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REGULATION OF AIRWAY SMOOTH MUSCLE RhoA/ROCK ACTIVITIES BY CHOLINERGIC AND BRONCHODILATOR STIMULI [1]

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Abstract:

We compared the temporal relationships of Rho activity, ROCK activity and tone following cholinergic stimulation in the presence and absence of three different bronchodilators. Bovine trachea challenged with a half-maximally effective concentration of CCh was flash-frozen at different times, then assayed for Rho (rhotekin pull-down assay) and ROCK activities (Western blot; radiometric assay). Rho was activated within 30s, followed by ROCK (peak at 2 min); both returned to baseline by 20 min, although tone continued to rise over that period. Increasing the concentration of CCh greatly increased the magnitudes and rates of stimulation of Rho, ROCK and tone. These CCh-induced changes were next compared in tissues pretreated with isoproterenol (ISO), salmeterol (SAL) or the nitric oxide donor SNAP. Neither time-course nor magnitude of Rho-activation were reduced by the β-agonists; SNAP, however, slowed Rho activation but did not alter the peak magnitude. These observations were mirrored in ROCK-activation and contraction. In tissues first preconstricted with CCh and then challenged with the bronchodilators, however, all three agonists reversed cholinergically-stimulated Rho, ROCK and myosin light chain kinase activities as well as tone. We conclude that bronchodilators can suppress RhoA and ROCK activities, although their major effect appears to be on MLCK activity.

Keywords: RhoA; ROCK; airway smooth muscle; contraction; relaxation; myosin; cholinergic; adrenergic; nitric oxide

INTRODUCTION

A great deal has been learned about the diverse signaling events underlying cholinergic excitation of airway smooth muscle. For example, M_2-receptors are coupled negatively to adenylate cyclase, leading to its inhibition (Jones et al; Madison and Yamaguchi; Roux et al). Stimulation of muscarinic M_3-receptors triggers the phosphoinositide signaling cascade via the heterotrimeric G-protein $G_{q,11}$ resulting in activation of protein kinase C and release of internally sequestered Ca^{2+} (via production of diacylglycerol and IP_3, respectively) (Chilvers et al; Kajita and Yamaguchi; Roux et al). Ca^{2+}-release stimulates myosin light chain kinase (MLCK) activity directly (Gerthoffer; Kotlikoff and Kamm), but may also open plasmalemmal Cl^--channels (Janssen and Sims; Janssen and Sims) leading to membrane depolarization and voltage-dependent Ca^{2+}-influx. The M_3-receptors are also coupled to $G_{12,13}$, which activates the monomeric G-protein Rho and its downstream effector Rho-kinase (ROCK) (Somlyo and Somlyo): one of the targets of ROCK is myosin light chain phosphatase (MLCP), leading to suppression of that latter activity.

There has been a great deal of interest recently in the contributions of the Rho/ROCK signaling pathway toward excitation-contraction coupling (Iizuka et al; Janssen et al; Janssen et al; Setoguchi et al; Smith et al; Togashi et al; Yoshii et al) and airway hyperresponsiveness (Chiba et al; Chiba and Misawa; Chiba et al; Hashimoto et al; Hunter et al). However, the data available to date are limited in many ways. First, most groups do not measure Rho/ROCK activities directly, but instead infer changes in ROCK activity on the basis of the pharmacological effect of the ROCK inhibitor Y27632 on contractile activity. Second, generally only a single supramaximal concentration of excitatory agonist is used and/or only a single time-point examined. There is a great deal of evidence to suggest that the contribution of various

signaling pathways to excitation-contraction coupling may vary depending upon the degree and/or duration of excitatory challenge; also, supramaximally-effective concentrations are generally not physiologically relevant. Third, the vast majority of the work has been done in vascular smooth muscle preparations (Bolz et al; Murthy et al; Sauzeau et al), which can employ the same cellular machinery in very different ways (witness the contrary dependence upon electromechanical coupling in these two smooth muscles, despite both possessing exactly the same voltage-dependent Ca^{2+}-channels). Finally, the regulation of the Rho/ROCK signaling pathway by inhibitory stimuli has remained unexplored (see next paragraph).

Our understanding of the signaling events underlying inhibitory regulation of airway smooth muscle — mediated in large part by adrenergic agonists and nitric oxide — is also well developed, but has lagged somewhat behind that pertaining to ASM excitation. Generally speaking, bronchodilators trigger signaling events opposite to those summarized above, including stimulation of the enzymatic activities of adenylate cyclase / protein kinase A (β-agonists) or of guanylate cyclase / protein kinase G (nitric oxide), Ca^{2+}-uptake and Ca^{2+}-extrusion, membrane hyperpolarization, inhibition of MLCK and stimulation of MLCP (Somlyo and Somlyo; Kotlikoff and Kamm; Tansey et al; Word et al; Janssen et al; Bai and Sanderson). Interestingly, some of those effects depend upon whether cholinergic stimulation precedes β-adrenoceptor stimulation, or vice versa (Tansey et al; Word et al; Kotlikoff and Kamm). However, a major gap in this picture is the interaction between excitatory and inhibitory inputs at the level of the Rho/ROCK signaling pathway.

In this study, we examined the interactions between excitatory (cholinergic) and inhibitory (β-adrenergic and nitric oxide) agonists with respect to the Rho/ROCK signaling pathway in bovine tracheal smooth muscle. Our objectives were to examine: (*i*) the kinetics of activation of RhoA, ROCK and the contractile apparatus following varying degrees of

cholinergic stimulation; and (*ii*) the effects of bronchodilator stimuli on these cholinergic changes. Comparisons were made between the classical β-agonist isoproterenol (ISO; non-selective), the more recently introduced long-acting β-agonist salmeterol (SAL; β_2-selective), and the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP). Comparisons were also made between prevention of cholinergic responses *versus* reversal of cholinergic responses by these agonists.

