Effects of 8-Bromo-Cyclic GMP on Membrane Potential of Single Swine Tracheal Smooth Muscle Cells¹

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ABSTRACT
Cyclic GMP relaxes swine tracheal smooth muscle. Relaxation occurs because of decreases in intracellular calcium concentration ([Ca⁺⁺]i) that are thought to occur through hyperpolarization which inhibits calcium influx. Activation of K⁺ channels has been suggested as the underlying mechanism for the hyperpolarization. In the present study, the effects of 8-bromo-guanosine 3',5'-cyclic monophosphate (8-Br-cGMP, a membrane-permeable analog of cyclic GMP) on acetylcholine (ACH)-induced increases in [Ca⁺⁺]i, were examined by laser scanning confocal microscopy in fluo 3-loaded single cells. Membrane potential and currents were measured by the perforated-configuration of patch-clamp method. 8-Bromo-cGMP (1 μM–0.1 mM) inhibited 0.1 μM ACh-induced oscillations in [Ca⁺⁺]i, in a concentration-dependent manner. Spontaneous changes in membrane potential were observed by the patch-clamp method. Acetylcholine (0.03 μM) did not affect the time-averaged mean potential. The spontaneous changes in membrane potential were reduced and the cells were depolarized by 0.1 μM ACh and to a greater degree by 1 μM ACh. This result is consistent with previous observations of ACh-induced depolarization in intact tissue. The application of 0.1 mM 8-Br-cGMP had no significant effects on spontaneous changes in membrane potential and did not induce changes in membrane potential in cells treated with 0.1 μM ACh. In voltage-clamped cells, ACh (0.1 μM) induced oscillations in calcium-activated K⁺ currents. 8-Bromo-cGMP (0.1 mM) inhibited these ACh-induced oscillations in currents, but had no significant effects on spontaneous changes in membrane current in unstimulated cells. These data indicate that 8-Br-cGMP inhibits ACh-induced increases in [Ca⁺⁺]i, by mechanisms other than regulation of membrane potential.

An increase in intracellular calcium concentration is necessary for agonist-induced contractions of tracheal smooth muscle. Elevation in [Ca⁺⁺]i, triggers myosin ATPase activation and cross-bridge cycling that leads to smooth muscle contraction (Miller-Hance et al., 1988; Somlyo and Somlyo, 1994). In fact, the degree of ACh-induced tension in swine tracheal smooth muscle is correlated with the magnitude of the initial rise in [Ca⁺⁺]i, (Shieh et al., 1991, 1995).

Acetylcholine-induced elevation in [Ca⁺⁺]i, consists of two components. Binding of ACh to its receptor activates G proteins that stimulate PLC and activated PLC degrades phosphatidylinositol 4,5-bisphosphate into IP₃ and diacylglycerol (Berridge and Irvine, 1984). Inositol 1,4,5-trisphosphate induces an initial burst of calcium release from intracellular stores by binding to the IP₃ receptor/channels located in the sarcoplasmic reticulum membrane. Then, a sustained elevation of [Ca⁺⁺]i, is observed in muscle strips during continuous application of agonist (Shieh et al., 1991, 1995). Extracellular calcium influx is involved in this sustained rise in [Ca⁺⁺]i, (Bourreau et al., 1993; Liu and Farley, 1996a; Tomasic et al., 1992), and in the case of ACh-induced increases in [Ca⁺⁺]i, calcium influx is believed to occur mainly through VDCC at ACh concentrations less than 1 μM (Liu and Farley, 1996a; Shieh et al., 1995). The activity of VDCC depends on the depolarization of cells and thus reducing cell depolarization may limit the activation of VDCC and the increase in [Ca⁺⁺]i.

