Am J Physiol Lung Cell Mol Physiol 290: L278–L283, 2006.
First published September 9, 2005; doi:10.1152/ajplung.00188.2005.

Cyclic nucleotide regulation of store-operated Ca^{2+} influx in airway smooth muscle

Binnaz Ay,[3] Adeyemi Iyanoye,[2] Gary C. Sieck,[2] Y. S. Prakash,[1] and Christina M. Pabelick[1]

Departments of [1]Anesthesiology, and [2]Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, Minnesota; and Department of Anesthesiology, [3]Marmara University, Istanbul, Turkey

Submitted 26 April 2005; accepted in final form 31 August 2005

Ay, Binnaz, Adeyemi Iyanoye, Gary C. Sieck, Y. S. Prakash, and Christina M. Pabelick. Cyclic nucleotide regulation of store-operated Ca^{2+} influx in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 290: L278–L283, 2006. First published September 9, 2005; doi:10.1152/ajplung.00188.2005.—Sarcoplasmic reticulum (SR) Ca^{2+} release and plasma membrane Ca^{2+} influx are key to intracellular Ca^{2+} ($[Ca^{2+}]_i$) regulation in airway smooth muscle (ASM). SR Ca^{2+} depletion triggers influx via store-operated Ca^{2+} channels (SOCC) for SR replenishment. Several clinically relevant bronchodilators mediate their effect via cyclic nucleotides (cAMP, cGMP). We examined the effect of cyclic nucleotides on SOCC-mediated Ca^{2+} influx in enzymatically dissociated porcine ASM cells. SR Ca^{2+} was depleted by 1 μM cyclopiazonic acid in 0 extracellular Ca^{2+} ($[Ca^{2+}]_o$), nifedipine, and KCl (preventing Ca^{2+} influx through L-type and SOCC channels). SOCC was then activated by reintroduction of $[Ca^{2+}]_o$ and characterized by several techniques. We examined cAMP effects on SOCC by activating SOCC in the presence of 1 μM isoproterenol or 100 μM dibutryl cAMP (cell-permeant cAMP analog), whereas we examined cGMP effects using 1 μM (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO nitric oxide donor) or 100 μM 8-bromoguanosine 3',5'-cyclic monophosphate (cell-permeant cGMP analog). The role of protein kinases A and G was examined by preexposure to 100 nM KT-5720 and 500 nM KT-5823, respectively. SOCC-mediated Ca^{2+} influx was dependent on the extent of SR Ca^{2+} depletion, sensitive to Ni^{2+} and La^{3+}, but not inhibitors of voltage-gated influx channels. cAMP as well as cGMP potently inhibited Ca^{2+} influx, predominantly via their respective protein kinases. Additionally, cAMP cross-activation of protein kinase G contributed to SOCC inhibition. These data demonstrate that a Ni^{2+}/La^{3+}-sensitive Ca^{2+} influx in ASM triggered by SR Ca^{2+} depletion is inhibited by cAMP and cGMP via a protein kinase mechanism. Such inhibition may play a role in the bronchodilatory response of ASM to clinically relevant drugs (e.g., β-agonists vs. nitric oxide).

capacitative calcium entry; trachea; adenosine 3′,5′-cyclic monophosphate; guanosine 3′,5′-cyclic monophosphate; nitric oxide; isoproterenol

AIRWAY SMOOTH MUSCLE (ASM) tone represents a balance between bronchoconstriction and bronchodilation. The goal of clinical therapy for pathophysiological states such as asthma, allergy, and inflammation is to prevent excessive bronchoconstriction, both in the acute and chronic setting, and to restore a balance by producing bronchodilation. Cytosolic (intracellular) Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key determinant of ASM tone (12, 23, 33). Elevation of $[Ca^{2+}]_i$ by bronchoconstrictors such as acetylcholine (ACh) and histamine involves both Ca^{2+} release from sarcoplasmic reticulum (SR) stores and plasma membrane Ca^{2+} influx. In ASM, Ca^{2+} influx is known to occur through both voltage-gated (36) and receptor-gated (11, 21) channels. In addition to these mechanisms, we recently demonstrated the existence of controlled Ca^{2+} influx in ASM (2, 22) that occurs via store-operated Ca^{2+} channels [SOCC; also termed capacitative Ca^{2+} entry (24, 29, 30)] in response to SR Ca^{2+} depletion, thus allowing for replenishment of intracellular Ca^{2+} stores (2, 22). SOCC-mediated influx in ASM appears to be Ni^{2+} and La^{3+} sensitive and is modulated by agonists such as ACh.

The cyclic nucleotides cyclic adenosine 3′,5′-cyclic monophosphate (cAMP) and cyclic guanosine 3′,5′-cyclic monophosphate (cGMP) mediate the effects of a variety of endogenous substances as well as clinically relevant drugs, e.g., β_2-agonists and nitric oxide (NO), used to produce relaxation of ASM (13, 25, 28). Cyclic nucleotide effects are mediated in part by downregulation of mechanisms that would normally elevate $[Ca^{2+}]_i$ in ASM. Previously, we demonstrated that salbutamol, a β_2-agonist (28), as well as NO donors (26) inhibit ACh-induced $[Ca^{2+}]_i$ oscillations in ASM that occur via repetitive SR Ca^{2+} release and reuptake. Both cAMP and cGMP also influence the plasma membrane via membrane hyperpolarization (9, 15) and inhibition of Ca^{2+} influx via L-type Ca^{2+} channels (16, 17). Given the relative novelty but somewhat ubiquitous expression of SOCC, there is currently limited and sometimes inconsistent data on cyclic nucleotide modulation of Ca^{2+} influx via this mechanism in tissues other than ASM. In rat aorta, cAMP inhibits SOCC (34) but enhances it in astrocytes (37). We hypothesize that cyclic nucleotides inhibit SOCC, preventing $[Ca^{2+}]_i$ elevation as well as SR Ca^{2+} store repletion. In the present study, we examined the effect of cAMP and cGMP on SOCC-mediated Ca^{2+} influx in porcine ASM cells.