METHODS

Preparation of isolated tissues Tracheae were obtained from cows (200-500 kg) euthanized at a local abattoir, and immediately put in ice-cold physiological solution for transport to the laboratory. TSM was isolated by removing connective tissue, vasculature, and epithelium, then cut into strips parallel to the muscle fibers (≈1 mm wide).

Muscle bath technique Tracheal strips were tied with silk suture (Ethicon 4-O) to a Grass FT.03 force transducer on one end, and to a plexiglass rod which served as an anchor on the other end. These were bathed in Krebs-Ringer's buffer (see below for composition) containing indomethacin (10^{-5} M) and N-ω-nitro-L-arginine (L-NNA; 10^{-4} M), bubbled with 95% O_2 / 5% CO_2, and maintained at 37°C; tissues were passively stretched to impose a preload tension of ≈1 g. Isometric changes in tension were amplified, digitized (2 samples per second) and recorded on-line (DigiMed System Integrator, MicroMed, Louisville, KY) for plotting on the computer. Tissues were equilibrated for 1 hour before commencing the experiments, during which time they were challenged with 65 mM KCl three times to assess the functional state of each tissue.

Assay for RhoA activity Tissues which had been flash frozen in liquid nitrogen were homogenized in ice-cold buffer (50mM Tris-HCl, pH 7.5, 0.1mM EDTA, 0.1mM EGTA, 750 mM NaCl, 5% Igepal CA-630, 50 mM $MgCl_2$, 10% glycerol, 10 ug/ml aprotinin, 10 ug/ml leupeptin, 1mM phenylmethylsulfonyl fluoride, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 2 mM sodium orthovanadate), total protein content determined (Bradford method) and adjusted (by addition of media) in order to make uniform. Tissue homogenates were incubated (60 min, at 4°C) with rhotekin coated-cellulose beads (rhotekin specifically binds activated RhoA and not inactive RhoA). The sample was then centrifuged (14,000g for 10 seconds, at

4°C) to "pull down" the beads, the supernate (unbound material) discarded, after which Rho was dissociated from the beads by incubating with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% β-mercaptoethanol, 0.01% bromophenol blue). Samples were boiled for 5 min, subjected to SDS polyacrylamide gel electrophoresis, then transferred to nitrocellulose membrane (blocked with 3% BSA / TBS-T). RhoA was visualized using a rabbit anti-Rho polyclonal antibody preparation (Upstate Biotechnology Inc.; Waltham, MA) followed by secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1:15000 dilution; Sigma). Blots were detected with enhanced chemiluminescence (Amersham).

ROCK assay (Western blot) Tissues were homogenized and protein content adjusted as outlined above. Tissue homogenates were incubated (10 min, at 30°C) with 0.5 ng MYPT (the myosin-targeting subunit of myosin light chain phosphatase), after which the reaction was terminated by addition of Laemmli sample buffer. Samples were then subjected to Western blot analysis, as outlined above. Phospho-MYPT was visualized using a rabbit anti-phospho-MYPT1 polyclonal antibody preparation (Upstate Biotechnology Inc.; Waltham, MA). While it is true that other kinases can also potentially phosphorylate MYPT (Hartshorne et al), we have shown previously that the changes in MYPT phosphorylation seen with airway smooth muscle homogenates were sensitive to Y27632.

ROCK assay (^{32}P incorporation) Tissue homogenates were centrifuged (10,000 g for 10 min at 4°C). The pellet was resuspended and an aliquot used to quantify the total protein content (Bradford method). The remainder was resuspended (1 mg/ml) in kinase assay buffer (20 mM 3-[N-morpholino]propane sulfonic, 25 mM β-glycerophosphate, 15 mM $MgCl_2$, 1 mM EGTA, 0.1 mM NaF, 1 mM Na_3VO_4, 1 mM DTT , pH 7.2) containing 50 μM MYPT as a substrate. The kinase reaction was started by adding 100 μM ATP (containing 10 μCi/ml γ^{32}P-ATP) and

incubated for 10 minutes at 30°C with agitation. Aliquots of reaction mixture were spotted on P81 paper, washed 5 times with ice cold 0.75% H_3PO_4 and then with acetone. Paper squares were dried and radioactivity counted (Cerenkov method).

MLCK assay Flash frozen bovine TSM tissues were homogenized in ice-cold buffer (50mM Tris-HCl, 0.1mM EDTA, 0.1mM EGTA, 0.1% β-mercaptoethanol, 25 ug/ml aprotinin, 25 ug/ml leupeptin, 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, pH 7.5), then centrifuged at 13,000g for 10 min at 4°C. Supernatants were collected and their protein concentrations determined by the Bradford method. Tissue homogenates (20 μg) were warmed for 5 min to 25°C in reaction buffer (30 mM Tris-HCl, 50 mM KCl, 0.1mM EDTA, 0.1% β-mercaptoethanol; pH 7.5), after which the phosphorylation reaction was initiated by adding myosin (10 μg; extracted from porcine stomach using protocols published elsewhere (Ikebe and Hartshorne) and ^{32}P-ATP (1 mM; 0.5 mCi), and allowed to proceed for 20 min at 25°C; Y27632 was added to prevent ROCK-mediated phosphorylation of myosin. The reaction was terminated by addition of trichloroacetic acid and bovine serum albumin (1 mg/ml), left on ice for 10 min, then centrifuged for 10 min at 13,000g. Aliquots of supernatant were added to scintillation fluid and counted.

Solutions and chemicals Tissues were studied using Krebs-Ringer's buffer containing (in mM): NaCl, 116; KCl, 4.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.6; $MgSO_4$, 1.2; $NaHCO_3$, 22; D-glucose, 11; bubbled to maintain pH at 7.4. N-ω-nitro-L-arginine (L-NNA; 10^{-4} M) and indomethacin (10 μM) were also added to prevent generation of nitric oxide and of cyclo-oxygenase metabolites of arachidonic acid, respectively.