Two cyclic nucleotide second messengers (cAMP and cGMP) decrease [Ca⁺⁺]i, in airway smooth muscle (Felbel et al., 1988; Nuttle and Farley, 1996). Nitric oxide increases cGMP by stimulating guanylyl cyclase (Murad, 1994). Beta-adrenergic agonists, the primary therapeutic bronchodilators, increase cAMP by activating adenyl cyclase (Torphy, 1994). Cyclic GMP activates cGMP-dependent protein kinase and cAMP activates cAMP-dependent protein kinase (Fran...
decreases in $[Ca^{2+}]_i$ at 10 ml PSS. The tissue was incubated at 37°C for 40 to 50 min in this solution. The cells were pelleted twice by centrifugation for 10 min at 100 x g in PSS to wash the cells free of enzymes and resuspended in PSS. Cells were then plated on glass coverslips for patch-clamp recording and on glass-bottomed dishes (MatTek Corp., Ashland, MA) for confocal microscopy. After removing unattached cells by gentle suction, cells were covered with culture medium. Cells were placed in an incubator (37°C, 5% CO₂) and used within 2 days.

**Confocal microscopy.** The culture medium was removed and the cells were incubated with 2 μM fluo-3 AM dissolved in PSS for 30 min at 37°C. Intracellular calcium concentration was measured with a Nera Odyssey confocal microscope system (Middleton, WI) including a Nikon Diaphot microscope (Garden City, NY) fitted with a 60× oil immersion lens. Excitation and emission wavelengths used were 488 and 510 nm, respectively. The laser intensity was set to 8% of maximum, and the photomultiplier amplification was set at 2,900 to 3,500 (4,096 maximum). A 100 μm confocal slit was used and 32 frame averaging with a sampling rate of 1 per second was performed. The brightness over time of selected cells was measured with MetaMorph™ (Universal Imaging Corporation, West Chester, PA). Data were stored as ASCII files and imported to Origin (Microcal Software Inc., Northampton, MA) for plotting and analysis. All experiments were performed during continuous perfusion at room temperature.

**Patch-clamp recordings.** Electrophysiological recordings of membrane potential and currents were obtained with the amphotericin perforated-patch configuration of the conventional patch-clamp recording technique (Hamill et al., 1981). Cells attached to glass coverslips were placed in the recording chamber that was perfused continuously with PSS (flow rate approximately 2 ml/min) at room temperature. Pipettes (3–5 meq/mols) were pulled from borosilicate glass (Dagan Corp., FMG 15, Minneapolis, MN) and fine polished immediately before filling with pipette solution. The tip of the pipette was then dipped into amphotericin-free pipette solution for 10 sec to permit easier giga-seal formation. Recordings were obtained with an Axopatch 200 B amplifier (Axon Instruments, Foster City, CA). Data were digitized and sampled at 1 kHz with a DigiData 1200 interface (Axon Instruments) and stored by an IBM-compatible PC system with pCLAMP6 data acquisition software (Axon Instruments). STORED data were analyzed with the pCLAMP module of Origin and Fetchan of pCLAMP6.

**Data analysis.** Time-averaged mean potential was obtained by integration of the membrane potential divided by the integration time, with Fetchan. Mean potential was obtained during the last 30 sec of each application period to avoid any delays in effect caused by the solution change. The mean potentials from more than two groups were compared by one-way repeated-measures ANOVA followed by Bonferroni’s or Student-Newman-Keuls method for multiple comparisons. The paired t test was used to compare mean potential before and after stimulation. Differences were considered to be statistically significant at P < .05. Data are reported as the mean ± S.E., and n indicates number of cells tested. Data in table 1 were expressed as the mean ± S.D. to show the variability of membrane potential in different cells.

**Methods**

**Chemicals.** The fluorescent dye fluo-3 AM was purchased from Molecular Probes Inc. (Eugene, OR). 8-Bromo-cGMP was purchased from Research Biochemicals International (Natick, MA). All the enzymes for cell dissociation, amphotericin and chemicals for the PSS and the pipette solution were purchased from Sigma (St. Louis, MO). Fetal bovine serum for the culture medium was purchased from HyClone Laboratories Inc. (Logan, UT).

