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8-Bromo-cAMP decreases the Ca\(^{2+}\) sensitivity of airway smooth muscle contraction through a mechanism distinct from inhibition of Rho-kinase

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Endou, Katsuki, Kunihiko Iizuka, Akihiro Yoshii, Hideo Tsukagoshi, Tamotsu Ishizuka, Kunio Dobashi, Tsugio Nakazawa, and Masatomo Mori. 8-Bromo-cAMP decreases the Ca\(^{2+}\) sensitivity of airway smooth muscle contraction through a mechanism distinct from inhibition of Rho-kinase. Am J Physiol Lung Cell Mol Physiol 287: L641–L648, 2004. First published April 30, 2004; 10.1152/ajplung.00287.2003.—To clarify whether cyclic AMP (cAMP)/cAMP-dependent protein kinase (PKA) activation and Rho-kinase inhibition share a common mechanism to decrease the Ca\(^{2+}\) sensitivity of airway smooth muscle contraction, we examined the effects of 8-bromoadenosine 3’5’-cyclic monophosphate (8-BrcAMP), a stable cAMP analog, and (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide dihydrochloride, monohydrate (Y-27632), a Rho-kinase inhibitor, on carbachol (CCh)-, guanosine 5’-O-(3-thiotriphosphate) (GTP\(\gamma\)S), 4β-phorbol 12,13-dibutyrate (PDBu)-, and leukotriene D\(_4\) (LT\(_D_4\))-induced Ca\(^{2+}\) sensitization in \(\alpha\)-toxin-permeabilized rabbit tracheal and human bronchial smooth muscle. In rabbit trachea, CCh-induced smooth muscle contraction was inhibited by 8-BrcAMP and Y-27632 to a similar extent. However, GTP\(\gamma\)S-induced smooth muscle contraction was resistant to 8-BrcAMP. In the presence of a saturating concentration of Y-27632, PDBu-induced smooth muscle contraction was completely reversed by 8-BrcAMP. Conversely, PDBu-induced smooth muscle contraction was resistant to Y-27632. In the presence of a saturating concentration of 8-BrcAMP, GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization was also reversed by Y-27632. The 8-BrcAMP had no effect on the ATP-triggered contraction of tracheal smooth muscle that had been treated with calyculin A in rigor solutions. The 8-BrcAMP and Y-27632 additively accelerated the relaxation rate of PDBu- and GTP\(\gamma\)S-treated smooth muscle under myosin light chain kinase-inhibited conditions. In human bronchus, LT\(_D_4\)-induced smooth muscle contraction was inhibited by both 8-BrcAMP and Y-27632. We conclude that cAMP/PKA-induced Ca\(^{2+}\) desensitization contains at least two mechanisms: 1) inhibition of the muscarinic receptor signaling upstream from Rho activation and 2) cAMP/PKA’s preferential reversal of PKC-mediated Ca\(^{2+}\) sensitization in airway smooth muscle.

\begin{align*}
\text{calcium sensitization; cAMP; leukotriene D\(_4\)}
\end{align*}

ALTHOUGH INTRACELLULAR calcium concentration Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is the primary regulator of smooth muscle contraction, Ca\(^{2+}\) sensitivity of the contractile apparatus can change in response to agonists. An increase and a decrease in muscle tension at a constant Ca\(^{2+}\) concentration are correspondingly referred to as Ca\(^{2+}\) sensitization and desensitization of smooth muscle contraction. These are believed to be the results of changes in the ratio of kinase and phosphatase activities toward the 20-kDa light chain of myosin (MLC\(_{20}\)) (22, 25). We have demonstrated that receptor-dependent, G protein-mediated Ca\(^{2+}\) sensitization occurs in canine, rabbit, and human airway smooth muscles (28) and that a small G protein, RhoA, and its target protein, Rho-kinase, play a key role in G protein-mediated Ca\(^{2+}\) sensitization of smooth muscle contraction (26), especially in the sustained phase. Rho/Rho-kinase signaling increases phosphorylation of MLC\(_{20}\) through the inhibition of myosin light chain phosphatase (MLCP)-associated mechanisms, but it does not directly phosphorylate the MLC\(_{20}\) of airway smooth muscle in situ (13).

Adenosine 3’5’-cyclic monophosphate (cAMP)-elevating agents such as β-adrenergic agonists and phosphodiesterase (PDE) inhibitors are most widely used clinically to relax airway smooth muscle. Elevated cAMP not only decreases net [Ca\(^{2+}\)]\(_i\), by enhancing Ca\(^{2+}\) extrusion to the extracellular space and Ca\(^{2+}\) sequestration to the [Ca\(^{2+}\)]\(_j\) store but also decreases calcium sensitization in airway smooth muscle.