METHODS

Cell preparation. The ASM cell preparation technique has been previously described in detail (14, 27). In brief, porcine tracheae were collected from a local abattoir. After removing the adventitia, we excised and minced the smooth muscle layer in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES (pH 7.4; Invitrogen, Carlsbad, CA). The tissue then was incubated first for 2 h with 20 U/ml papain and 2,000 U/ml DNase (Worthington Biochemical, Lakewood, NJ), and subsequently for 1 h at 37°C with 1 mg/ml type IV collagenase (Worthington). After incubation, the sample was triturated and centrifuged, and the freshly dissociated cells were resuspended in minimum essential medium with 10% fetal calf serum. After plating the isolated cells on collagen-coated glass coverslips, we

Address for reprint requests and other correspondence: C. M. Pabelick, 4-184 W Jos SMH, Mayo Clinic College of Medicine, Rochester, MN 55905 (e-mail: pabelick.christina@mayo.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

followed with another incubation (for 1 h in 5% CO_2 at 37°C) to allow the cells to attach to coverslips.

Real-time fluorescence Ca^{2+} imaging. Cells attached to coverslips were loaded with 5 μM fura-2 AM (Molecular Probes, Eugene, OR) for 30–45 min at 37°C in HBSS, washed, mounted on an open slide chamber (RC-25F; Warner Instruments, Hamden, CT), and perfused with HBSS at room temperature. Fura-2-loaded cells were visualized using a MetaFluor real-time fluorescence imaging system (Universal Imaging, Downingtown, PA) on a Nikon Diaphot inverted microscope (Fryer Instruments, Edina, MN). Pairs of images for excitation wavelengths 340 and 380 nm were obtained once every second, and the ratiometric data was calibrated for Ca^{2+} levels by previously published techniques (8).

Store-operated Ca^{2+} influx. The protocol for activation of SOCC in ASM has been recently published (2, 22) and is only summarized here. Whereas in our previous work on SOCC we demonstrated that Ca^{2+} influx occurs following SR Ca^{2+} depletion either with the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA) or with caffeine, it must be noted that caffeine depletes only ryanodine-sensitive SR Ca^{2+} stores. Furthermore, CPA has a more pronounced effect on SOCC compared with caffeine (2). Finally, caffeine is known to inhibit phosphodiesterase activity in cells, which would certainly alter cAMP or cGMP levels in the present study. Accordingly, we selected CPA over caffeine for the present study.

ASM cells were washed with HBSS and exposed to zero-Ca^{2+} HBSS (5 mM EGTA) for 5 min to remove extracellular Ca^{2+} ($[Ca^{2+}]_o$). Cells were also exposed to 1 μM nifedipine and 10 mM KCl during continued absence of $[Ca^{2+}]_o$ to inhibit L-type Ca^{2+} channels. The SR was then passively depleted by 1 μM CPA in zero-Ca^{2+} HBSS (in the continued presence of nifedipine and KCl), likely due to continued SR Ca^{2+} leak via both inositol 1,4,5-trisphosphate (IP_3)- and ryanodine-sensitive SR stores. $[Ca^{2+}]_i$ levels gradually increased and eventually either reached a plateau level or started to trend down (likely reflecting increasing Ca^{2+} efflux via the plasma membrane). At this point, 2.5 mM $[Ca^{2+}]_o$ was rapidly reintroduced (in the continued presence of CPA, nifedipine, and KCl), and the observed $[Ca^{2+}]_i$ response was measured. In a subset of cells, 5 mM caffeine in zero $[Ca^{2+}]_o$ was used instead of 2.5 mM $[Ca^{2+}]_o$ to verify a lack of $[Ca^{2+}]_i$ response (indicating at least functional SR depletion).

Characterization of SOCC-mediated Ca^{2+} influx has also been previously reported (2, 22). In addition to the previously published techniques, in the present study we determined whether the extent of SOCC-mediated Ca^{2+} influx was dependent on the extent of CPA-induced SR Ca^{2+} depletion. After exposure to nifedipine and KCl, cells were exposed to CPA (with nifedipine and KCl) for 2, 5, or 15 min, and SOCC-mediated Ca^{2+} influx was then activated.

Effect of cyclic nucleotides on store-operated Ca^{2+} influx. As mentioned above, we first established SOCC in ASM cells by performing a control protocol with CPA. After SOCC-mediated Ca^{2+} influx was observed and the amplitude of the response recorded, the cells were washed for 15–20 min with HBSS to replenish SR Ca^{2+} stores and wash out the CPA. $[Ca^{2+}]_o$ was then removed, nifedipine and KCl added, and the cells reexposed to CPA (in the continued presence of nifedipine and KCl). Once an $[Ca^{2+}]_i$ response was observed (thus ensuring SR Ca^{2+} depletion first), the cells were exposed for 5 min to one of the following drugs: 1 μM isoproterenol, 100 μM dibutryl cAMP (dbcAMP; a membrane-permeable cAMP analog), 1 μM (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate (DETA-NO, a NO donor) or 100 μM 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP, cell-permeable cGMP analog). CPA, nifedipine, and KCl were present throughout this part of the protocol. In the continued presence of CPA, nifedipine, KCl, and one of the above drugs, $[Ca^{2+}]_o$ was rapidly reintroduced, and the $[Ca^{2+}]_i$ response was recorded. In other experiments to determine whether cyclic nucleotide effects on the observed influx represent effects on SOCC per se, cells were also preexposed to 1 μM La^{3+} following CPA, but before dbcAMP or 8-BrcGMP exposure. SOCC was then activated in the presence of CPA, nifedipine, KCl, La^{3+}, and dbcAMP (or 8-BrcGMP). We used only dbcAMP and 8-BrcGMP for these experiments to ensure that cAMP and cGMP, respectively, were elevated without any additional effects of indirect activation by isoproterenol or DETA-NO.