**Solutions.** Physiological saline solution contained (in mM): 140, NaCl; 5, KCl; 1, CaCl₂; 5.5, glucose; 10, HEPES. The pH was adjusted to 7.4 with NaOH. An amphotericin stock solution of 60 mg/ml in DMSO was prepared daily and diluted to a final concentration of 120 μg/ml in filtered pipette solution every 2 to 3 hr. The pipette solution contained (in mM): 45, K₂SO₄; 50, KCl; 10, NaCl; 10, HEPES; 50, mannitol (pH 7.2 with KOH). The culture medium consisted of Dulbecco’s Modified Eagle’s Medium/F12 (Sigma, D 8900) supplemented with 18 mM sodium bicarbonate, 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Fluo-3 AM stock solution (2 mM) was prepared in DMSO, kept at −20°C and diluted to 2 μM in PSS just before use.

**Cell isolation.** Male pigs (Yorkshire white, 4–6 weeks old, 20–30 kg) were purchased from local suppliers and kept at the animal facility for 3 to 5 days until use. They were anesthetized with 5% isoflurane (Ohio Medical Products, Madison, WI) and sacrificed by exsanguination. The trachea was removed and transported in PSS to wash the cells free of enzymes and resuspended in PSS with antibiotics. The smooth muscle was cleaned of epithelia, gland cells and connective tissue at room temperature. The smooth muscle was then minced and incubated with protease (0.5 mg/ml, type XIV, 6.6 units/mg solid) dissolved in 10 ml PSS for 1 hr at 37°C. The tissue was removed from the protease-containing solution by centrifugation at 100 × g for 10 min at room temperature and resuspended in an enzyme solution consisting of collagenase (1 mg/ml, type I, 300 units/mg solid) and trypsin inhibitor (1 mg/ml, type II-S) dissolved in 10 ml PSS. The tissue was incubated at 37°C for 40 to 50 min in this solution. The cells were pelleted twice by centrifugation for 10 min at 100 × g in PSS to wash the cells free of enzymes and resuspended in PSS. Cells were then plated on glass coverslips for patch-clamp recording and on glass-bottomed dishes (MatTek Corp., Ashland, MA) for confocal microscopy. After removing unattached cells by gentle suction, cells were covered with culture medium. Cells were placed in an incubator (37°C, 5% CO₂) and used within 2 days.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Before Stimulation</th>
<th>ACh 0.03 μM</th>
<th>ACh 0.1 μM</th>
<th>ACh 1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base (mV)</td>
<td>−18 ± 4</td>
<td>−16 ± 4</td>
<td>−14 ± 4</td>
<td>−7 ± 3*</td>
</tr>
<tr>
<td>Peak (mV)</td>
<td>−54 ± 8</td>
<td>−56 ± 7</td>
<td>−53 ± 7</td>
<td>−34 ± 11*</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>36 ± 9</td>
<td>40 ± 7</td>
<td>39 ± 6</td>
<td>27 ± 10*</td>
</tr>
<tr>
<td>Mean potential (mV)</td>
<td>−25 ± 5</td>
<td>−25 ± 5</td>
<td>22 ± 5*</td>
<td>−16 ± 4*</td>
</tr>
</tbody>
</table>
Results

