Ca$^{2+}$-dependent Cl$^-$ channels in mouse and rabbit aortic smooth muscle cells: regulation by intracellular Ca$^{2+}$ and NO

YOJI HIRAKAWA, MARION GERICKE, RICHARD A. COHEN, AND VICTORIA M. BOLOTINA

Vascular Biology Unit, Whitaker Cardiovascular Institute, Boston Medical Center, Boston, Massachusetts 02118

Hirakawa, Yoji, Marion Gericke, Richard A. Cohen, and Victoria M. Bolotina. Ca$^{2+}$-dependent Cl$^-$ channels in mouse and rabbit aortic smooth muscle cells: regulation by intracellular Ca$^{2+}$ and NO. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1732–H1744, 1999.—Ca$^{2+}$-dependent Cl$^-$ (Cl$_{Ca}$) channels and their regulation by intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) and nitric oxide (NO) were characterized in mouse and rabbit aortic smooth muscle cells (SMC) using patch clamp and fura 2 imaging. Single channels (1.8 pS) and whole cell Cl$_{Ca}$ currents were activated by caffeine-induced Ca$^{2+}$ release. Single Cl$_{Ca}$ channels were also activated by >200 nM Ca$^{2+}$ in inside-out membrane patches and remained active for >5 min in ≤1 µM Ca$^{2+}$ but showed rapid rundown in 2 mM Ca$^{2+}$. Authentic NO or S-nitroso-N-acetylpenicillamine (SNAP) did not affect their activation or rundown in inside-out patches. In the whole cell, SNAP (100 µM) and 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (50 µM) did not affect Cl$_{Ca}$ current, but at a higher concentration SNAP (1 mM) induced a sustained [Ca$^{2+}$]$_i$ rise, accompanied by a dramatic decrease in caffeine-induced Ca$^{2+}$ release and Cl$_{Ca}$ current. These results indicate that 1) mouse and rabbit aortic SMC possess 1.8-pS Cl$_{Ca}$ channels that are activated by Ca$^{2+}$ release from the stores, 2) both activation and rundown of single Cl$_{Ca}$ channels depend on [Ca$^{2+}$], and 3) NO does not affect Cl$_{Ca}$ channels directly or via cGMP but can inhibit their activation indirectly by decreasing Ca$^{2+}$ release from the stores.

Single chloride channel; rundown; noise analysis; caffeine-induced calcium release; nitric oxide

CalCIum (Ca$^{2+}$)-dependent chloride (Cl$_{Ca}$) currents can be activated by Ca$^{2+}$ sparks (39) and by global Ca$^{2+}$ release from intracellular stores triggered by a variety of contractile agonists in vascular smooth muscle cells (SMC) (for review see Refs. 5, 22). Under physiological conditions, activation of Cl$_{Ca}$ channels produces inward current and membrane depolarization that can activate L-type Ca$^{2+}$ channels, Ca$^{2+}$ influx, and contraction of vascular SMC (2, 20, 26). Inactivation or inhibition of Cl$_{Ca}$ current could cause membrane repolarization and inhibition of Ca$^{2+}$ influx, and this mechanism has been proposed to mediate endothelium-dependent hyperpolarization and relaxation of SMC (9, 24, 32). However, it is still unclear whether nitric oxide (NO), the main endothelium-derived hyperpolarizing and relaxing factor (8, 30), can affect Cl$_{Ca}$ channels.

Aortic SMC are widely used in studies of the mechanisms of endothelium-dependent vascular relaxation, but Cl$_{Ca}$ channels have not been studied in freshly dispersed aortic SMC. Thus, before addressing the questions about the effects of NO on Cl$_{Ca}$ channels, we first characterized single Cl$_{Ca}$ channels and whole cell Cl$_{Ca}$ currents in a novel preparation of SMC from mouse aorta. To the best of our knowledge, this is the first study of ion channels performed in freshly dispersed mouse aortic SMC. This preparation is a valuable tool for electrophysiological studies of smooth muscle in a growing variety of genetically modified mouse models. Because NO-induced relaxation has been extensively studied in rabbit aorta, we also characterized Cl$_{Ca}$ channels and their regulation by Ca$^{2+}$ release from intracellular stores in SMC freshly dispersed from rabbit aorta.