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We obtained the desired concentration of free Ca\(^{2+}\) by mixing the GTP\(\gamma\)S-, and 4\(\beta\)-phorbol 12,13-dibutyrate (PDBu)-induced Ca\(^{2+}\) sensitization. We wanted to determine the following: whether 8-BrcAMP blocks CCh- or GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization in a similar manner to Y-27632; whether 8-BrcAMP affects myosin light chain kinase (MLCK)-associated mechanisms or MLCP-associated mechanisms; whether 8-BrcAMP reverses PDBu-induced Ca\(^{2+}\) sensitization in the presence of a saturating concentration of Y-27632, because the inhibitory effect of Y-27632 on GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization is affected by 8-BrcAMP; and whether the Y-27632- and 8-BrcAMP-responsive mechanisms are involved in leukotriene D\(_4\) (LTD\(_4\))-induced Ca\(^{2+}\) sensitivity in human bronchial smooth muscle.

**MATERIALS AND METHODS**

**Tissue preparation and isometric force measurement.** The tissue preparation and force measurement have been reported elsewhere (9, 10, 12). We administered the anesthesia by placing the animals in an anesthetic chamber until the animals became anesthetized and unresponsive to corneal stimulation. When the tracheal tissue had been removed, the animals were killed by rapid exsanguination through the carotid artery, in accordance with the recommendations of the Animal Care and Experimentation Committee, Gunma University, Showa Campus. The airways were first cut longitudinally at the center of the cartilage opposite the smooth muscle. Small strips of tracheal smooth muscle were then carefully separated from connective tissue, epithelium, and cartilage with a razor blade under a binocular microscope.

Human bronchial smooth muscle was prepared from a macroscopically normal section of lung tissue that was obtained during surgery for lung cancer. Consent was obtained from each patient before surgery. The surgically resected tissue was put in ice-cold Dulbecco’s modified Eagle’s medium, and small bronchi with an outer diameter of 2–4 mm were carefully dissected as previously described (28). Cartilage was removed to the greatest extent possible. Small strips of rabbit tracheal smooth muscle (200–300 \(\mu\)m wide, 40–50 \(\mu\)m thick, 3 mm long) and human bronchial smooth muscle (150–200 \(\mu\)m wide, 20–30 \(\mu\)m thick, 3 mm long) were mounted on a bubble plate (400 ml per bubble), and isometric force development was measured with a force transducer (AE801; SensoNor, Horten, Norway). The developed force was normalized to an initial pCa 5.0 (10\(^{-5}\) M) response in the same strip (12, 28). Consent was obtained from each patient before surgery. These protocols were approved by the Institutional Review Board, Gunma University Faculty of Medicine, School of Medicine. **Solutions and permeabilization with \(\alpha\)-toxin.** The method of permeabilization with \(\alpha\)-toxin has been described previously (12, 28). The trachea was permeabilized with \(\alpha\)-toxin (16.4 \(\mu\)g/ml) for 30 min at 30°C. We added a Ca\(^{2+}\) ionophore, A-23187 (10 \(\mu\)M), to the trachea during \(\alpha\)-toxin permeabilization to block the sarcoplasmic reticulum. After permeabilization, all experiments except for that depicted in Fig. 5 were performed at 24°C (9, 10, 12).

The normal relaxing solution (G1) contained (in mM) 74.1 potassium methanesulfonate, 2 Mg\(^{2+}\), 4.5 ATP (Mg\(^{2+}\) salt), 1 EGTA, 10 creatine phosphate, and 30 PIPES-KOH (pH 7.1 at 24°C, ionic strength 0.2). The same solution containing 10 mM EGTA rather than 1 mM EGTA and various amounts of calcium methanesulfonate was used to achieve the desired concentration of free Ca\(^{2+}\). According to Zimmermann et al. (29), we prepared EGTA (10 mM)-buffered ATP-free (rigor) Ca\(^{2+}\)-free solution (G10 rigor) and ATP-free high Ca\(^{2+}\) solution (pCa 4.5 rigor). These rigor solutions contained 50 \(\mu\)M P\(^{1}\)P\(^{3}\)di(adenosine-5') pentaphosphate, an inhibitor of kinase activity. We estimated the desired concentration of free Ca\(^{2+}\) by mixing Y-27632 with G10 rigor and pCa 4.5 rigor solutions. All solutions contained ibuprofen (2 \(\mu\)M) to avoid cyclooxygenase activity that may attenuate airway smooth muscle tone.

