractions, suggesting that the Ca\textsuperscript{2+}-sensitizing effects of ET-1 may be mediated more exclusively by the G\textsubscript{i} pathway than are the effects of ACh. However, comparisons of drug effects on ACh and ET-1 responses in this study might be misleading because the magnitude of the contractions induced by these agonists was not comparable. We therefore sought more direct evidence for a G\textsubscript{i}-independent pathway in the Ca\textsuperscript{2+}-sensitizing effects of ACh.

Resistance of ACh-induced contractions at a constant [Ca\textsuperscript{2+}]\textsubscript{i} to inhibition by GP Ant-2A could reflect involvement of a G\textsubscript{i}-mediated pathway. We tested this possibility with tissues incubated overnight in the absence or presence of pertussis toxin, which is known to ADP-ribosylate and irreversibly inactivate G\textsubscript{i} (10). Tracheal smooth muscle strips treated with pertussis toxin showed smaller ACh-induced contractions at a constant [Ca\textsuperscript{2+}]\textsubscript{i}, whereas the responses to ET-1 and GTP\textsubscript{y}S were unaltered (Fig. 5). These data indicate a novel function for G\textsubscript{i} in mediating increases in Ca\textsuperscript{2+} sensitivity and, hence, indicate that G\textsubscript{i} contributes directly to contraction. This conclusion contradicts the generally held belief that the M\textsubscript{2} receptor-G pathway contributes to contraction only indirectly via inhibition of cAMP-mediated relaxation (6). In fact, our results agree with studies of the M\textsubscript{2}-selective antagonist methoctramine in guinea pig airways that yielded Schild plots with slopes significantly less than unity (17), consistent with contraction through a heterogeneous population of receptors. The lack of effect of pertussis toxin on ET-1 responses indicates that ET couples primarily to G\textsubscript{i} in porcine tracheal smooth muscle, consistent with other data from this laboratory showing stimulation of inositol trisphosphate production by ET-1 but no inhibition of GTP-stimulated adenylyl cyclase activity (8).

Studies (12, 16, 18, 20, 28) in a variety of nonairway smooth muscles have indicated that the Rho p21 family of small G proteins mediates, at least in part, the Ca\textsuperscript{2+}-sensitizing responses to activation of large G protein-coupled receptors. Because ACh and ET-1 appeared to differ with regard to the relative importance of G\textsubscript{i} and G\textsubscript{q} in their Ca\textsuperscript{2+}-sensitizing effects, we questioned whether inhibition of Rho family G proteins by C3 exoenzyme would have differential effects on the responses to these agonists. C3 exoenzyme treatment reduced the contractile responses to ACh, ET-1, and GTP\textsubscript{y}S (Fig. 6), demonstrating that small G proteins of the Rho family are important mediators of Ca\textsuperscript{2+} sensitivity in airway smooth muscle. However, the proportional inhibition was similar for all three stimuli. Hence, our data are consistent with a convergent pathway in which G\textsubscript{i} and G\textsubscript{q} both lead to activation of Rho family monomeric G proteins. Incomplete inhibition by C3 exoenzyme of agonist-induced contractions in this study could result either from incomplete inactivation of Rho by our treatment or from the presence of a pathway for Ca\textsuperscript{2+} sensitization that does not involve Rho.

A previous study (33) demonstrated that a constitutively active mutant of H-Ras p21 produced contraction of β-escin-permeabilized mesenteric microvessels at a constant [Ca\textsuperscript{2+}]. This result, in combination with recent data that G protein-coupled receptors can activate Ras (36), suggests that Ras may mediate, in part, the Ca\textsuperscript{2+}-sensitizing effects of contractile agonists. To investigate this possibility, we studied tracheal smooth muscle strips preincubated in the presence or absence of a farnesyltransferase inhibitor, FPT III. Farnesyltransferase catalyzes a posttranslational modification that is important for activation of Ras (26). Our data showed consistently less contraction by ET-1 in FPT III-treated strips than in control strips (Fig. 7). Hence, a farnesylated protein, likely Ras, contributes to the acute potentiation by ET-1 of Ca\textsuperscript{2+}-induced contraction. Because ET-1 responses were not affected by pertussis toxin, we suggest that G\textsubscript{i} may mediate the activation of Ras. Partial mediation of cholinergic effects via a G\textsubscript{i} pathway could lessen the contribution of G\textsubscript{i} to ACh responses and account for the lack of effect of FPT III on contractile responses to that agonist in these experiments. Ras may promote contraction by stimulation of mitogen-activated protein kinase, which can phosphorylate and activate myosin light chain kinase (23), or by activation of a Rho G protein (4) that inhibits myosin light chain phosphatase (28).

Although GTP\textsubscript{y}S is presumed to activate all G proteins and hence all of the pathways examined in this study, we found that the contractile responses to GTP\textsubscript{y}S...
were inhibited by only some of the drugs used. C3 exoenzyme was effective, indicating that a substantial portion of the GTPγS-induced response is mediated by Rho. GP Ant-2A also inhibited contraction by GTPγS at a constant [Ca2+]i, suggesting that GTPγS activation of Gαi may be more effective than its activation of small G proteins or that Gαi may activate some Ca2+-sensitizing pathway that does not involve small G proteins. However, an alternative explanation is that GP Ant-2A is not entirely selective for large G proteins at this concentration. Despite their ability to attenuate agonist-induced responses, neither pertussis toxin nor farnesyltransferase inhibition significantly reduced the contractile effects of GTPγS. Thus, whereas Gαi- and Ras-mediated pathways may be important for receptor-mediated responses, they appear to account for only a small fraction of the Ca2+-sensitization that can be induced by indiscriminate activation of all G proteins by GTPγS.

In summary, the signal transduction mechanisms of airway smooth muscle that lead to enhanced myofilament Ca2+-sensitivity involve both large and small G proteins, as has been demonstrated in other smooth muscle types. In this tissue, the effects of ET-1 are mediated primarily by Gαi, whereas both Gαq and Gα11 contribute to the effects of ACh. Studies with pertussis toxin indicate that Gαi enhances airway smooth muscle contraction directly, in addition to its known ability to inhibit relaxation. Rho family small G proteins contribute to the Ca2+-sensitizing effects of both ACh and ET-1. Hence, the M3 receptor-Gαi pathway and the M2 receptor-Gα11 pathway appear to operate in parallel to enhance Ca2+-sensitivity of airway smooth muscle via Rho. This functional redundancy may contribute to the durability of some agonist-induced contractions of airway smooth muscle. At least for ET, mediation of Ca2+-sensitization also involves a farnesylated protein, most likely a monomeric G protein of the Ras family.

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