To determine whether cyclic nucleotide effects on SOCC were mediated via protein kinases, we repeated the above experiments (in separate sets of cells) additionally using protein kinase inhibitors. To examine the role of protein kinase A, cells already exposed to CPA in zero $[Ca^{2+}]_o$ (and thus SR depleted) were first exposed to 100 nM KT-5720 (protein kinase A inhibitor) and then to 100 μM dbcAMP. Extracellular Ca^{2+} was then reintroduced to activate SOCC-mediated Ca^{2+} influx. In other experiments examining the role of protein kinase G, CPA-exposed cells in zero $[Ca^{2+}]_o$ were exposed to 500 nM KT-5823 (protein kinase G inhibitor) and then to 100 μM 8-BrcGMP. SOCC-mediated influx was then activated.

In non-ASM cells, there is evidence for cross-activation of protein kinases A and G by cGMP and cAMP, respectively (3, 9, 32, 35). To determine whether such protein kinase cross-activation occurs in ASM, we performed two sets of experiments. In the first set, cells already exposed to CPA in zero $[Ca^{2+}]_o$ were exposed to 500 nM KT-5823 and then to 100 μM dbcAMP, before activation of SOCC-mediated Ca^{2+} influx. In the other set, cells in CPA and zero $[Ca^{2+}]_o$ were exposed to 100 nM KT-5720 (protein kinase A inhibitor) and then to 100 μM 8-BrcGMP, followed by activation of SOCC-mediated Ca^{2+} influx.

Statistical analysis. Comparisons were done for the same ASM cell before and after exposure to a drug. Student's paired *t*-test was used for such comparisons. All experimental protocols were not performed in all cells. Results were replicated in at least five cells obtained from at least four animals (paired comparisons within cells, independent testing across cells). Statistical significance was tested at $P < 0.05$ level. Values are reported as means ± SE.

RESULTS

Store-operated Ca^{2+} influx in ASM. Baseline $[Ca^{2+}]_i$ levels in ASM cells ranged from 80 to 110 nM (89 ± 8 nM, $n = 164$) and either did not change or decreased with removal of $[Ca^{2+}]_o$ or with addition of nifedipine and KCl (presumably reflecting transient Ca^{2+} efflux via plasma membrane mechanisms). In the absence of $[Ca^{2+}]_o$ but in the presence of nifedipine and KCl, 1 μM CPA increased $[Ca^{2+}]_i$ levels, which eventually reached a plateau (325–522 nM). At this point, rapid reintroduction of $[Ca^{2+}]_o$ resulted in a further, sustained elevation of $[Ca^{2+}]_i$ (211–491 nM, Fig. 1, $P < 0.05$ compared with the first plateau). Characterization of SOCC-mediated Ca^{2+} influx in ASM has been recently published (2, 22). In the present study, several of these characterization techniques were repeated to ensure that the Ca^{2+} influx observed in the experimental protocols was indeed mediated by SOCC. The results are summarized in Fig. 1. In addition, SOCC-mediated Ca^{2+} influx increased with 5 min of CPA exposure vs. 2 min (presumably reflecting greater extents of SR Ca^{2+} depletion; Fig. 1). However, 15-min CPA exposure did not result in greater influx compared with the 5-min CPA value.

Effect of cAMP on store-operated Ca^{2+} influx. In control ASM cells, the observed SOCC-mediated Ca^{2+} influx was reproducible to within 10% with repetitions of the protocol in the same cells (rundown control). In absence of $[Ca^{2+}]_o$, exposure to 1 μM isoproterenol (in the presence of CPA) did not significantly alter the $[Ca^{2+}]_i$ levels (Fig. 2). Subsequent