All chemicals were obtained from Sigma Chemical Company and prepared as 10 mM stock solutions, either as aqueous solutions (carbachol; ISO; SNAP) or in absolute EtOH

(salmeterol; (+)-(R)-trans-4-(1-aminoethyl)-N-(pyridyl) cyclohexanecarboxamide dihydrochloride [Y27632]) or DMSO ((+)-(R)-trans-4-(1-aminoethyl)-N-(pyridyl) cyclohexanecarboxamide dihydrochloride 11-2[[2-(diethylamino)methyl1]-1-piperidinyl] acetyl-5,11-dihydro-6H-pyrido-[2,3-b]-benzodiazepine-6-one [AFDX-116]). Aliquots were then added to the muscle baths, the final bath concentration of DMSO and EtOH did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

Data analysis Cholinergic contractions were expressed as a percentage of the response to 60 mM KCl added during the equilibration period (immediately before onset of the experiment), whereas adrenergic relaxations were expressed as reversals of pre-existing tone (evoked by CCh or KCl). Enzymatic activities are expressed as a percent of the activity observed in tissues flash-frozen immediately before addition of CCh (for cholinergic activation study) or those not receiving any inhibitory agonist (adrenergic inhibition study). Data are reported as mean ± S.E.M; n refers to the number of animals. Statistical comparisons were made using ANOVA with Student-Newman-Keuls *post hoc* test; $P<0.05$ was considered statistically significant.

RESULTS

Cholinergic and adrenergic concentration-response relationships in bovine TSM

We first sought to ascertain a concentration of carbachol (CCh) which was half-maximally effective in producing contraction, as well as the optimal concentrations of bronchodilators to be used in this study.

The concentration-response relationship for carbachol (CCh) in bovine tracheal strips was examined by challenging the tissues with increasing concentrations (10^{-10} to 10^{-5} M, in 10-fold increments) of carbachol in cumulative fashion. These cumulative challenges resulted in sustained contractions with threshold, half-maximally effective and maximally-effective CCh concentrations of 10^{-9} M, $1.6x10^{-7}$ M, and 10^{-5} M, respectively; these values correspond closely to those published previously (Kirkpatrick and Rooney) (Fig. 1).

In another set of tissues preconstricted with 10^{-7} M CCh for 20 minutes, we compared the concentration-response relationships for 3 different bronchodilators. ISO (10^{-6} M) is a short-acting, non-selective β-agonist with full agonist activity, whereas SAL (10^{-6} M) is a long-acting, β_2-selective agonist with only partial agonist activity; both signal through stimulation of adenylate cyclase activity (Kotlikoff and Kamm). SNAP (10^{-5} M), on the other hand, is a nitric oxide donor which acts through stimulation of guanylate cyclase. Since the relaxations evoked by SAL were much more delayed and slower in development than those evoked by ISO or SNAP (see Fig. 2A), we only examined the responses to 10^{-9} and 10^{-7} M SAL, but probed a more complete range of concentrations for ISO and SNAP (10^{-9} to 10^{-5} M, in 10-fold increments). The concentration-response relationships obtained in this way are given in Fig. 2B.

Relaxations to submaximal concentrations of ISO and SNAP exhibited a degree of tachyphylaxis. However, reversal of cholinergic tone was complete and sustained at micromolar concentrations (10^{-6} in the case of ISO, and 10^{-5} M in the case of SNAP).

SAL-evoked relaxations, on the other hand, developed much more slowly and did not exhibit tachyphylaxis over the duration of our experiments. This agent exerted just over 50% reversal of cholinergic tone when applied at 10^{-7} M; higher concentrations were not tested in this study.

Cholinergic regulation of Rho and ROCK activities and tone

Next, we investigated the temporal relationships between cholinergic stimulation of Rho and ROCK activities, as well as tone. Following the equilibration period, bovine tracheal strips were challenged with a half-maximally effective concentration of CCh ($2x10^{-7}$ M) and flash-frozen at various times ranging from 30 sec to 20 min, then assayed for Rho and ROCK activities. The data are given in Fig. 3A. Rho activity was markedly and significantly increased even 30 sec after addition of the cholinergic agonist, and reached a peak at 2 min, after which it fell toward baseline. As should be expected, changes in ROCK activity paralleled those in Rho activity, albeit with somewhat of a delay: this was not significantly elevated at 30 sec, reached a peak at 2 min, then decreased by half at 20 min following cholinergic stimulation (though this was still significantly above baseline level). The increase in mechanical tone lagged even further: this was negligible at 30 seconds, and less than a third of maximal at 2 min (when Rho and ROCK activities had peaked), reaching a stable plateau by 20 min. In contrast to the decay toward baseline in the enzymatic activities (above), peak contraction was followed by very little reversal of tone.

Increasing the concentration of CCh from $2x10^{-7}$ M to 10^{-6} M increased the magnitudes and rates of increase of Rho or ROCK activities, with parallel changes in tone (Fig. 3B). In particular, Rho activity was more than doubled above baseline by 2 minutes, and remained stimulated to this level by 20 minutes, whereas ROCK activity was increased approximately 60%

above baseline by 2 minutes and then declined somewhat over the next 15-20 minutes. As described above, tone was negligibly affected at 30 seconds (despite substantial enhancement of Rho and ROCK activities), but was nearly maximal by 10 minutes, with marginal decay in tone by 20 minutes.

Regulation of Rho and ROCK by ∃-agonists versus SNAP

We next compared cholinergic stimulation of Rho and ROCK activities and tone in tissues pretreated with ISO (10^{-6} M), SAL (10^{-7} M) or SNAP (10^{-5} M); given the longer time-course for SAL-mediated changes (Fig. 2A), tissues were pretreated for 30 minutes in the case of ISO or SNAP but 60 min in the case of SAL. Following this pretreatment, tissues were challenged with $2x10^{-7}$ M CCh, then flash frozen at different time points as described above. The data so obtained are summarized in Figure 4.