**Results**

Effects of 8-BrcAMP and Y-27632 on CCh- or GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization in \(\alpha\)-toxin-permeabilized tracheal smooth muscle. After obtaining the maximum contraction at pCa 5.0, we incubated the tracheal smooth muscle with 8-BrcAMP (100 \(\mu\)M) in G10 (containing ATP) for 10 min. In the continued presence of 8-BrcAMP, the tracheal smooth muscle was quickly transferred to a G10 rigor solution containing calyculin A (300 nM) for 55 min to inactivate MLCP without contraction. After incubation of the tracheal smooth muscle in a pCa 5.0 rigor solution containing 8-BrcAMP and calyculin A for 5 min, ATP-triggered contraction was initiated. If 8-BrcAMP inhibits MLCP-dependent contraction, the rate of ATP-triggered contraction would be slowed by 8-BrcAMP. Time-matched, ATP-triggered experiments were carried out in the absence of 8-BrcAMP, as controls.

Comparison of relaxation rates between PKA activation and Rho inhibition of \(\alpha\)-toxin-permeabilized tracheal smooth muscle. To compare the effects of PKA activation and Rho-kinase inhibition on MLCK-dependent contraction and relaxation in situ, we measured the relaxation rate of fully contracted tracheal smooth muscle in the presence of 8-BrcAMP (100 \(\mu\)M), Y-27632 (3 \(\mu\)M), or both at 10°C. The low temperature conditions were required for comparing relaxation rates, because the relaxation rate at 24°C was too fast to evaluate the reagent effects on relaxation (19, 20). The tracheal smooth muscle was fully contracted by high Ca\(^{2+}\) with PDBu or GTP\(\gamma\)S and then relaxed by combined treatment with Ca\(^{2+}\) removal and 1-(5-chlonoraphthalene-1-sulfonfyl) homopiperadine-HCl (ML-9), an MLCK inhibitor. We measured the half-time of the relaxation rate (time required to reach 50% of maximum relaxation induced by a given inhibitor) in the absence or presence of 8-BrcAMP. Y-27632, or both (Fig. 5).

Inhibitory effect of 8-BrcAMP and Y-27632 on LTD\(_4\)-induced Ca\(^{2+}\) sensitization in \(\alpha\)-toxin-permeabilized human bronchial smooth muscle. When submaximum contraction induced by pCa 6.8 plus GTP (3 \(\mu\)M) was stable, LTD\(_4\) (1 \(\mu\)M) was added to the \(\alpha\)-toxin-permeabilized human bronchial smooth muscle. At the peak of additional contractions, Y-27632 (3 \(\mu\)M), 8-BrcAMP (100 \(\mu\)M), or both were added to the strip.

Reagents. **Staphylococcus aureus** \(\alpha\)-toxin was obtained from RBI (Natick, MA); P\(^{1}\)P\(^{3}\)di(adenosine-5') pentaphosphate was from Sigma (St. Louis, MO). The Y-27632 was a gift from Mitsubishi Pharma (Osaka, Japan). The Y-27632 was dissolved in distilled water to create a 10 mM stock solution, which was stored at −20°C until use. The GTP\(\gamma\)S was purchased from Boehringer Mannheim (Indianapolis, IN). The 8-BrcAMP, calyculin A, and PDBu were purchased from Calbiochem (La Jolla, CA). The LTD\(_4\) was purchased from Sigma. All other chemicals were of reagent grade.

Statistical analysis. Data were normalized to the pCa 5.0 response measured before the reagent treatment of each strip and are shown as means ± SE of the indicated numbers of experiments. Data were compared by the Mann-Whitney U-test or Student’s t-test with the Bonferroni correction for multiple comparisons. A P value of <0.05 was considered to be statistically significant.