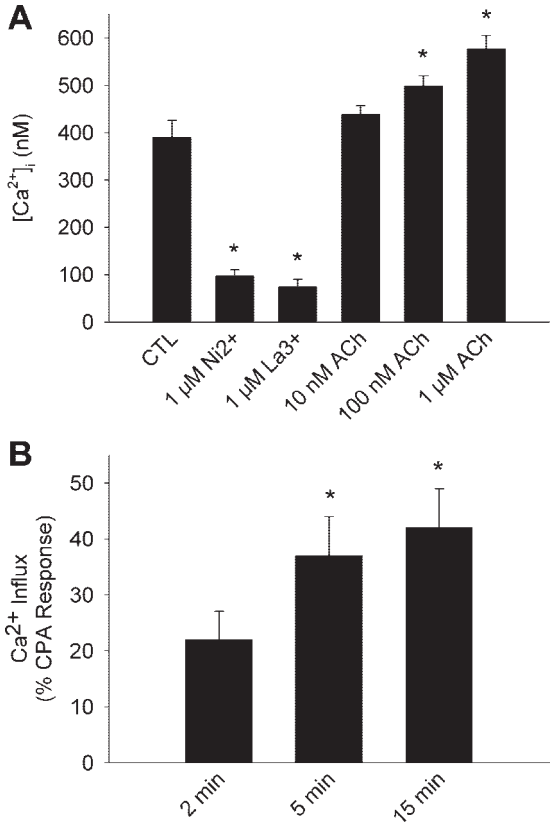

Fig. 1. Characterization of store-operated Ca^{2+} channel (SOCC)-mediated Ca^{2+} influx in airway smooth muscle (ASM). SOCC-mediated Ca^{2+} influx was triggered following cyclopiazonic acid (CPA)-induced sarcoplasmic reticulum (SR) Ca^{2+} depletion as described previously (2, 22). To characterize the influx, cells in which SOCC was verified were washed, and the protocol was repeated in the presence of the agents in *A*. The resulting SOCC-mediated Ca^{2+} influx (measured as a percentage of the response to CPA) was significantly decreased by Ni^{2+} and La^{3+} and enhanced by ACh. The extent of SOCC-mediated Ca^{2+} influx was decreased when SR depletion was limited by only brief exposure to CPA (*B*). *Significant difference from control ($P < 0.05$).

introduction of $[Ca^{2+}]_o$ resulted in a significantly smaller SOCC-mediated Ca^{2+} influx compared with the control responses in the same cells ($P < 0.05$, Fig. 2). In fact, in some cells, Ca^{2+} influx was completely inhibited, whereas in remaining cells, a significantly reduced influx was observed. Similarly, 100 μM dbcAMP also attenuated the observed Ca^{2+} influx compared with control ($P < 0.05$, Fig. 2). The inhibitory effect of dbcAMP on Ca^{2+} influx was significantly greater compared with isoproterenol ($P < 0.05$). Preexposure to 1 μM La^{3+} resulted in only a small amount of Ca^{2+} influx following reintroduction of $[Ca^{2+}]_o$ even when dbcAMP was present (11 ± 4% of CPA response).

Effect of cGMP on store-operated Ca^{2+} influx. In absence of $[Ca^{2+}]_o$, exposure to 1 μM DETA-NO in the presence of CPA did not significantly alter $[Ca^{2+}]_i$ (Fig. 3). Subsequent introduction of $[Ca^{2+}]_o$ resulted in significantly lesser SOCC-mediated Ca^{2+} influx compared with the control responses in the same cells ($P < 0.05$, Fig. 3). Exposure to 100 μM 8-BrcGMP decreased SOCC-mediated Ca^{2+} influx in some but not all cells (minimal effect in ~40% of cells). In contrast to isoproterenol vs. dbcAMP, the effect of DETA-NO was significantly greater compared with 8-BrcGMP ($P < 0.05$). Furthermore, the inhibitory effects of isoproterenol and dbcAMP on SOCC-mediated Ca^{2+} influx were both lesser than those of DETA-NO but not 8-BrcGMP ($P < 0.05$). Preexposure to 1 μM La^{3+} resulted in negligible Ca^{2+} influx following reintroduction of $[Ca^{2+}]_o$ even in the presence of 8-BrcGMP (7 ± 4% of CPA response).

Protein kinases A and G and store-operated Ca^{2+} influx. In cells preexposed to the protein kinase A inhibitor KT-5720, the inhibitory effect of dbcAMP on SOCC-mediated Ca^{2+} influx was significantly less compared with inhibition in the absence of KT-5720 ($P < 0.05$, Fig. 4). However, only one or two cells showed complete lack of cAMP-mediated influx inhibition in the presence of KT-5720. In cells preexposed to the protein kinase G inhibitor KT-5823, the inhibitory effect of 8-BrcGMP on SOCC-mediated Ca^{2+} influx was also significantly less compared with inhibition in the absence of KT-5823 ($P < 0.05$, Fig. 4).

In ASM cells already exposed to CPA in zero $[Ca^{2+}]_o$, KT-5823 did not significantly alter $[Ca^{2+}]_i$ levels. In the presence of protein kinase G inhibition by KT-5823, inhibition of SOCC-mediated Ca^{2+} influx by dbcAMP was significantly less ($P < 0.05$, Fig. 4), albeit not to the extent induced by the protein kinase A inhibitor KT-5720. In contrast to these effects, in other cells the inhibitory effects of 100 μM 8-BrcGMP on