Neither the time-course nor the magnitude of Rho-activation were significantly affected by either of the two β-adrenoceptor agonists (Fig. 4A). SNAP, on the other hand, slowed the activation of Rho: peak activation now occurred at 5 min, compared to 2 min in the control tissues. Despite this marked slowing of Rho-activation, the overall peak magnitude of activation was not significantly different from that seen in the control tissues. Furthermore, deactivation of Rho did not appear to be affected by ISO nor SNAP: at 20 min, this too decreased by about half and was not significantly different from control.

The changes in Rho-activation summarized above were generally mirrored in the changes in ROCK-activation (Fig. 4B). Overall, neither of the two β-adrenoceptor agonists suppressed the rate nor magnitude of ROCK-activation compared to control tissues; in fact, at some time points these were greater than control tissues (Fig. 4B and 4C), although these differences did not reach statistical significance. In the SNAP-pretreated tissues, ROCK-activation was once

again slowed compared to control, with peak activation now occurring at 5 min, followed by nearly complete decay back to baseline by 20 min; however, the absolute magnitude of activation at peak and at 20 min was not significantly different from the control tissues.

Finally, the initial rate of development of tone was unaltered by either of the β-adrenoceptor agonists (Fig. 4C): tone in these tissues was not significantly different from control tissues at any time point during the first 5 minutes, but was significantly less than control at 20 minutes. SNAP pretreatment appeared to have no effect on development of cholinergic tone: there was no statistically significant difference at any time point.

Reversal of cholinergically-evoked responses

It has long been recognized that the relaxant effect of β-adrenoceptor-stimulation can vary depending on whether it precedes or follows excitatory stimulation of the smooth muscle (Tansey et al; Word et al; Kotlikoff and Kamm). We therefore re-examined inhibitory responses in tissues which were first preconstricted with CCh ($2x10^{-7}$ M), then challenged with ISO (10^{-7} M) or SNAP (10^{-5} M) for 20 min, or with SAL (10^{-7} M) for 30 minutes (because of its slower onset of action); comparisons were made with control tissues which were challenged with CCh ($2x10^{-7}$ M) alone for 40 min. All tissues were pretreated for 20 minutes prior to cholinergic stimulation with the M_2-selective cholinergic antagonist AFDX-116 (10^{-6} M) in order to obviate functional antagonism of the adrenergic responses via the M_2-receptors (Janssen and Daniel). All three agonists reversed cholinergically-stimulated Rho activation approximately 20%, but reversed ROCK activity by 30-40% (Fig. 5); the effects on ROCK activity were statistically significant. On the other hand, all three agonists significantly reversed cholinergically-stimulated MLCK activity approximately 75%, and nearly abolished cholinergic tone.

DISCUSSION

While many studies of excitation-contraction coupling in airway smooth muscle have focused on excitatory mechanisms, relatively fewer have scrutinized those underlying bronchodilator responses. Recently, a great deal of attention has been focused upon the RhoA/ROCK signaling pathway and its regulation by cholinergic agonists. Most of these have only used sensitivity of contractile responses to ROCK inhibitors such as Y27632 as indirect indices of Rho/ROCK activities (*i.e.*, these activities were not measured directly), and/or only used supramaximally-effective concentrations of cholinergic agonist. Few have examined RhoA/ROCK activities *per se* in ASM, and to our knowledge, none have done so during sub-maximal stimulation (which is more physiologically relevant) nor in the presence of bronchodilators (which is more clinically relevant). In this study, we examined the temporal relationships between RhoA and ROCK activities (measured directly) and tone using two submaximally-effective concentrations of CCh with and without simultaneous stimulation with two different β-agonists and a nitric oxide donor. Although we would like to have done this using the small airways (given their more important role in determining airflow resistance and in asthma), we found the considerable amounts of connective tissue, cartilage and epithelium to be problematic (data not shown): these made it difficult to standardize the amount of muscle prior to homogenization, especially when this involved flash-freezing, and the Rho/ROCK activities in the non-muscle cell types (particularly the epithelium) complicated interpretation of the data. For this reason, we instead used the trachealis smooth muscle, which has been shown to differ in some structural and functional respects from the small airways.

During half-maximally effective cholinergic stimulation, RhoA and ROCK activities were both enhanced markedly above baseline levels. Rho activity was rapidly increased (half of the overall response developed within the first 30 seconds), followed shortly thereafter by

increased ROCK activity; both activities peaked 2 minutes after onset of cholinergic stimulation, then decayed roughly half way back toward baseline by 20 minutes. The consequent changes in tone lagged considerably after these: development of tone was negligible at one minute (when Rho and ROCK activities were markedly increased), and took 20 minutes to reach a peak value (at which point Rho and ROCK activities decayed to roughly 50% of their peak values). This cholinergic tone is quite stable, being sustained for well over an hour (in other studies, we have seen this tone sustained for several hours; data not shown).

These cholinergic effects were dose-dependent in that increasing the concentration of CCh five-fold (still less than maximally-effective with respect to contractions) enhanced RhoA and ROCK activities nearly twice as much as did $2x10^{-7}$ M CCh. Moreover, the kinetics were accelerated, peak activation now occurring by 1 minute after cholinergic stimulation. Interestingly, we found Rho- and ROCK-activities then began to decrease toward baseline levels over the next 15-20 minutes, despite maintained stimulation with the nonhydrolyzable cholinergic agonist, and despite continued development of tone; this finding diverges from what has been shown in vascular and intestinal smooth muscles (Murthy et al; Sakurada et al), wherein Rho-/ROCK-stimulation is sustained (*i.e.*, does not exhibit a decrease toward baseline). Other excitation-contraction coupling mechanisms, such as Ca^{2+}-release (Bai and Sanderson), may become more important during these later periods of prolonged contraction. It could be argued that these measures of Rho/ROCK activities were underestimated, since the data were obtained in the presence of the non-steroidal anti-inflammatory steroid indomethacin (used here to inhibit endogenous prostanoid synthesis), which others have shown may inhibit Rho/ROCK signaling in some cell types (Zhou et al;Weggen et al). However, that novel effect of indomethacin requires concentrations of indomethacin considerably higher than those which we employed (100 μM (Weggen et al) *versus* 10 μM, respectively). That notwithstanding, the changes in Rho/ROCK

activities presented here using submaximal excitatory stimulation were on the same order as those shown previously in other cell types maximally-stimulated by other means (Murthy et al; Yoneda et al).