Activation of whole cell Cl$_{Ca}$ current in a variety of SMC has been shown to strictly depend on intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) rise caused by contractile agonists (for review, see Refs. 5, 22). The mechanism of inactivation of the whole cell Cl$_{Ca}$ current or rundown of single Cl$_{Ca}$ channels is not that clear. Ca$^{2+}$ regulation of Cl$_{Ca}$ channels has been studied mostly at the level of whole cell Cl$_{Ca}$ currents because of extremely small conductance and rapid rundown of single Cl$_{Ca}$ channels in excised membrane patches. Some authors suggested that Cl$_{Ca}$ current strictly follows [Ca$^{2+}$] (25), whereas others observed that Cl$_{Ca}$ current decreased faster than [Ca$^{2+}$] after the release of Ca$^{2+}$ from the stores (34). It was proposed that Ca$^{2+}$/calmodulin-dependent protein kinase can induce inactivation of Cl$_{Ca}$ currents in equine tracheal SMC by uncoupling channel activity from [Ca$^{2+}$] (34). However, it is unclear whether this process could be responsible for rundown or inactivation of single Cl$_{Ca}$ channels. In the present study, we provide a detailed description of activation and rundown of single Cl$_{Ca}$ channels in excised membrane patches. We believe that our finding of the dependence of the rundown of single Cl$_{Ca}$ channels on intracellular Ca$^{2+}$ will open new possibilities for their prolonged recording in excised membrane patches, which was a nonresolvable problem for many years.

Many different targets for NO have been found in SMC, but it is unknown whether single Cl$_{Ca}$ channels can be affected by NO directly, similar to Ca$^{2+}$-dependent K$^+$ (K$_{Ca}$) channels (1, 4, 28). Also, one could propose the existence of some indirect regulation of Cl$_{Ca}$ channels by NO through its effects on Ca$^{2+}$ stores, because Ca$^{2+}$ released from the stores activates Cl$_{Ca}$ channels. NO has been shown to affect ryanodine receptors in caffeine-sensitive stores of cardiac and skeletal myocytes (23, 29, 35, 36), although it is unclear how this might affect Cl$_{Ca}$ channels. In the present...
study, we address these questions and show that NO does not inhibit single Cl\(_{\text{Ca}}\) channels in excised membrane patches but an NO donor, S-nitroso-N-acetylpenicillamine (SNAP), can suppress caffeine-induced activation of Cl\(_{\text{Ca}}\) currents by decreasing Ca\(^{2+}\) release from the stores. Preliminary results have been published in abstract form (14).

**MATERIALS AND METHODS**

Preparations

Mouse aortic SMC. Freshly isolated mouse aortic SMC (mSMC) were obtained by a modified method using papain digestion (6) as follows. C57BL/6 mice (15–20 g) were anesthetized by inhalation of diethyl ether and killed by cervical dislocation. The thoracic aorta was rapidly removed, cleaned of connective tissue, cut into small pieces, and rinsed in dissociation medium (DM) containing (in mM) 110 NaCl, 5 KCl, 10 NaHCO\(_3\), 0.5 NaH\(_2\)PO\(_4\), 0.5 K\(_2\)HPO\(_4\), 2 MgCl\(_2\), 10 taurocholic acid, 10 HEPES, and 11 glucose and 0.02% bovine serum albumin (pH 7.2). Pieces of aorta were incubated in DM with papain (40 U/ml; Fluka, Buchs, Switzerland) and dithiothreitol (DTT, 2 mM; Sigma, St. Louis, MO) for 15 min at 37°C with constant stirring with a small magnetic stirrer (Fisher, Pittsburgh, PA). After incubation, they were rinsed twice in fresh DM and then gently triturated with a heat-polished Pasteur pipette. Isolated cells were stored at 4°C until use for up to 4 h. A small drop of the cell suspension was placed in a 35-mm polystyrene tissue culture dish (Corning) or a 0.15-mm glass-bottom chamber (Bioptechs, Butler, PA), and SMC were allowed to adhere to the bottom before they were washed with normal bath solution.