**Results**

Effects of 8-BrcAMP and Y-27632 on CCh- or GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization in \(\alpha\)-toxin-permeabilized tracheal smooth muscle. After obtaining the maximum contraction at pCa 5.0, we incubated the strip in G1 containing 8-BrcAMP, Y-27632, or saline for 20 min. In the continuous presence of the reagents, the tracheal smooth muscle was precontracted in

Effects of 8-BrcAMP and Y-27632 on CCh- or GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization in \(\alpha\)-toxin-permeabilized tracheal smooth muscle. After obtaining the maximum contraction at pCa 5.0, we incubated the strip in G1 containing 8-BrcAMP, Y-27632, or saline for 20 min. In the continuous presence of the reagents, the tracheal smooth muscle was precontracted in
a pCa 6.5 solution containing GTP (3 μM); CCh (100 μM) was then applied to the strip. In the experiments of GTPγS-induced Ca\(^{2+}\) sensitization, the tracheal smooth muscle was precontracted in a pCa 6.5 solution without GTP, followed by the application of GTPγS (10 μM) to the tracheal smooth muscle. As shown in Fig. 1A, CCh increased the contractile force from the steady-state level at pCa 6.5 (15.8 ± 2.2%) to 82.2 ± 5.0% (n = 6). Pretreatment with 8-BrcAMP dose dependently inhibited the agonist-induced smooth muscle contraction. The effect of 8-BrcAMP was saturated at 100 μM, and the extent of the maximum inhibition by 8-BrcAMP was comparable with that by Y-27632 at 100 μM. Similarly, GTPγS caused rapid contractions from 4.99 ± 1.3 to 97.4 ± 3.5% (n = 8) at pCa 6.5, and the GTPγS response was inhibited by Y-27632 (100 μM). The inhibitory effect of 8-BrcAMP was only partial in GTPγS-induced smooth muscle contraction, and the resultant contraction was 60.6 ± 9.1% in the presence of 8-BrcAMP at 300 μM (n = 6, Fig. 1B). Thus the GTPγS response was relatively more resistant to 8-BrcAMP.

Lack of effect of 8-BrcAMP on ATP-triggered contraction of calyculin A-treated tracheal smooth muscle. As shown in Fig. 2A, with or without 8-BrcAMP (100 μM), ATP elicited rapid contractions of tracheal smooth muscle that had been treated with calyculin A in the rigor solutions. The final force developments were not different between the two groups (80 ± 7.2% in the control, 80.9 ± 5.3% in the 8-BrcAMP-treated group, n = 4), and the values of T\(_{1/2}\) (time required to reach 50% of maximum force induced by a given stimulant) in the control and the 8-BrcAMP-treated strips were also comparable (63.1 ± 3.6 and 61.9 ± 7.6 s, respectively; n = 4). Thus 8-BrcAMP did not affect the MLCK-associated mechanisms.

Involvement of a distinct mechanism between 8-BrcAMP- and Y-27632-induced decreases in Ca\(^{2+}\) sensitivity. To find a qualitative difference between 8-BrcAMP- and Y-27632-induced changes in Ca\(^{2+}\) sensitivity, we attempted inhibition of PDBu-induced Ca\(^{2+}\) sensitization by 8-BrcAMP in the presence of a saturating concentration of Y-27632. As shown in Fig. 3A, after obtaining the maximum contraction at pCa 5.0, we treated the tracheal smooth muscle with Y-27632 (30 μM) for 20 min, and then PDBu (10 μM)-induced Ca\(^{2+}\) sensitization was evoked at pCa 6.5. We verified that the concentration of Y-27632 was saturated, because an increase in the concentration of Y-27632 from 30 to 100 μM had no effect on force. In the saturated concentration of Y-27632, 8-BrcAMP dose dependently reversed contraction of the tracheal smooth muscle. Time-matched experiments were carried out in the absence of Y-27632 as controls. As shown in Fig. 3B, although Y-27632 showed a tendency to decrease PDBu-induced contractions, the contractions before the application of 8-BrcAMP were not significantly different from those in the controls.

Fig. 1. Inhibitory effect of 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP) or Y-27632 on carbachol (CCh)- or guanosine 5′-O-(3-thiotriphosphate) (GTPγS)-induced Ca\(^{2+}\) sensitization. After a stable pCa 5.0 response was obtained, the a-toxin-permeabilized tracheal smooth muscle was incubated in G1 containing saline, 8-BrcAMP (0.1–300 μM), or Y-27632 (100 μM) for 20 min. In the continuous presence of reagents, the tracheal smooth muscle was precontracted in a pCa 6.5 solution containing GTP (3 μM); then CCh (100 μM) was applied to the strips (n = 3–6, A). In the experiments of GTPγS-induced Ca\(^{2+}\) sensitization, tracheal smooth muscle was precontracted in a pCa 6.5 solution, followed by the application of GTPγS (10 μM) to the trachea (n = 3–8, B). Relative force was normalized to saline.