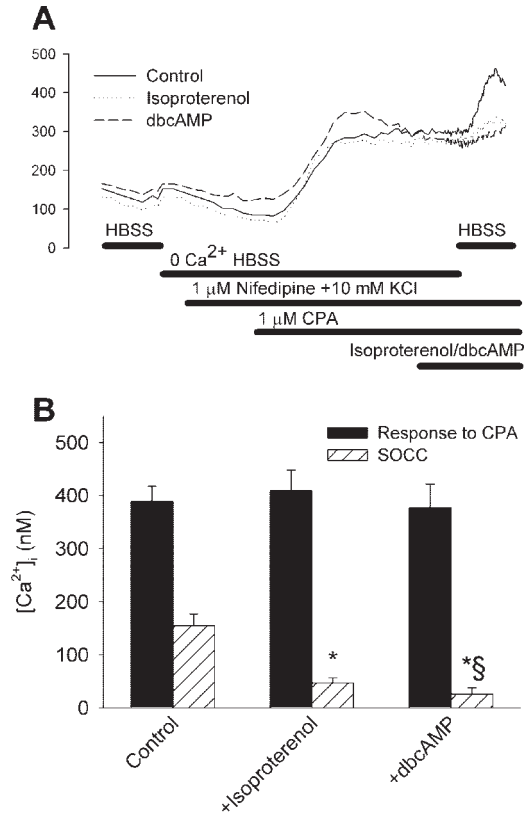

Fig. 2. Effect of cAMP on SOCC-mediated Ca^{2+} influx. After a control verification of SOCC-mediated Ca^{2+} influx, ASM cells were washed and reexposed to CPA, followed by either 1 μM isoproterenol or 100 μM dibutryl cAMP (dbcAMP, cell-permeant cAMP analog) (*A*). Neither isoproterenol nor dcAMP significantly altered $[Ca^{2+}]_i$ levels during CPA exposure (*B*). In the continued presence of CPA and isoproterenol or dbcAMP, extracellular Ca^{2+} was reintroduced. The resulting SOCC-mediated Ca^{2+} influx was significantly decreased in the presence of both isoproterenol and dbcAMP. *Significant difference from control, §between isoproterenol and dbcAMP ($P < 0.05$).

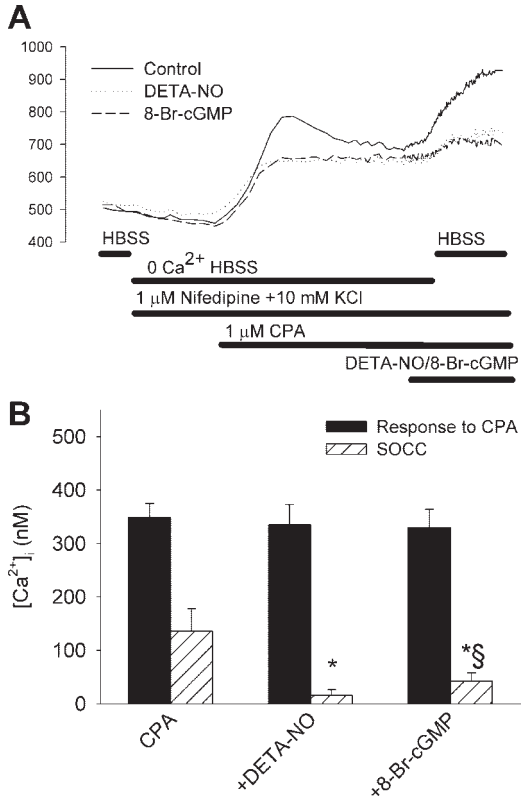

Fig. 3. Effect of cGMP on SOCC-mediated Ca^{2+} influx. As with cAMP, following a control evaluation of Ca^{2+} influx, ASM cells were washed and reexposed to CPA, followed by either 1 μM (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO, a nitric oxide donor) or 100 μM 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP, cell-permeant cGMP analog) (*A*). In the continued presence of CPA and DETA-NO or 8-BrcGMP, SOCC-mediated Ca^{2+} influx following reintroduction of extracellular Ca^{2+} was significantly decreased by both DETA-NO and 8-BrcGMP (*B*). *Significant difference from control, §between DETA-NO and 8-BrcGMP ($P < 0.05$).

SOCC-mediated Ca^{2+} influx were largely unaffected by preexposure to KT-5720.

DISCUSSION

Cyclic nucleotides (cAMP and cGMP) relax ASM (13, 25) via several mechanisms that would normally elevate $[Ca^{2+}]_i$ in different smooth muscle types (9, 15–17, 26, 28, 31). In the present study, we demonstrate that cAMP and cGMP also inhibit SOCC-mediated Ca^{2+} influx. Given the fact that SR Ca^{2+} release is an important contributor to total $[Ca^{2+}]_i$ in ASM, inhibition of SOCC-mediated influx would only lead to lesser available SR Ca^{2+} release during agonist stimulation and thus to enhanced relaxation of ASM.

SOCC and $[Ca^{2+}]_i$ regulation in ASM. In ASM, both extracellular Ca^{2+} influx and SR Ca^{2+} release are important in regulation of $[Ca^{2+}]_i$. The initial $[Ca^{2+}]_i$ response to agonists such as ACh involves SR Ca^{2+} release; however, SR Ca^{2+} release continues to occur, sometimes displaying an oscillatory pattern (27). Regardless, maintenance of $[Ca^{2+}]_i$ likely involves sustained Ca^{2+} influx, at least serving to maintain and replenish SR Ca^{2+} and to compensate for plasma membrane Ca^{2+} efflux. In this regard, Ca^{2+} influx occurs via both voltage-gated (36) and receptor-gated channels (11, 21). In recent studies, we (2, 22) and others (10) found that Ca^{2+} influx in ASM occurs in response to SR Ca^{2+} depletion. Such store-operated Ca^{2+} influx (i.e., SOCC) has now been demonstrated in several smooth muscle types (1, 5–7, 19). In ASM, such influx is blocked by Ni^{2+} and La^{3+} (2, 19, 22) and is triggered by Ca^{2+} release via IP_3 receptor channels and ryanodine receptor channels (2). A novel finding in the present study was that the extent of SOCC-mediated Ca^{2+} influx was correlated to the extent of SR Ca^{2+} depletion (as determined by the timing of CPA exposure). However, we did not directly measure the state of SR Ca^{2+} stores. Nonetheless, these data suggest that SOCC-mediated influx may play a role even during agonist stimulation resulting in only partial SR depletion. It remains to be determined whether certain thresholds of SR Ca^{2+} stores exist for triggering of SOCC-mediated Ca^{2+} influx.