Rabbit aortic SMC. Male New Zealand White rabbits (2–2.5 kg) were exsanguinated after injection of pentobarbital sodium (30 mg/kg) and heparin (150 U/kg). A segment of thoracic aorta was rapidly removed, cleaned of connective tissues, cut into small pieces, and rinsed in DM. Pieces of aorta were incubated in DM with papain (50 U/ml) and DTT (2 mM) for 30 min at 37°C in a shaking water bath. After incubation, they were rinsed twice in fresh DM and then gently triturated with a heat-polished Pasteur pipette. Isolated cells were stored at 4°C until use for up to 4 h. A small drop of the cell suspension was placed in a 35-mm polystyrene tissue culture dish (Corning) or a 0.15-mm glass-bottom chamber (Bioptechs, Butler, PA), and SMC were allowed to adhere to the bottom before they were washed with normal bath solution.

**Electrophysiological Studies**

Single-channel recording. Single-channel currents were recorded using the standard patch-clamp technique (12) in cell-attached or inside-out membrane patches. Pipettes were pulled from borosilicate glass capillaries with a filament (1B150F; WPI, Sarasota, FL) on a horizontal puller (model P-87; Sutter Instrument, Novato, CA) and heat polished with a microforge (model MF-9; Narishige, Tokyo, Japan). To improve signal-to-noise ratio during single-channel recording, pipettes with tip resistance of 20–25 M\(\Omega\) were coated with Sylgard (Dow Corning, Midland, MI). The currents were recorded with a low-noise patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA). Data were filtered at 1 kHz and stored on digital audio tapes (DT-1200A; Sony, Tokyo, Japan) using a digital tape recorder (DTR-1203; Bio-Logic, Claix, France) for later analysis. Representative current traces were additionally filtered at 200–500 Hz for better visual resolution on the figures. pClamp6 software (Axon Instruments) was used for data acquisition and analysis. Probability of the channels being open (NP\(_o\), where N = no. of channels in the patch) was analyzed and plotted over time to illustrate the time course of channel activity in representative experiments. The original traces of single-channel currents are shown at different times of the experiment as defined in Figs. 1, 2, 4, and 11.

Whole cell recording. Whole cell currents were recorded using an amphotericin B-perforated patch-clamp technique unless otherwise indicated. Amphotericin B (300 µg/ml) was included in the pipette solution, sonicated for 2–3 min, and then kept in the dark and used within 12 h. Heat-polished glass pipettes (1B150; WPI) with tip resistance of 1–3 M\(\Omega\) were used. The very tip of the pipette was filled with amphotericin B-free pipette solution, and then the pipette was backfilled with amphotericin B-containing solution. Electrical connection with series resistance of 20–30 M\(\Omega\) was achieved within 5 min after making gigahm seal contact. With this technique, whole cell currents could be recorded without changing cytosolic Ca\(^{2+}\) concentration because the pores formed by amphotericin B are not permeable for Ca\(^{2+}\) and intracellular messenger molecules (21). The currents were recorded with Axopatch 200B, filtered at 1–2 kHz, and stored on a computer hard disk for later analysis using pClamp6 software. The cell capacitance and the series resistance were compensated. Leakage currents were not subtracted in any current traces. Whole cell currents were evoked by ramp depolarizations (from −100 to +50 mV for 150 or 750 ms, every 1 or 2 s) from a holding potential of −30 or −60 mV (as specified in the legends to Figs. 5, 7, 8, and 10). All experiments were performed at room temperature (20–21°C).

**Noise Analysis of Whole Cell Current**

Nonstationary noise analysis was done using standard methods (13) as described earlier (37). Whole cell Cl\(_{\text{Ca}}\) current was recorded at constant holding potentials (−30, −60, and/or −100 mV) during application of caffeine (n = 8). Data were filtered at 2 kHz and sampled at 10 kHz. Mean current (\(I\)) and current variance (\(\sigma^2\)) were determined during 25- to 50-ms intervals. During the sampling intervals, I changes by <10%. Analysis of the dependence of \(\sigma^2\) on I was performed using Microcal Origin (Microcal Software, Northampton, MA). The single-channel current (\(i\)) was estimated for each experiment by fitting the equation \(\sigma^2(I) = I_0 + (N_b + B)\), where \(\sigma^2(I)\) is the variance, N is the number of channels, and B is a free parameter reflecting the background noise. Single-channel conductance was estimated from the slope of the single-channel current-voltage relationship.