Fig. 2. Lack of effect 8-BrcAMP on kinase activity toward 20-kDa myosin light chain (MLC\(_{20}\)). After obtaining the maximum contraction at pCa 5.0, we relaxed the tracheal smooth muscle in GI0 solution. To activate PKA, we incubated the tracheal smooth muscle with 8-BrcAMP (100 μM) in GI0 (containing ATP) for 10 min. In the continued presence of 8-BrcAMP, tracheal smooth muscle was quickly transferred to a GI0 ATP-free (rigor) solution containing calyculin A (300 nM) for 55 min to inactivate myosin light chain phosphatase (MLCP) without contraction. After incubation of tracheal smooth muscle in a pCa 6.5 rigor solution containing 8-BrcAMP and calyculin A for 5 min, ATP-triggered contraction was initiated (A). The final force developments were not different between the two groups, and the values of the time required to reach 50% of maximum force induced by a given stimulant in the control strips (c) and the 8-BrcAMP-treated strips (○) were also comparable (n = 4, B). Relative force was normalized to the initial pCa 5.0 (10\(^{-5}\) M) response in the same strip.
were not statistically significant \((P = 0.147, \text{Mann-Whitney test})\), and the IC\(_{50}\) values with or without Y-27632 were comparable; the amplitude of the PDBu response and IC\(_{50}\) values for 8-BrcAMP in the Y-27632-treated group were 53.1 ± 7.1\% and 5.24 ± 0.8 \(\mu M\) \((n = 7)\), whereas those in the control group were 70.0 ± 8.1\% and 8.21 ± 1.0 \(\mu M\) \((n = 11)\), respectively.\(\text{Relative force was normalized to the initial pCa 5.0 (10}^{-5} \text{M)}\) response in the same strip.

Next, we verified inhibition of GTP\(_\text{yS}\)-induced \(\mathrm{Ca}^{2+}\) sensitization by Y-27632 in the presence of 8-BrcAMP (Fig. 4). The amplitude of the GTP\(_\text{yS}\) response and IC\(_{50}\) values for Y-27632 in the 8-BrcAMP-treated group were 81.4 ± 7.0\% and 3.06 ± 0.5 \(\mu M\) \((n = 6)\), whereas those in the control group were 104.9 ± 7.0\% and 2.24 ± 0.7 \(\mu M\) \((n = 5)\), respectively.

**Time course of relaxation of \(\alpha\)-toxin-permeabilized trachea in the presence of 8-BrcAMP, Y-27632, or both.** We measured the relaxation rates of fully contracted tracheal smooth muscle in the presence of 8-BrcAMP, Y-27632, or both at 10\(^\circ\)C to estimate the effect of MLCP-associated mechanisms. Figure 5 shows relaxation by ML-9/G10 after treatment with Y-27632, 8-BrcAMP, or both in \(\alpha\)-toxin-permeabilized rabbit tracheal smooth muscle following PDBu- or GTP\(_\text{yS}\)-induced \(\mathrm{Ca}^{2+}\) sensitization. After obtaining the maximum contraction at pCa 5.0 at 10\(^\circ\)C, we precontracted the strips in a pCa 6.5 solution containing PDBu (10 \(\mu M\)) or GTP\(_\text{yS}\) (100 \(\mu M\)). The strips were then treated with a pCa 4.5 solution containing either PDBu or GTP\(_\text{yS}\) and the inhibitors 8-BrcAMP (100 \(\mu M\)) or Y-27632 (3 \(\mu M\)) or both as indicated in Fig. 5. In the continuous presence of all reagents, the strips were moved into a G10 solution containing ML-9 (100 \(\mu M\)). The 8-BrcAMP accelerated the relaxation rate of PDBu-treated strips but not that of GTP\(_\text{yS}\)-treated strips. In contrast, Y-27632 was effective in GTP\(_\text{yS}\)-treated strips, but not in PDBu-treated strips (Table 1).

**Inhibitory effects of 8-BrcAMP and Y-27632 on LTD\(_4\)-induced \(\mathrm{Ca}^{2+}\) sensitization in \(\alpha\)-toxin-permeabilized human bronchial smooth muscle.** To estimate the relative contributions of PKA activation and Rho-kinase inhibition to LTD\(_4\)-mediated \(\mathrm{Ca}^{2+}\) sensitization, we treated strips of permeabilized human bronchial smooth muscle with 8-BrcAMP (100 \(\mu M\)), Y-27632 (3 \(\mu M\)), or both. Sensitization to \(\mathrm{Ca}^{2+}\) was evoked with a saturated solution of 1 \(\mu M\) LTD\(_4\). The pCa 5.0-induced contractions before \(\mathrm{Ca}^{2+}\) sensitization were 1.81 ± 0.17 \(\text{mN}\) in human bronchial smooth muscle \((n = 8)\). As shown in Fig. 6, in the presence of GTP (3 \(\mu M\)), LTD\(_4\) (1 \(\mu M\)) induced an