Regarding the second objective of the current study — the regulation of Rho and ROCK by bronchodilators — we found cholinergic stimulation of Rho and ROCK activities to be largely insensitive to pretreatment with either of the two β-adrenoceptor agonists: neither magnitude nor kinetics of RhoA/ROCK signaling were suppressed, although tone was markedly decreased. In fact, there was a suggestion that pretreatment with the β-agonists might slightly increase cholinergically-induced ROCK-activity (Fig. 4B): this could account in part for the finding that long-term or excessive use of β-agonists may actually worsen asthma (Sears). In contrast to their inability to prevent or slow the increase in Rho- and ROCK-activities induced by subsequent cholinergic challenge, both β-agonists did markedly reverse the levels of activity of these enzymes during the peak of cholinergic stimulation. In addition to their effect on RhoA/ROCK signaling, the β–agonists and SNAP markedly suppressed MLCK activity (Fig. 5), an effect which has been described in more detail previously (Kotlikoff and Kamm) and is beyond the scope of the current study. Others have previously documented this paradox — that the interactions between β-adrenergic and cholinergic stimulation in airway smooth muscle depends on the order in which these functionally opposing stimuli are delivered — and have attributed it to interactions between protein kinase A, myosin light chain kinase and/or other Ca^{2+}/calmodulin-dependent enzymes (Tansey et al; Word et al; Kotlikoff and Kamm). As such, the prophylactic use of bronchodilators may be less justified than their use as a rescue medication (Sears).

The nitric oxide donor SNAP, on the other hand, markedly slowed the activation of Rho

and ROCK upon cholinergic stimulation (although the peak and sustained levels of Rho-stimulation were not significantly different from the control tissues), in addition to its ability to suppress their activities following cholinergic stimulation. The effects of nitric oxide on RhoA translocation *per se* remain to be investigated.

The signaling events which couple activation of β-adrenoceptors or guanylate cyclase with changes in Rho-/ROCK-activities are as yet unclear. We have previously shown that Rho/ROCK activities are in part Ca^{2+}-dependent (Janssen et al; Liu et al), and β-agonists and nitric oxide are known to decrease $[Ca^{2+}]_i$ in airway smooth muscle: we did not control for changes in $[Ca^{2+}]_i$ in this study. Future studies of the mechanisms by which β-adrenoceptors couple to the Rho/ROCK signaling pathway will require a careful pharmacological dissection using agents which abrogate one or another of the excitation-contraction coupling pathways. In addition to cAMP and/or PKA, it is also possible that cGMP/PKG are involved, given the well-documented phenomenon of cross-talk between these two signaling pathways: several recent studies using vascular smooth muscle have documented an inhibition of Rho/ROCK via a cGMP-dependent pathway (Sauzeau et al; Murthy et al). As such, it will be necessary to use selective blockers of the two pathways to resolve this question. Another candidate for this coupling is telokin, a substrate of PKA and PKG, which has been shown to directly stimulate MLCP in vascular smooth muscle (Somlyo and Somlyo; Choudhury et al), but may also act by suppressing Rho/ROCK; there have been no investigations of this protein in the regulation of ROCK in ASM.

In conclusion, we have described for the first time the temporal relationships in airway smooth muscle between cholinergic stimulation of RhoA and ROCK activities and tone, and shown these to be inhibited by β-agonists and a nitric oxide donor, although the major effect of the bronchodilators appears to be on MLCK activity.