**Intracellular Ca\(^{2+}\) Measurement**

Freshly dispersed individual SMC were placed in a chamber with a 0.15-mm glass bottom (Bioptechs) and incubated with fura 2-AM (5 µM) for 30 min at room temperature. After being washed with regular bath solution (in a shaking water bath at 37°C for 15 min), the cells were transferred to the stage of an inverted microscope (Olympus IX70, Tokyo, Japan) equipped with a ×40 fluorescence objective (Olympus Uapo/340, NA 0.9).

Fluorescence was measured at room temperature (20–21°C) using a dual-excitation fluorescence imaging system from IonOptix (Milton, MA). Cells were illuminated at 340 and 380 nm, and the emitted light was collected at 510 nm by an intensified charge-coupled device (CCD) camera. [Ca\(^{2+}\)] was calculated from the measured ratio of 340-nm to 380-nm signals (R) using the formula (10) [Ca\(^{2+}\)] = \(K_d \cdot \beta \cdot (R - R_{\min})/R_{\max} - R\), where \(R_{\min}\), \(R_{\max}\), and \(\beta\) were determined from in vitro calibration and a dissociation constant (\(K_d\)) of 145 nM for fura 2 was used. After each experiment, cells were permeabilized with ionomycin (5 µM) and fura 2 was quenched with Mn\(^{2+}\) (10–20 mM). The resulting image was used as a background image and was subtracted from the experimental
traces. IonWizard software (IonOptics) was used for data acquisition and analysis. For simultaneous measurement of fluorescence and whole cell currents, IonWizard was synchronized with pClamp6.

Solutions

The normal bath solution contained (in mM) 130 NaCl, 10 tetraethylammonium chloride (TEA), 2 CaCl₂, 2.8 KCl, 2 MgCl₂, 10 HEPES, and 5.5 glucose. The pH was adjusted to 7.4 with NaOH. In some experiments, Na⁺ or Cl⁻ was replaced by equimolar N-methyl-D-glucamine (NMDG) or glutamate, respectively. For Ca²⁺-free solution, Ca²⁺ was not added and the solution contained 2 mM EGTA to buffer the residual Ca²⁺. For low-Ca²⁺ (100, 200, and 400 nM and 1 µM) solutions, 1 mM EGTA was included together with 390, 550, 720 and 870 µM Ca²⁺, respectively. The normal pipette solution for perforated patch recordings contained (in mM) 100 cesium aspartate, 40 CsCl, 3 MgCl₂, 0.1 EGTA, and 10 HEPES. The pH was adjusted to 7.2 with CsOH. For conventional whole cell recordings, MgCl₂ was replaced by equimolar MgATP. For single-channel recordings, the normal pipette solution contained (in mM) 140 NaCl, 2 CaCl₂, and 10 HEPES (pH 7.2). As indicated in some experiments, cation currents were eliminated by substituting all membrane-permeant cations with impermeant NMDG, and the pipette solution contained (in mM) 140 NMDG and 10 HEPES (pH 7.2 with HCl).

Preparation of NO Solution

A standard 1-liter intravenous solution bag was filled with distilled water (750 ml) that had been bubbled with nitrogen gas to remove oxygen. Approximately 30 mg of Bio-Rad analytical grade anion exchange resin were mixed in the water before the bag was filled. The resin retains any nitrates or nitrites that may be formed by NO reacting with oxygen. The contents of the bag were bubbled with nitrogen gas for another 30 min. Any dead space nitrogen gas was expelled from the bag before it was filled to capacity with NO gas. The contents were then mixed thoroughly, and the bag was placed in a refrigerator at 4°C. The concentration of NO in the solution equilibrated to give a 3.1 ± 0.6 mM (n = 5) saturated solution at least for 1 wk as measured by a chemiluminescent NO analyzer (Sievers NOA model 270, Boulder, CO). At the time of use, subsequent dilutions were made from this stock by simply drawing off the solution from the bag with a syringe. Serial dilutions were prepared in 100 x 16-mm Vacutainer blood collection tubes filled with 9 ml of bath solution that was deoxygenated by bubbling for 1 h with nitrogen gas on ice.

Drugs

Caffeine, ionomycin, niflumic acid, amphotericin B, DTT, NMDG, TEA, SNAP, 8-(4-chlorophenylthio)-guanosine 3’,5’-cyclic monophosphate (CPT-cGMP), MgATP, R-24571, calyculin A, and bovine serum albumin (fatty acid, endotoxin free) were purchased from Sigma. Acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA-AM) and fura 2-AM were from Molecular Probes (Eugene, OR). Papain was from Fluka. SNAP, papain, DTT, bovine albumin, and amphotericin B were prepared from powder at the time of each experiment. Other drugs were prepared as a stock solution and were diluted to the final concentration with the bath solution at the time of the experiment.