![Image](https://example.com/image1.png)

**Fig. 3.** Inhibition of 4\(\beta\)-phorbol 12,13-dibutyrate (PDBu)-induced \(\mathrm{Ca}^{2+}\) sensitization by 8-BrcAMP in the presence of Y-27632. After obtaining the maximum contraction at pCa 5.0, we treated tracheal smooth muscle with Y-27632 (30 \(\mu M\)) or saline for 20 min, and then PDBu (10 \(\mu M\))-induced \(\mathrm{Ca}^{2+}\) sensitization was evoked at pCa 6.5. After we verified that the concentration of Y-27632 was saturated, 8-BrcAMP dose dependently reversed contraction of the trachea \((A)\), although Y-27632 tended to decrease PDBu-induced contractions. The contractions before 8-BrcAMP application were not statistically significant \((P = 0.147, \text{Mann-Whitney } U\text{-test})\), and the IC\(_{50}\) values with or without Y-27632 were also comparable; the amplitude of the PDBu response and the IC\(_{50}\) values for 8-BrcAMP in the Y-27632-treated group \((\circ)\) were 53.1 ± 7.1\% and 5.24 ± 0.8 \(\mu M\) \((n = 7)\); those in the control group \((\odot)\) were 70.0 ± 8.1\% and 8.21 ± 1.0 \(\mu M\) \((n = 11)\), respectively.**

**Fig. 4.** Inhibition of GTP\(_\text{yS}\)-induced \(\mathrm{Ca}^{2+}\) sensitization by Y-27632 in the presence of 8-BrcAMP. After obtaining the maximum contraction at pCa 5.0, we treated the trachea with 8-BrcAMP (30 \(\mu M\)) for 20 min, and then GTP\(_\text{yS}\) (10 \(\mu M\))-induced \(\mathrm{Ca}^{2+}\) sensitization was evoked at pCa 6.5. After we verified that the concentration of 8-BrcAMP was saturated, 8-BrcAMP dose dependently reversed contraction of the tracheal smooth muscle \((A)\). The amplitude of the GTP\(_\text{yS}\) response and the IC\(_{50}\) values for Y-27632 in the 8-BrcAMP-treated group \((\circ)\) were 81.4 ± 7.0\% and 3.06 ± 0.5 \(\mu M\) \((n = 6)\); those in the control group \((\odot)\) were 104.9 ± 7.0\% and 2.24 ± 0.7 \(\mu M\) \((n = 5)\), respectively.**
additional contraction at a fixed free Ca\(^{2+}\) concentration of pCa 6.8 in α-toxin-permeabilized human bronchial smooth muscle. The peak force achieved was 70.4 ± 4.7\% (n = 8) of the initial contraction at pCa 5.0. The LTD\(_4\) response was reversed by 8-BrcAMP to 43.6 ± 3.7\%, by Y-27632 to 33.7 ± 6.0\%, and by their combination to 9.14 ± 2.3\% (n = 4–8, Table 1).

**Table 1. Effects of 8-BrcAMP, Y-27632, or both in α-toxin-permeabilized rabbit tracheal smooth muscle with PDBu-, GTP\(\gamma\)S-, or LTD\(_4\)-induced Ca\(^{2+}\) sensitization**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Half-Time of Relaxation, s</th>
<th>Maximum Contraction at pCa 5.0, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDBu</td>
<td>GTP(\gamma)S</td>
</tr>
<tr>
<td>Control</td>
<td>411 ± 37.4</td>
<td>373 ± 37.5</td>
</tr>
<tr>
<td>8-BrcAMP</td>
<td>274 ± 15.3*</td>
<td>382 ± 22.2</td>
</tr>
<tr>
<td>Y-27632</td>
<td>352 ± 8.95</td>
<td>269 ± 7.51\†</td>
</tr>
<tr>
<td>8-BrcAMP + Y-27632</td>
<td>247 ± 15.7*</td>
<td>253 ± 1.52\†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Half-times of relaxation of the strips pretreated with 8-bromoadenosine 3’,:5’-cyclic monophosphate (8-BrcAMP, 100 μM) alone, Y-27632 (3 μM) alone, and both followed by 4β-phorbol 12,13-dibutyrate (PDBu)-induced (10 μM) or guanosine 5’-O-(3-thiotriphosphate) (GTP\(\gamma\)S)-induced (100 μM) contraction. Percentage of maximum contraction at pCa 5.0 showed that force development of 8-BrcAMP (100 μM), Y-27632 (3 μM), and both added at the peak of leukotriene D\(_4\) (LTD\(_4\))-induced contraction was normalized to the initial pCa 5.0 response. Data are given as Bonferroni corrections for multiple comparisons: control (saline) vs. 8-BrcAMP, Y-27632, or both, \* and \†, \‡ and \§ and \‡P < 0.05 vs. control (bullet, n = 4–6). Relative force was normalized to the initial pCa 5.0 (10\(^{-5}\) M) response in the same strip.