Effect of cyclic nucleotides on $[Ca^{2+}]_i$ in ASM. Cyclic nucleotides (cAMP and cGMP) are known to produce smooth muscle relaxation (13, 25). cAMP is activated by β-adrenoreceptor agonists such as albuterol, terbutaline, isoproterenol, and some prostaglandins (e.g., PGE_2). cAMP activation is coupled to adenylate cyclase, which in turn activates protein kinase A. cAMP-mediated actions in smooth muscle reflect downregulation of mechanisms that would normally elevate $[Ca^{2+}]_i$, thus leading to relaxation. For example, in the presence of ACh and histamine stimulation, cAMP increases IP_3 hydrolysis, reducing IP_3-induced SR Ca^{2+} release. In vascular smooth muscle, cAMP increases calcium uptake by internal stores (31). In a previous study, we demonstrated that cAMP activation via salbutamol, a β_2-agonist, inhibits ACh-induced $[Ca^{2+}]_i$ oscillations in ASM that occur via repetitive SR Ca^{2+} release and reuptake (28). In that study, we also found that salbutamol as well as a cell-permeant cAMP analog increase the rate of decline in $[Ca^{2+}]_i$ within each oscillation, suggesting accelerated SR Ca^{2+} reuptake. Other studies in non-ASM cell types have found that cAMP activates plasma membrane K^+ channels, leading to membrane hyperpolarization (15), thus leading to inhibition of Ca^{2+} influx via L-type Ca^{2+} channels (16, 17). Thus there is considerable previous evidence

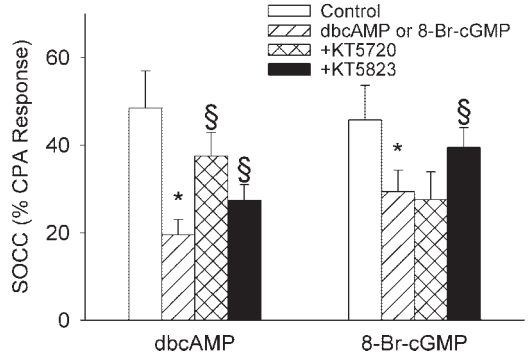

Fig. 4. Role of protein kinases A and G in cyclic nucleotide inhibition of SOCC-mediated Ca^{2+} influx. In the presence of a protein kinase A inhibitor (KT-5720), dbcAMP produced less inhibition of SOCC-mediated Ca^{2+} influx. However, even in the presence of a protein kinase G inhibitor (KT-5823), dbcAMP inhibition of SOCC was partially reversed, suggesting cross-activation of protein kinase G. 8-Br-cGMP inhibition of SOCC-mediated influx was partially reversed by KT-5823 but was not affected by KT-5720. *Significant difference from control, §between influx in the presence vs. absence of protein kinase inhibitor ($P < 0.05$).

(albeit not all in ASM) for cAMP modulation of several mechanisms that increase $[Ca^{2+}]_i$. However, data on cyclic nucleotide modulation of SOCC in smooth muscle are relatively novel and limited. For example, in rat aorta, cAMP has been found to inhibit SOCC (34), whereas in astrocytes there appears to be enhancement of SOCC-mediated Ca^{2+} influx by cAMP (37). However, Liu et al. (18) recently reported that in portal vein myocytes, β-adrenoceptor stimulation profoundly inhibits SOCC-induced Ca^{2+} currents. In agreement with results in vascular smooth muscle (18, 34), the present study found that cAMP (either by β-adrenoceptor activation or by direct elevation) inhibits SOCC in porcine ASM. Furthermore, the present study demonstrates that cAMP effects on SOCC in ASM are largely mediated via protein kinase A. Given the well-recognized bronchodilatory role of β-adrenoceptor agonists, inhibition of SOCC would be expected to lead to lesser available SR Ca^{2+} release during agonist stimulation and thus to enhanced relaxation of ASM. The significant difference in the inhibitory effect of cAMP on SOCC by β-adrenoceptor activation (isoproterenol) vs. direct cAMP elevation (dbcAMP) is also interesting. The relatively greater effect of dbcAMP was somewhat surprising since both compounds elevate cAMP, albeit with different mechanisms. However, we did not directly measure intracellular cAMP levels following the concentrations of isoproterenol or dbcAMP used. It is possible that dbcAMP produced a relatively greater quantity of cAMP, thus resulting in greater SOCC inhibition. Regardless, the fact that both compounds produce SOCC inhibition suggests that under conditions where β-adrenoceptor activation is less effective (e.g., during acidosis), indirect cAMP activation may still be a potential technique for producing bronchodilation.

The effects of cGMP in ASM function have been recently reviewed (9). NO and atrial natriuretic peptide relax ASM via guanylyl cyclase activation, cGMP production, and protein kinase G activation. In vascular smooth muscle, protein kinase G activation increases calcium uptake by internal stores (e.g., SERCA) and activation of maxi-K^+ channels (membrane hyperpolarization), leading to decreased $[Ca^{2+}]_i$. In previous studies, we found that NO donors inhibit ACh-induced $[Ca^{2+}]_i$ oscillations in ASM cells (26). Furthermore, as with cAMP, both NO and cGMP analogs increase the rate of decline in $[Ca^{2+}]_i$ during ACh-induced oscillations, suggesting accelerated Ca^{2+} reuptake. There is currently little data on cGMP effects on SOCC-mediated Ca^{2+} influx. Studies in vascular smooth muscle (20) and endothelial cells (4) show cGMP inhibition of SOCC, as do the results of the present study in ASM. Furthermore, our experiments demonstrate that cGMP inhibition of SOCC is mediated via protein kinase G. Interestingly, compared with cAMP, cGMP (at least an NO donor) appears to be more potent in inhibiting SOCC. Whether this difference translates to differential bronchodilatory responses of drugs acting via the cAMP vs. cGMP pathways remains to be determined.