Statistics

Data are shown as means ± SE, with n indicating the number of experiments. Statistical significance between groups was assessed using Student’s paired or unpaired t-test. Values of P < 0.05 were considered to be significant.

RESULTS

Single Cl⁻ Ca²⁺ Channels in Mouse and Rat Aortic SMC

General characteristics. Single Cl⁻–channel currents were recorded and compared in aortic SMC freshly isolated from mouse (mSMC) and rabbit (rSMC). Figure 1 shows a typical recording of inward single-channel currents and analysis of NP₀ in cell-attached and inside-out configurations of the same membrane patch from mSMC. There were virtually no channel openings in resting mSMC (Fig. 1A), but caffeine (10 mM) applied to the cell activated small inward currents (Fig. 1B; n = 6). The channel activation was always transient and lasted 30 s even in the continuous presence of caffeine. Exposing an inside-out membrane patch into 2 mM Ca²⁺-containing bath solution acti-
activated the same channel (Fig. 1C; n = 7). In rSMC we found similar channels that were activated either by caffeine in cell-attached membrane patches (Fig. 2A; n = 11) or by excising inside-out membrane patch into 2 mM Ca\(^{2+}\) (Fig. 2B; n = 19). In both preparations of mSMC and rSMC, the single-channel currents (Fig. 3, A and B) in inside-out membrane patches under symmetrical Cl\(^{-}\) conditions had a linear current-voltage (I-V) relationship with a reversal potential of 0 mV and an identical single-channel conductance of 1.8 ± 0.2 pS.

Substitution of all permeable cations with impermeable NMDG in the pipette did not affect the amplitude or appearance of inward single-channel currents in mSMC (Fig. 1; n = 63) or rSMC (Fig. 2C; n = 106). Inward currents disappeared when the concentration of Cl\(^{-}\) in the bath solution was reduced from 149 to 9 mM by substitution with glutamate (Fig. 2C; n = 3). Ni\(^{2+}\) (5 mM; n = 10) or TEA (10 mM; n = 9) did not affect single-channel currents (Fig. 2B), but the currents were absent when niflumic acid (100 µM), a Cl\(^{-}\)-channel inhibitor, was included in the pipette solution (Fig. 2D; n = 9). These data are consistent with the presence of single Cl\(^{-}\) channels of 1.8-pS conductance in aortic SMC from mouse and rabbit.

Ca\(^{2+}\)-dependent activation. Ca\(^{2+}\) dependence of single Cl\(^{-}\) channels was studied in excised inside-out mem-

Fig. 2. Single Cl\(_{\text{Ca}}\)-channel currents in rabbit aortic SMC (rSMC). Typical traces of single-channel currents recorded at −100 mV applied to membrane. Membrane patch configuration and main components of pipette and bath solutions are shown in insets. Inward currents are shown as downward deflections; zero current is shown by arrow at end of each trace. A: activation of single-channel currents in cell-attached membrane patch during application of caffeine (10 mM) to whole cell. B: single-channel currents before and after inside-out membrane patch was excised into 2 mM Ca\(^{2+}\)-containing bath solution. Moment of excising patch is shown by vertical bar interrupting time course of recording for −2 s to eliminate mechanical artifacts. Bottom inset shows distinct levels of single-channel currents at higher time resolution. Tetraethylammonium chloride (TEA, 10 mM) and Ni\(^{2+}\) (5 mM) are present in pipette. C: single-channel currents recorded before and after an inside-out membrane patch was excised in 2 mM Ca\(^{2+}\). Inward currents recorded with NMDG (140 mM) in pipette disappear after reduction of Cl\(^{-}\) concentration in bath solution from 149 to 9 mM (at time indicated). D: single-channel current recording before and after inside-out membrane patch was excised into Ca\(^{2+}\) (2 mM)-containing bath solution. Niflumic acid (Niflu A, 100 µM) and TEA (10 mM) were present in pipette solution.