**DISCUSSION**

β-Adrenergic agonists can be understood as a cascade involving activation of adenyl cyclase, elevation of cytoplasmic cAMP levels, and PKA activation leading to phosphorylation of target proteins. However, the precise mechanisms are still unknown. In Fig. 2, the contractile rate (T\(_{1/2}\)) was comparable between 8-BrcAMP-treated strips and control strips. The T\(_{1/2}\) of the strips treated with a phosphatase inhibitor (such as microcystin-LR or calyculin A) is dependent on the MLCK-
associated mechanisms in the presence of Ca\(^{2+}\) (13, 16). These results suggest that cAMP/PKA signaling preferentially affects MLCP-associated mechanisms of Ca\(^{2+}\) sensitization in rabbit tracheal smooth muscle. The relaxing rate was mainly dependent on MLCP under our experimental conditions (19, 20). We considered that cellular events other than MLCK/MLCP-induced mechanisms might be involved in our experiment conditions. Previous studies have suggested that MLCK/MLCP-associated mechanisms play a key role in at least the initiation of contraction and early phase of maintenance of force (19, 20). Many cellular events other than MLCK/MLCP-induced mechanisms may be slower than changes in the phosphorylation state of myosin and thus rate limiting. However, precise time-course studies suggest that initiation of contraction occurred within milliseconds and that the early phase of maintenance of force occurred within several seconds (25). Furthermore, we have demonstrated that MLCK inhibition with wortmannin but not Rho-kinase inhibition with Y-27632 slowed force developments under the same experimental conditions (13). The final amplitudes were not different. Therefore, other cellular events appear to precede the development of maximum force by minutes.

The 8-BrcAMP accelerated the relaxation rate in PDBu-treated strips but not in GTP\(\gamma\)S-treated strips (Fig. 5). In contrast, Y-27632 accelerated the relaxation rate in GTP\(\gamma\)S-treated strips but not in PDBu-treated strips. It is difficult to assess cellular events that occur much faster than the force changes. A simple delay mechanism may explain the longer half-time of relaxation but not the shorter half-time of relaxation. This is why we decreased the temperature conditions in the relaxation experiments. These results suggest that cAMP/ PKA signaling impairs the inhibition of MLCP-mediated responses by PKC signaling but not by the Rho/Rho-kinase signaling. Alternatively, this observation could have mechanistic implications; for example, one of many interpretations would be that 8-BrcAMP modulates activity of MLCP-associated mechanisms whereas Y-27632 modulates activation of MLCP-associated mechanisms, because 8-BrcAMP accelerates the rate of relaxation without changing the time of onset, whereas Y-27632 prolongs the onset of relaxing rate without changing its rate (Fig. 5, B and C).

Muscarinic receptor signaling for airway smooth muscle contraction contains Ca\(^{2+}\) sensitization mechanisms mediated by Rho/Rho-kinase (14, 28). Although CCh-induced Ca\(^{2+}\) sensitization was inhibited by both 8-BrcAMP and Y-27632 to a similar extent, GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization was resistant to 8-BrcAMP. In permeabilized canine tracheal smooth muscle, both PDBu and acetylcholine induce Ca\(^{2+}\) sensitization (3, 4). Rho/Rho-kinase-mediated signaling may be distinct from the PKC system because the effects of saturating concentrations of GTP\(\gamma\)S and PDBu were additive (9). Eto et al. (5) reported that PDBu-induced Ca\(^{2+}\) sensitization was partially inhibited by Y-27632 in \(\alpha\)-toxin-permeabilized rabbit femoral artery. However, we previously reported only a minor contribution of PKC to GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization in rabbit trachea. In our present study, PDBu-induced Ca\(^{2+}\) sensitization was not inhibited by Y-27632 (13), although the reason for the discrepancy is unknown.