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FIGURE LEGENDS

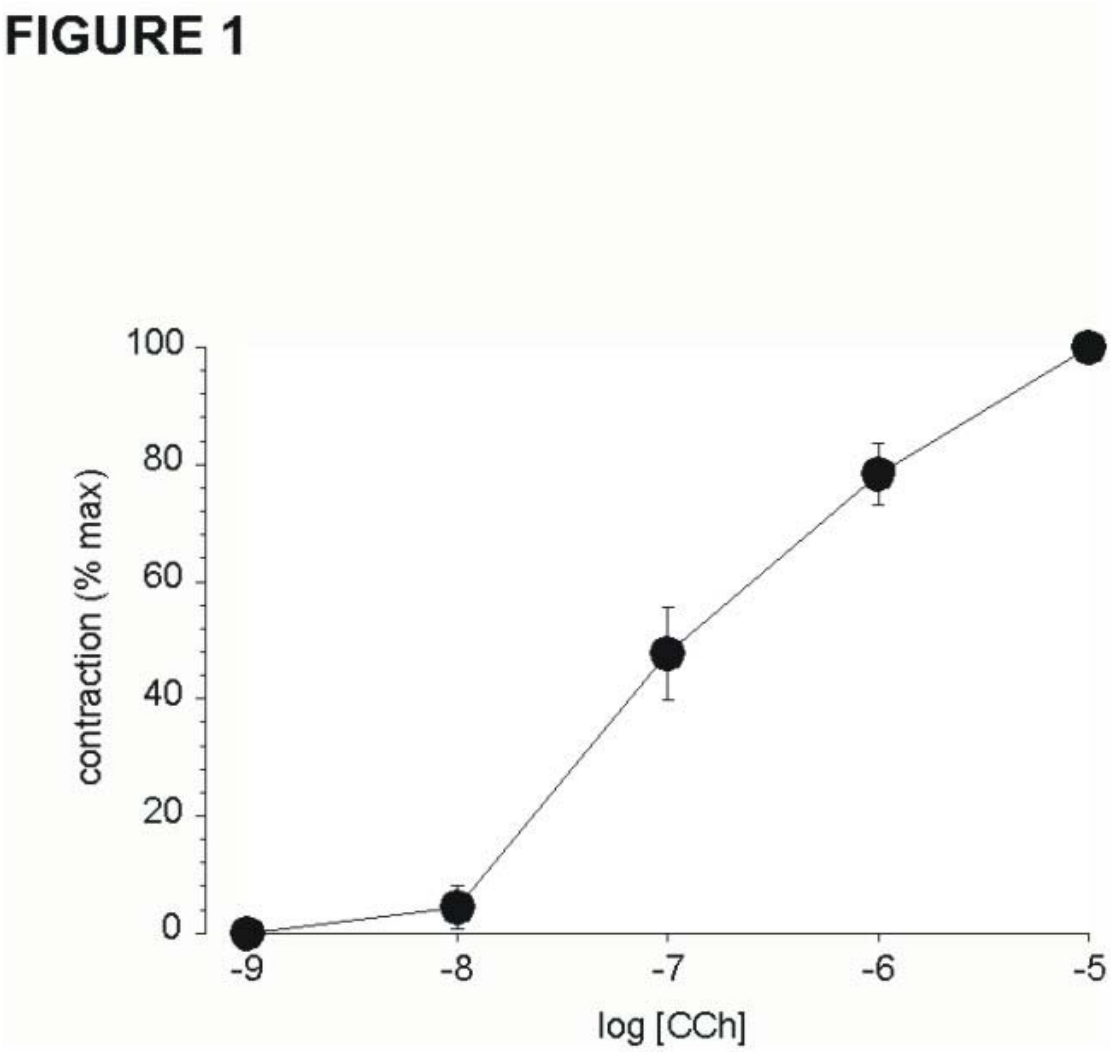

Figure 1 Cholinergic and adrenergic concentration-response relationships Mean concentration-response relationships for CCh-evoked contractions in bovine trachealis (n=5).

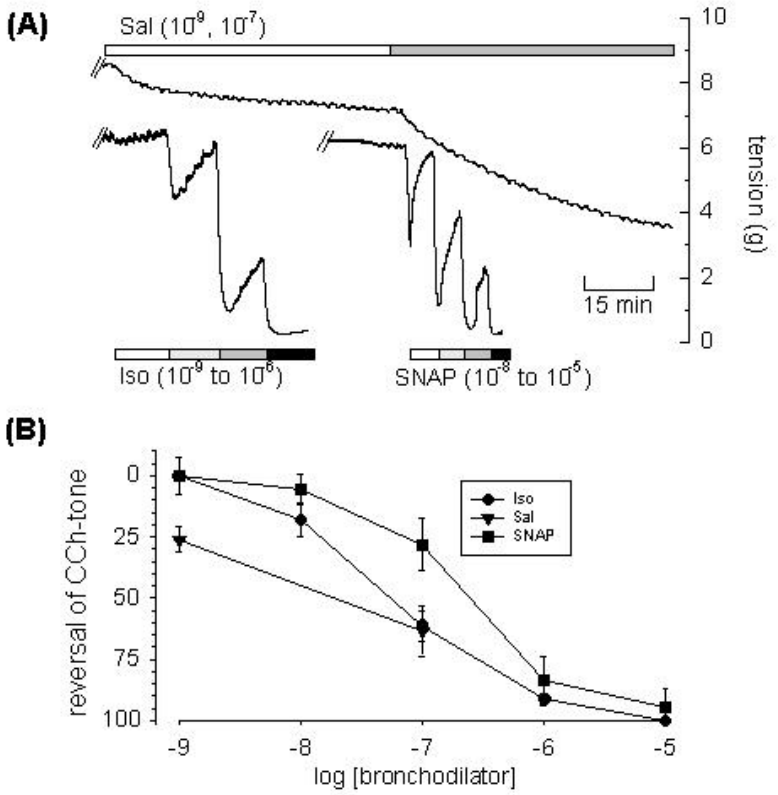

Figure 2 Comparison of relaxations evoked by ISO, SAL and SNAP **(A)** Representative tracings of the relaxations evoked by ISO, SAL and SNAP (concentrations as indicated by boxes) in bovine TSM preconstricted with 10^{-7} M CCh: the cholinergic constrictor response *per se* has been omitted to emphasize the much slower onset of SAL-mediated relaxation. Tachyphylaxis was often seen when submaximal concentrations of ISO or SNAP were used, but not at higher concentrations, or in the case of SAL. **(B)** Mean concentration-response relationships for ISO (●), SAL (▼) and SNAP (■) obtained using protocol illustrated in A) (n>5 for all)).