Inhibition of ACh-induced increases in $[Ca^{2+}]_i$ by 8-Br-cGMP. Changes in $[Ca^{2+}]_i$ caused by ACh and/or 8-Br-cGMP were measured by laser scanning confocal microscopy. Acetylcholine, at a concentration of 0.1 $\mu$M, increased $[Ca^{2+}]_i$, as reported previously (Liu and Farley, 1996a; Nuttle and Farley, 1996). As shown in figure 1A, 0.1 $\mu$M ACh induced a transient increase followed by sustained oscillations in $[Ca^{2+}]_i$, in single isolated tracheal smooth muscle cells. The effect of 8-Br-cGMP on the ACh-induced increases in $[Ca^{2+}]_i$ was examined in cells treated with this submaximal concentration of ACh (0.1 $\mu$M) (Liu and Farley, 1996a; Shieh et al., 1991). Increasing concentrations of 8-Br-cGMP (0.001–0.1 mM, fig. 1B) or 0.1 mM alone (data not shown) was applied. As shown in figure 1B, 8-Br-cGMP inhibited ACh-induced increases in $[Ca^{2+}]_i$, in a concentration-dependent manner in a pattern similar to the inhibitory effect of cAMP (Nuttle and Farley, 1996). The effect of 8-Br-cGMP was observed within 1 min after its application. The response to ACh did not recover quickly after removal of 8-Br-cGMP. In all the cells exposed to 0.1 $\mu$M ACh ($n = 11$), 1 mM 8-Br-cGMP inhibited ACh-induced increases in $[Ca^{2+}]_i$, whereas 1 $\mu$M 8-Br-cGMP reduced the frequency of oscillations in 6 of 11 cells. In 22 of 24 cells, 0.1 mM 8-Br-cGMP inhibited oscillations, and thus this concentration was used in further experiments to investigate the mechanism of $[Ca^{2+}]_i$ inhibition.

Spontaneous changes in membrane potential and ACh-induced depolarization. The effects of ACh and 8-Br-cGMP on membrane potential were investigated to test the hypothesis that these agents regulate $[Ca^{2+}]_i$ by modulating membrane potential. We used the perforated-patch method to prevent the loss of cytosolic proteins and second messengers into the pipette solution. Spontaneous changes in membrane potential as shown in figure 2 were observed in all single cell recordings. The time-averaged membrane potential was $-25 \pm 5$ mV (S.D., $n = 10$ cells from five different animals), with a basal potential of $-18 \pm 4$ mV and peak changes to $-54 \pm 8$ mV. These spontaneous changes in membrane potential were inhibited by ACh $\geq 0.1$ $\mu$M (fig. 2) and the cells were depolarized by $3 \pm 2$ mV (time-averaged mean membrane potential) with 0.1 $\mu$M ACh and by $9 \pm 4$ mV with 1 $\mu$M ACh (table 1).

Effect of 8-Br-cGMP on the spontaneous and ACh-induced changes in membrane potential. The effect of 8-Br-cGMP on membrane potential was examined in both ACh-treated and unstimulated cells. Acetylcholine (0.1 $\mu$M) inhibited spontaneous changes in membrane potential and induced depolarization (fig. 3A). As shown in figure 3A, 0.1 $\mu$M ACh depolarized cells as long as ACh was applied. The mean membrane potential after 4 min of ACh stimulation was not significantly different from the mean potential after 1 min ACh application (fig. 3B). Acetylcholine-induced changes in membrane potential were not affected by 0.1 mM 8-Br-cGMP (fig. 4, A and B), a concentration that inhibited
ACh-induced increases in \([\text{Ca}^{2+}]_i\) (fig. 1B). 8-Bromo-cGMP also had no significant effects on the spontaneous changes in membrane potential (fig. 5, A and B).

**Effect of 8-Br-cGMP on the outward currents.** Changes in membrane currents were examined to further investigate other possible mechanisms for the effects of 8-Br-cGMP on \([\text{Ca}^{2+}]_i\). Cells were voltage-clamped at the estimated equilibrium potential for Cl current, \(-23\) mV, to measure changes in outward current. Spontaneous transient outward currents (figs. 6A and 7) were observed at a holding potential of \(-23\) mV. The STOC are caused by the activation of \(K_{\text{ca}}\) (Saunders and Farley, 1991). The effects of 8-Br-cGMP on STOC and ACh-induced changes in outward currents were examined. In amphotericin-perforated whole cells where the cytosolic compartment was kept relatively intact, 0.1 \(\mu\)M ACh inhibited STOC and induced large oscillations in outward current (fig. 6A). Acetylcholine-induced oscillations were rhythmic compared with the irregular burst pattern of STOC, and the amplitudes of the ACh-induced oscillations were five to seven times larger than that of STOC. As shown in figure 6C, 8-Br-cGMP (0.1 mM) inhibited ACh-induced oscillations in outward current. 8-Bromo-cGMP (0.1 mM) did not induce any apparent changes in STOCs in unstimulated cells (fig. 7). The time-averaged mean currents before and after 0.1 mM 8-Br-cGMP application were 13 \(\pm\) 3 and 14 \(\pm\) 5 pA (\(n = 5\) from three animals), respectively, and not significantly different from each other.