In the present study, we demonstrated leukotriene-induced Ca\(^{2+}\) sensitization in human bronchial smooth muscle. Leukotrienes, as well as CCh, increased Ca\(^{2+}\) sensitivity in human bronchial smooth muscle, and leukotriene-induced Ca\(^{2+}\) sensitivity is reversed by 8-BrcAMP and Y-27632 (Fig. 6). Leukotrienes evoke a potent, sustained contraction of intact human airway smooth muscle (2, 24). Leukotrienes transiently increased Ca\(^{2+}\) sensitivity in \(\alpha\)-toxin-permeabilized porcine tracheal smooth muscle. Therefore, MLCP is not the only Ca\(^{2+}\) sensitization mechanism. In permeabilized trachea, the effect of MLCP inhibition on Ca\(^{2+}\) sensitization was smaller than changes in the phosphorylation state of myosin and thus rate limiting. However, precise time-course studies suggest that initiation of contraction occurred within milliseconds and that the early phase of maintenance of force occurred within several seconds (25). Furthermore, we have demonstrated that MLCK inhibition with wortmannin but not Rho-kinase inhibition with Y-27632 slowed force developments under the same experimental conditions (13). The final amplitudes were not different. Therefore, other cellular events appear to precede the development of maximum force by minutes.

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chel smooth muscle (23). However, it is not known whether mechanisms of Ca\(^{2+}\) sensitization in the sustained contraction were evoked by leukotrienes. Leukotrienes increased contractile force by \(-25\%\), and the LTD\(_4\)-induced Ca\(^{2+}\) sensitization was inhibited by 8-BrCAMP and Y-27632 together in an additive manner. This is the first report stating that Ca\(^{2+}\) sensitization is present in human bronchial smooth muscle contraction induced by leukotrienes and that mechanisms of both 8-BrCAMP sensitivity and Y-27632 sensitivity are involved. The LTD\(_4\)-induced contraction in human bronchial smooth muscle is at least in part independent of increases in Ca\(^{2+}\) sensitivity through activation of PKC (1). Thus inhibition of Rho/Rho-kinase signaling may become a second-line bronchodilator (in addition to \(\beta_2\)-agonists) for resolving limited airflow in asthma. Further studies are required to determine the relative contributions of Rho/Rho-kinase and PKC/CPI-17 in human tissue (27).

In smooth muscle, receptor-dependent, trimetric G protein-mediated Ca\(^{2+}\) sensitization was inhibited by both 8-BrCAMP and Y-27632 (Fig. 1B). Theoretically, GTP\(\gamma\)S may activate both trimetric and monometric G proteins, resulting in direct activation of Rho/Rho-kinase signaling and indirect activation of trimetric G protein-mediated PKC signaling. However, practically, PKC plays a minor role in GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization in rabbit tracheal smooth muscle (13). Hepler et al. (8) showed that purified G\(\alpha_i\) had only a slight binding affinity to GTP\(\gamma\)S. Additionally, AIF\(_3\) is known to activate the trimetric G protein. Contraction induced by AIF\(_3\) but not that induced by GTP\(\gamma\)S was insensitive to GDP\(\alpha\)S (15, 28). These findings suggest that GTP\(\gamma\)S mainly activates small G proteins, although the precise mechanism is still unknown.

The next question was why 8-BrCAMP effectively blocked CCh- or LTD\(_4\)-induced Ca\(^{2+}\) sensitization, in which PKC signaling might be involved. In fibroblasts, 8-BrCAMP blocks Rho activation by phosphorylation of heterotrimeric G proteins (7, 18). Thus the cAMP/PKA system may inhibit both trimetric and monometric G proteins, resulting in direct activation of Rho/Rho-kinase signaling through two steps. First, the cAMP/PKA system may inhibit the muscarinic receptor signaling upstream of Rho activation. Second, the cAMP/PKA system might be present downstream of the PKC pathway (Fig. 7).

In conclusion, cAMP/PKA preferentially reverses PKC-mediated Ca\(^{2+}\) sensitization to Rho/Rho-kinase-mediated Ca\(^{2+}\) sensitization. Activation of the MLCP-associated system is a mechanism of cAMP/PKA-induced Ca\(^{2+}\) desensitization, and this is distinct from Rho/Rho-kinase inhibition in airway smooth muscle. Human bronchial smooth muscle has Ca\(^{2+}\) sensitivity mechanisms that respond not only to contractile agonists but also to cAMP/PKA elevating agents.

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