Figure 3

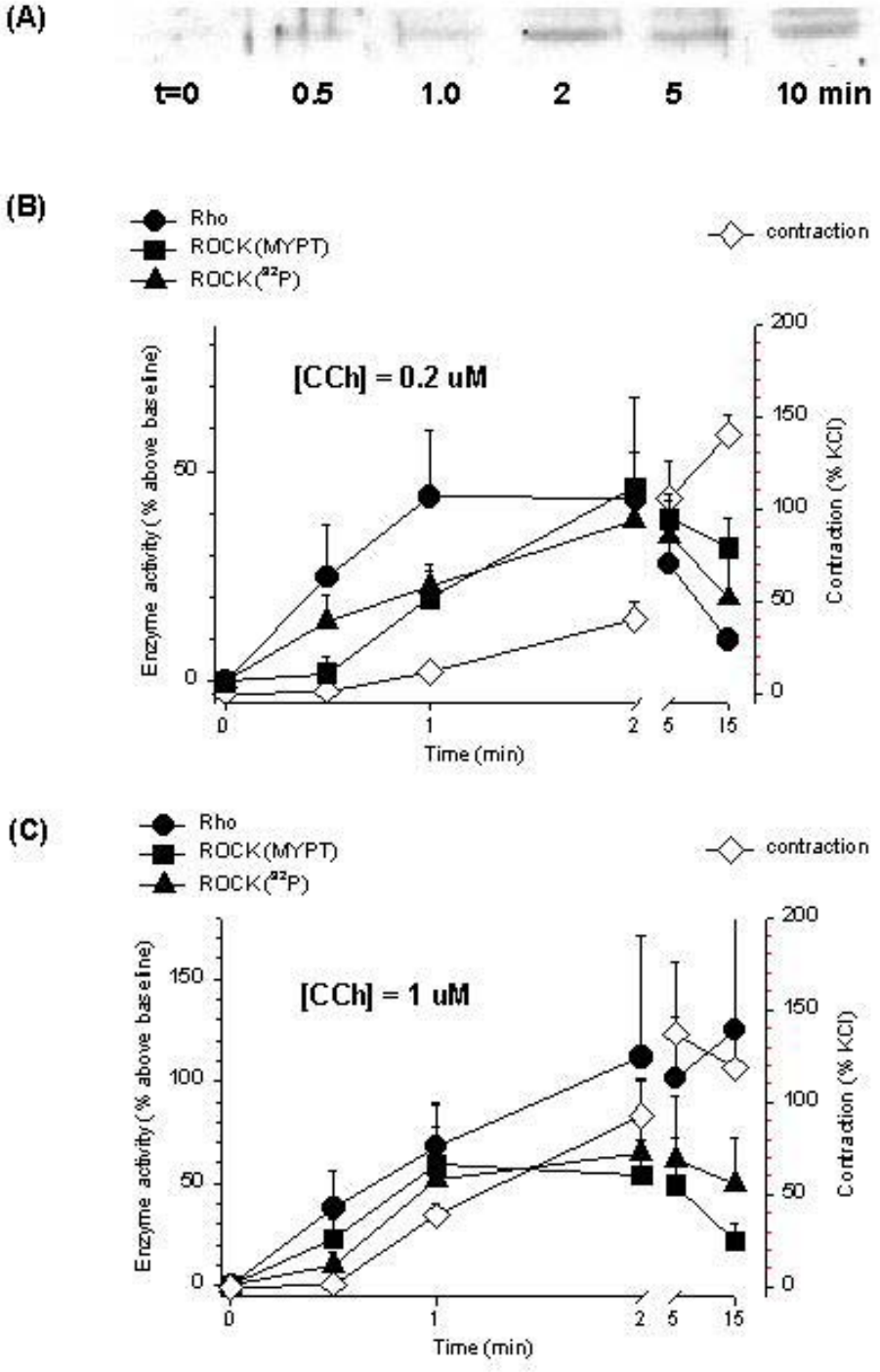
(A)
t=0
0.5
1.0
2
5
10 min
(B)
Rho
ROCK(MYPT)
ROCK(32P)
contraction
[CCh] = 0.2 uM
Enzyme activity (% above baseline)
Contraction (% KCl)
Time (min)
(C)
Rho
ROCK(MYPT)
ROCK(32P)
contraction
[CCh] = 1 uM
Enzyme activity (% above baseline)
Contraction (% KCl)
Time (min)

Figure 3 Kinetics of CCh-stimulated RhoA, ROCK and mechanical activities Tissues were challenged with either $2x10^{-7}$M (**A**; n=4) or 10^{-6} M (**B**; n=3) CCh, then flash frozen at times ranging from 0 to 20 minutes. Symbols indicate mean values (±SEM) of RhoA activity evaluated using rhotekin pull-down assay and Western blot (●), ROCK activity measured using either Western blot (phosphorylation of exogenous MYPT; ■) or by radiometric assay (incorporation of ^{32}P into MYPT; ▲), and tone (◇); see Methods for experimental details. Enzymatic effects are expressed as a % change above baseline; contractile responses are expressed as a percentage of the response evoked by 60 mM KCl during the equilibration period.

Figure 4

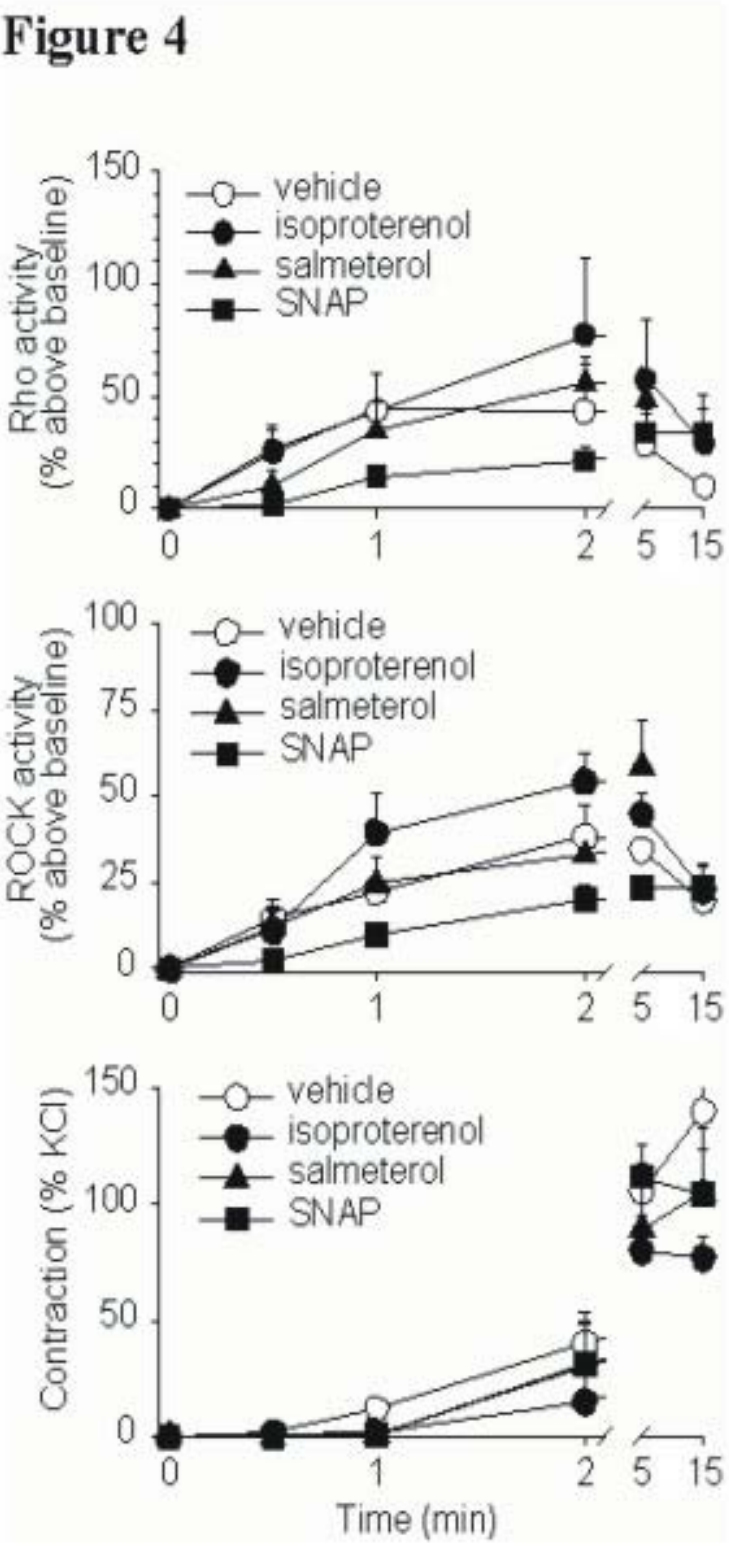
Rho activity
(% above baseline)
150
100
50
0
vehicle
isoproterenol
salmeterol
SNAP
0
1
2
5
15
ROCK activity
(% above baseline)
100
75
50
25
0
vehicle
isoproterenol
salmeterol
SNAP
0
1
2
5
15
Contraction (% KCl)
150
100
50
0
vehicle
isoproterenol
salmeterol
SNAP
0
1
2
5
15
Time (min)