**Discussion**

**Spontaneous changes in membrane potential.** Many types of smooth muscle including swine tracheal smooth muscle cells have STOC and spontaneous transient inward currents (Benham and Bolton, 1986; Janssen and Sims, 1994; Saunders and Farley, 1991, 1992). These are believed to occur because of spontaneous release of calcium from intracellular stores and activation of \(K_{\text{ca}}\) and \(Cl_{\text{rat}}\), respectively (Bolton and Imaizumi, 1996; Janssen and Sims, 1994; Nelson et al., 1995; Saunders and Farley, 1991). In this study, spontaneous changes in membrane potential were shown in single isolated tracheal smooth muscle cells. The spontaneous changes in membrane potential appear to be
correlated with STOC and spontaneous transient inward currents. Activation of transient outward currents should cause transient hyperpolarizations to the $K^+$ equilibrium potential ($-84$ mV in this study). However, the transient hyperpolarizations do not reach $K^+$ equilibrium potential, probably because both $K_{Ca}$ and $Cl_{Ca}$ are activated during the spontaneous release of calcium from the sarcoplasmic reticulum that drives this response. The $Cl^-$ equilibrium potential is about $-23$ mV, thus the hyperpolarization should reach a potential between $-23$ and $-84$ mV.

Time-averaged membrane potential in single tracheal smooth muscle cells was $-25 \pm 5$ mV with use of perforated patch recording. However, the range in a single cell was $-18$ to $-54$ mV (table 1). In intact muscle strips, membrane potential at 37°C is about $-60$ mV in canine and swine trachea (Farley and Miles, 1977; Murali Mohan et al., 1988; Shieh et al., 1992) and $-50$ mV in human trachea (Honda and Tomita, 1987). The membrane potential recorded in intact tissue is generally quite stable. However, this potential represents the average membrane potential across many cells connected electrically through gap junction channels. The length constant of the muscle is 3 to 4 mm (H.-M. H. Saunders and J. M. Farley, unpublished observations), but the lengths of smooth muscle cells are only 75 to 100 $\mu$m. Thus the potentials of individual cells are modulated by a large volume of tissue. This effect will moderate changes in potential in any single cell. Because of the random nature of the spontaneous currents in each cell, the spatially averaged membrane potential of all cells is stable. Other studies with enzyme-dissociated smooth muscle cells have reported somewhat lower membrane potentials of about $-35$ mV in human bronchi (Janssen, 1996) and $-40$ mV in guinea pig trachea (Nakajima et al., 1995). Another possible explanation for the lack of transient spontaneous changes in membrane potential in intact tissue is the minor contribution of the $BK_{Ca}$ to the resting membrane potential in intact tissue. Previous studies with charybdotoxin, an inhibitor of $BK_{Ca}$, did not increase resting $[Ca^{++}]_i$ or tension in tracheal smooth muscle (Shieh et al., 1996). Voltage-dependent delayed rectifier channels have been suggested to be the main channel respon-
sible for maintaining resting membrane potential in canine airway smooth muscle cells (Kotlikoff, 1990). In addition, most membrane potential measurements in intact smooth muscle were made at 37°C (Farley and Miles, 1977; Murali Mohan et al., 1988; Sheib et al., 1992).