An important finding in vascular smooth muscle cells has been that cAMP and cGMP cross-activate protein kinases A and G, resulting in common effects on smooth muscle (3, 9, 32, 35). However, cross-activation has not been well studied in ASM but is of significant interest to clinical therapy given the potential for pathways activated by NO donors or ANP to enhance effects of β-adrenoceptor agonists in the treatment of bronchoconstriction. Furthermore, drugs that activate one pathway may still be effective in decreasing $[Ca^{2+}]_i$ in ASM, even when the other pathway is inhibited by other drugs. In the present study, we were able to demonstrate that cAMP-mediated inhibition of Ca^{2+} influx via SOCC involves, at least in part, cross-activation of protein kinase G by cAMP. However, it must be noted a considerable portion of the cAMP effect is in fact mediated via protein kinase A, suggested by the almost complete lack of cAMP inhibition of SOCC in the presence of a protein kinase A inhibitor. These data on SOCC are consistent with data on L-type Ca^{2+} channels in other smooth muscle types (3, 35).

In contrast to cAMP effects, studies have shown that cGMP inhibits protein kinase A (9). However, we were unable to demonstrate cGMP effects on protein kinase A. This does not necessarily rule out any cGMP effects on the cAMP pathway. Given the potency of DETA-NO and cGMP effects on SOCC, any inhibition of cAMP effects (and thus reduced effects on SOCC) may have been masked. Further studies on these issues are warranted.

In summary, we demonstrate that cAMP and cGMP inhibit SOCC-mediated Ca^{2+} influx in ASM cells, predominantly via their respective protein kinases, with additional contribution from protein kinase cross-activation. Given the role of SR Ca^{2+} release in ASM $[Ca^{2+}]_i$ regulation, inhibition of SOCC-mediated influx would only lead to lesser available SR Ca^{2+} release during agonist stimulation and thus to enhanced relaxation of ASM. The effects of cyclic nucleotides on SOCC have clinical potential in that several classes of bronchodilating drugs may be used in isolation or in combination to produce ASM relaxation under conditions where a single drug may be less effective.

ACKNOWLEDGMENTS

The authors greatly appreciate the superb technical assistance of Thomas Keller.

GRANTS

This work was supported by the Foundation for Anesthesia Education and Research (C. M. Pabelick), National Institutes of Health Grants GM-56686 and HL-74309 (B. Ay, A. Iyanoye, C. M. Pabelick, Y. S. Prakash, G. C. Sieck), the Mayo Clinic College of Medicine, Rochester, MN (Y. S. Prakash, C. M. Pabelick), and Marmara University, Istanbul, Turkey (B. Ay).

REFERENCES

1. **Albert AP and Large WA.** Activation of store-operated channels by noradrenaline via protein kinase C in rabbit portal vein myocytes. *J Physiol* 544: 113–125, 2002.
2. **Ay B, Prakash YS, Pabelick CM, and Sieck GC.** Store-operated Ca^{2+} entry in porcine airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 286: L909–L917, 2004.
3. **Barman SA, Zhu S, Han G, and White RE.** cAMP activates BK_{Ca} channels in pulmonary arterial smooth muscle via cGMP-dependent protein kinase. *Am J Physiol Lung Cell Mol Physiol* 284: L1004–L1011, 2003.
4. **Bischof G, Brenman J, Bredt DS, and Machen TE.** Possible regulation of capacitative Ca^{2+} entry into colonic epithelial cells by NO and cGMP. *Cell Calcium* 17: 250–262, 1995.
5. **Dalrymple A, Slater DM, Beech D, Poston L, and Tribe RM.** Molecular identification and localization of Trp homologues, putative calcium channels, in pregnant human uterus. *Mol Hum Reprod* 8: 946–951, 2002.
6. **Flemming R, Cheong A, Dedman AM, and Beech DJ.** Discrete store-operated calcium influx into an intracellular compartment in rabbit arteriolar smooth muscle. *J Physiol* 543: 455–464, 2002.
7. **Gibson A, McFadzean I, Wallace P, and Wayman CP.** Capacitative Ca^{2+} entry and the regulation of smooth muscle tone. *Trends Pharmacol Sci* 19: 266–269, 1998.