Figure 4 Prevention of cholinergic effects on RhoA, ROCK, and mechanical activities by bronchodilators Tissues were pretreated with ISO (10^{-6}) or SNAP(10^{-5}) for 20 minutes, or with SAL (10^{-6}) for 30 minutes, then constricted with $2x10^{-7}$ Cch, then flash frozen at times ranging from 0 to 20 minutes before assaying RhoA and ROCK activities (**A** and **B**, respectively) and tone (**C**), in the same fashion as summarized in Fig. 2. Symbols indicate mean values (±SEM); n=4-6 for all groups.

FIGURE 5

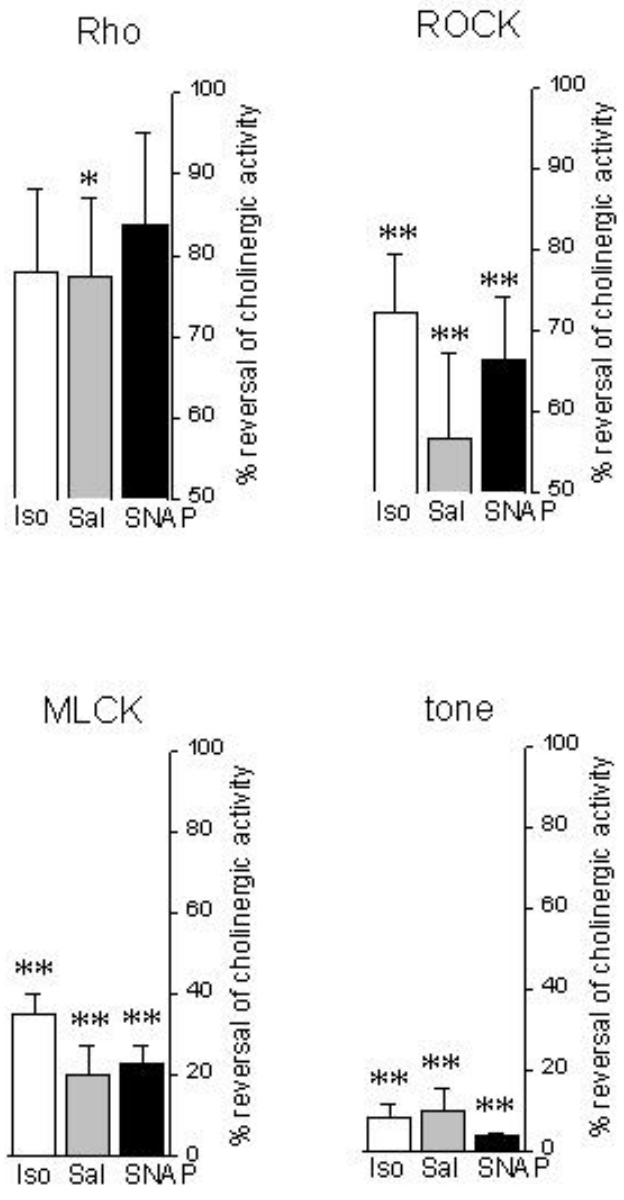
Rho
ROCK
MLCK
tone
% reversal of cholinergic activity
100
90
80
70
60
50
40
20
0
*
**
Iso
Sal
SNAP

Figure 5 Reversal of cholinergically-enhanced RhoA, ROCK, MLCK and mechanical activities by bronchodilators Tissues were pretreated for 20 minutes with the M_2-selective antagonist AFDX-116 (10^{-6} M), then preconstricted with CCh ($2x10^{-7}$ M) for another 20 minutes before addition of vehicle, ISO (10^{-7} M), or SNAP (10^{-5} M) for 20 min, or with SAL (10^{-7} M) for 30 minutes. Tissues were then flash frozen and assayed for Rho- ROCK- or MLCK- activities: the latter were expressed as a % of the activities measured in matched tissues challenged with CCh alone for 40 min. Tone existing immediately prior to flash-freezing was standardized as a percent of that existing immediately prior to addition of the bronchodilators. Bars indicate the mean changes in enzyme activities or tone induced by ISO (open bars), SNAP (filled bars) or SAL (shaded bars); n>5 for all. ANOVA was used to determine whether the decrease from control levels were statistically significant (*, $p<0.05$; **, $p<0.01$).