Effects of 8-Br-cGMP on ACh-induced changes in membrane potential. It has been reported that calcium influx is required to maintain resting [Ca$$^{2+}$$], or ACh-induced increases in [Ca$$^{2+}$$], in smooth muscle cells (Liu and Farley, 1996b; Sheib et al., 1991, 1995). In swine tracheal smooth muscle, calcium influx is mediated by VDCC (Liu and Farley, 1996a; Sheib et al., 1995; Tomasic et al., 1992) and other non-voltage-mediated calcium influx (Liu and Farley, 1996a; Sheib et al., 1995). Calcium influx through VDCC depends on the depolarization of cells and thus several hypotheses propose that changes in [Ca$$^{2+}$$]i might be accomplished by modulation of membrane potential. Earlier studies have shown that cAMP and cGMP decrease [Ca$$^{2+}$$], and activate BKCa (Bialecki and Stinson-Fisher, 1995; Kume et al., 1994; Yamakage et al., 1996). The activation of BKCa should hyperpolarize cells leading to decreased activation of VDCC and calcium influx. This has been a primary mechanism suggested for the action of cAMP and cGMP-induced decreases in [Ca$$^{2+}$$]. However, we found that 8-Br-cGMP did not change membrane potential significantly at a concentration that inhibited ACh-induced increases in [Ca$$^{2+}$$]. These data are consistent with a previous report that cAMP inhibited ACh-induced oscillations in Cl$$^{-}$$ currents in cells under voltage-clamp at −80 mV (Nuttle and Farley, 1996). The fact that ACh-induced changes in membrane potential were unaffected during exposure of the cells to 8-Br-cGMP could be caused by several factors. First, the activity of BKCa is reduced by muscarinic receptor activation (Kotlikoff, 1993; Kume and Kotlikoff, 1991). Thus, activation of KCa by 8-Br-cGMP may not overcome the inhibition. Second, Cl$$^{-}$$ activity is increased by muscarinic receptor activation (Janssen and Sims, 1992; Nuttle and Farley, 1996). This would tend to drive the cell potential to the Cl$$^{-}$$ equilibrium potential and the increased conductance would decrease the relative importance of K$$^{+}$$ channel opening in membrane potential control. Thus, the overall result is cell depolarization that is less sensitive to KCa channel opening (Robertson et al., 1993).

Effects of 8-Br-cGMP on the spontaneous changes in membrane potential. Cyclic GMP has been demonstrated to increase the single-channel activity of BKCa (Robertson et al., 1993; Yamakage et al., 1996). Robertson et al. (1993) demonstrated that cGMP caused a 40- to 50-fold increase in the open probability of BKCa at −10 mV. In our study, the average current carried by STOC at −23 mV was not altered by 8-Br-cGMP, that is STOC amplitude was not greatly increased as would be predicted by Robertson et al. (1993). This discrepancy may be explained partly by the fact that Nelson et al. (1995) demonstrated that the STOC in the vascular smooth muscle are completely inhibited by iberiotoxin indicating they are caused solely by BKCa. In airway, STOC are not totally inhibited by either charybotoxin or iberiotoxin (J. Choi and J. M. Farley, unpublished observation). Saunders and Farley (1991) suggested that STOC are composed of multiple types of K$$^{+}$$ channels. Thus, changes in the activity of BKCa may not be sufficient to alter the amplitude of STOC.

In addition, the measurements of Robertson et al. (1993) were made with [Ca$$^{2+}$$] buffered to 300 nM. This is approximately the concentration estimated by Nelson et al. (1995) to occur during a calcium spark associated with STOC. In our studies, [Ca$$^{2+}$$] varied with time during the STOC (or hyperpolarization). How this difference might be important is not known.

Other possibilities for cyclic nucleotide-induced inhibition of [Ca$$^{2+}$$]i, such as direct modulation of calcium channels (Mery et al., 1991), accelerated reuptake of calcium into stores (McGrogan et al., 1995), or inhibition of calcium release from stores (Komalavilas and Lincoln, 1994) have been suggested but have not been investigated further in detail.

In conclusion, the present study shows that 0.1 mM 8-Br-cGMP did not change membrane potential significantly in untreated and 0.1 μM ACh-treated cells. These data suggest that ACh-induced increases in [Ca$$^{2+}$$]i, are inhibited by 8-Br-cGMP by mechanisms other than regulation of membrane potential. Further investigations are needed to define mechanisms to understand how [Ca$$^{2+}$$]i is regulated by 8-Br-cGMP.

References


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