8. **Grynkiewicz G, Poenie M, and Tsien RY.** A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985.
9. **Hamad AM, Clayton A, Islam B, and Knox AJ.** Guanylyl cyclases, nitric oxide, natriuretic peptides, and airway smooth muscle function. *Am J Physiol Lung Cell Mol Physiol* 285: L973–L983, 2003.
10. **Ito S, Kume H, Yamaki K, Katoh H, Honjo H, Kodama I, and Hideharu H.** Regulation of capacitative and noncapacitative receptor-operated Ca^{2+} entry by rho-kinase in tracheal smooth muscle. *Am J Respir Cell Mol Biol* 26: 491–498, 2002.
11. **Ito Y, Takagi K, and Tomita T.** Relaxant actions of isoprenaline on guinea-pig isolated tracheal smooth muscle. *Br J Pharmacol* 116: 2738–2742, 1995.
12. **Janssen LJ, Wattie J, Lu-Chao H, and Tazzeo T.** Muscarinic excitation-contraction coupling mechanisms in tracheal and bronchial smooth muscles. *J Appl Physiol* 91: 1142–1151, 2001.
13. **Jones KA, Lorenz RR, Warner DO, Katusic ZS, and Sieck GC.** Changes in cytosolic cGMP and calcium in airway smooth muscle relaxed by 3-morpholinosydnonimine. *Am J Physiol Lung Cell Mol Physiol* 266: L9–L16, 1994.
14. **Kannan MS, Fenton AM, Prakash YS, and Sieck GC.** Cyclic ADP-ribose stimulates sarcoplasmic reticulum calcium release in porcine coronary artery smooth muscle. *Am J Physiol Heart Circ Physiol* 270: H801–H806, 1996.
15. **Knox AJ and Tattersfield AE.** Airway smooth muscle relaxation. *Thorax* 50: 894–901, 1995.
16. **Koh SD and Sanders KM.** Modulation of Ca^{2+} current in canine colonic myocytes by cyclic nucleotide-dependent mechanisms. *Am J Physiol Cell Physiol* 271: C794–C803, 1996.
17. **Liu H, Xiong Z, and Sperelakis N.** Cyclic nucleotides regulate the activity of L-type calcium channels in smooth muscle cells from rat portal vein. *J Mol Cell Cardiol* 29: 1411–1421, 1997.
18. **Liu M, Large WA, and Albert AP.** Stimulation of beta-adrenoceptors inhibits store-operated channel currents via a cAMP-dependent protein kinase mechanism in rabbit portal vein myocytes. *J Physiol* 562: 395–406, 2005.
19. **McDaniel SS, Platoshyn O, Wang J, Yu Y, Sweeney M, Krick S, Rubin LJ, and Yuan JX.** Capacitative Ca^{2+} entry in agonist-induced pulmonary vasoconstriction. *Am J Physiol Lung Cell Mol Physiol* 280: L870–L880, 2001.
20. **Moneer Z, Dyer JL, and Taylor CW.** Nitric oxide co-ordinates the activities of the capacitative and non-capacitative Ca^{2+}-entry pathways regulated by vasopressin. *Biochem J* 370: 439–448, 2003.
21. **Murray RK and Kotlikoff MI.** Receptor-activated calcium influx in human airway smooth muscle cells. *J Physiol* 435: 123–144, 1991.
22. **Pabelick CM, Ay B, Prakash YS, and Sieck GC.** Effects of volatile anesthetics on store-operated Ca^{2+} influx in airway smooth muscle. *Anesthesiology* 101: 373–380, 2004.
23. **Pabelick CM, Sieck GC, and Prakash YS.** Invited review: significance of spatial and temporal heterogeneity of calcium transients in smooth muscle. *J Appl Physiol* 91: 488–496, 2001.
24. **Parekh AB and Putney JW Jr.** Store-operated calcium channels. *Physiol Rev* 85: 757–810, 2005.
25. **Pfitzer G, Hofmann F, DiSalvo J, and Ruegg JC.** cGMP and cAMP inhibit tension development in skinned coronary arteries. *Pflügers Arch* 401: 277–280, 1984.
26. **Prakash YS, Kannan MS, and Sieck GC.** Nitric oxide inhibits ACh-induced intracellular calcium oscillations in porcine tracheal smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 272: L588–L596, 1997.
27. **Prakash YS, Kannan MS, and Sieck GC.** Regulation of intracellular calcium oscillations in porcine tracheal smooth muscle cells. *Am J Physiol Cell Physiol* 272: C966–C975, 1997.
28. **Prakash YS, van der Heijden HF, Kannan MS, and Sieck GC.** Effects of salbutamol on intracellular calcium oscillations in porcine airway smooth muscle. *J Appl Physiol* 82: 1836–1843, 1997.
29. **Putney JW.** TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. *Proc Natl Acad Sci USA* 96: 14669–14671, 1999.
30. **Putney JW and McKay RR.** Capacitative calcium entry channels. *Bioessays* 21: 38–46, 1999.
31. **Raeymaekers L, Eggermont JA, Wuytack F, and Casteels R.** Effects of cyclic nucleotide dependent protein kinases on the endoplasmic reticulum Ca^{2+} pump of bovine pulmonary artery. *Cell Calcium* 11: 261–268, 1990.
32. **Ruiz-Velasco V, Zhong J, Hume JR, and Keef KD.** Modulation of Ca^{2+} channels by cyclic nucleotide cross activation of opposing protein kinases in rabbit portal vein. *Circ Res* 82: 557–565, 1998.
33. **Sanders KM.** Invited review: mechanisms of calcium handling in smooth muscles. *J Appl Physiol* 91: 1438–1449, 2001.
34. **Van der Zypp A, Rechtman M, and Majewski H.** The role of cyclic nucleotides and calcium in the relaxation produced by amrinone in rat aorta. *Gen Pharmacol* 34: 245–253, 2000.
35. **White RE, Kryman JP, El-Mowafy AM, Han G, and Carrier GO.** cAMP-dependent vasodilators cross-activate the cGMP-dependent protein kinase to stimulate BK_{Ca} channel activity in coronary artery smooth muscle cells. *Circ Res* 86: 897–905, 2000.
36. **Worley JF and Kotlikoff MI.** Dihydropyridine-sensitive single calcium channels in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 259: L468–L480, 1990.
37. **Wu ML, Chen WH, Liu IH, Tseng CD, and Wang SM.** A novel effect of cyclic AMP on capacitative Ca^{2+} entry in cultured rat cerebellar astrocytes. *J Neurochem* 73: 1318–1328, 1999.