Fig. 3. Current-voltage (I-V) relationship of single Cl\(_{\text{Ca}}\)-channel current in mSMC (A) and rSMC (B) in inside-out membrane patches obtained from mSMC and rSMC under symmetrical Cl\(^{-}\) conditions. Each point represents mean ± SE from 3–29 different experiments (no. of experiments shown for each point). Single-channel conductance γ is calculated from slope of I-V relationship.
brane patches by exposing the intracellular surface of the membrane to different concentrations of Ca$^{2+}$. Channel activation did not occur when patches were excised from rSMC in Ca$^{2+}$-free (n = 41) or 100 nM Ca$^{2+}$-containing (n = 29) bath solution (Fig. 4A). The channels could be activated only when the patch was excised in [Ca$^{2+}$] $\geq$200 nM (Fig. 4, B–D; n = 53 for mSMC, and n = 97 for rSMC). The initial activity of the channels (immediately after the patch was excised) in 200 nM [Ca$^{2+}$] was lower than in >400 nM [Ca$^{2+}$] (Fig. 4, B–D).

Ca$^{2+}$-dependent rundown. After inside-out membrane patches were excised in 2 mM Ca$^{2+}$, activation of single Cl$_{Ca}$ channels was followed by rapid rundown, which was dependent on the Ca$^{2+}$ concentration at the intracellular surface of the membrane patches. In 2 mM Ca$^{2+}$, the channel activity completely disappeared within 1–2 min after the patches were excised (Fig. 4D; 72 ± 14 s (n = 12) for rSMC and 63 ± 12 s (n = 4) for mSMC). Rundown was significantly slower at lower [Ca$^{2+}$]. Indeed, as shown in Fig. 4C, the channel could be active for >10 min when patches were excised in 1 µM Ca$^{2+}$ and the maximum time of the single-channel recording was primarily determined by the lifetime of the membrane patches and varied from 5 to 11 min (n = 14). Interestingly, when the patches were excised from rSMC and exposed to Ca$^{2+}$-free solution (with 2 mM EGTA added) for 3 min, subsequent application of Ca$^{2+}$ (1 µM) failed to activate Cl$_{Ca}$ channels (n = 4), indicating that along with Ca$^{2+}$-dependent rundown, there is also some Ca$^{2+}$-independent rundown of single Cl$_{Ca}$ channels.

Excising the membrane patch from rSMC in 2 mM Ca$^{2+}$ in the presence of ATP (3 mM) did not affect activation of Cl$_{Ca}$ channels and did not change the time required for their rundown (71 ± 14 s (n = 9) compared

---

**Fig. 4.** Ca$^{2+}$-dependent activation and rundown of single Cl$_{Ca}$ channels in inside-out membrane patches. NP$_o$ and original traces of single-channel currents during different periods of experiment are shown before and after inside-out membrane patches from rSMC were excised into bath with different concentrations of Ca$^{2+}$ (100 and 400 nM (A), 200 and 400 nM (B), 1 µM (C), and 2 mM (D)). Pipette solution contained 140 mM NMDG. Inward currents (shown as downward deflections) were recorded at −100 mV applied to membrane; zero current level is shown by a dotted line.
with control $72 \pm 14$ s ($n = 12$; $P > 0.1$). Similar activation and rundown of $\text{Cl}_{\text{Ca}}$ channels were also observed in the combined presence of ATP and the calmodulin inhibitor R-24571 (3 $\mu$M). The time required for rundown under these conditions was $58 \pm 20$ s ($n = 4$, $P > 0.1$). A high concentration of the protein phosphatase 1 and 2A inhibitor calyculin A (100 $\mu$M) in the bath neither prevented nor accelerated the rundown of $\text{Cl}_{\text{Ca}}$ channels. When patches were excised into 1–2 $\mu$M $\text{Ca}^{2+}$ in the presence of calyculin, the channels remained active for $>5$ min ($n = 7$), which was similar to the duration observed under control conditions.

**WholeCell $\text{Ca}^{2+}$-Dependent $\text{Cl}^-$ Current**

**General characteristics.** There was no difference in the basal characteristics and the regulation of $\text{Cl}_{\text{Ca}}$ currents between mSMC and rSMC, although the cell capacitance of fully relaxed mSMC ($10.5 \pm 2.1$ pF; $n = 74$) was about one-half that of rSMC ($20.3 \pm 3.8$ pF; $n = 95$). Under conditions in which $\text{K}^+$ channels were inhibited by $\text{Cs}^+$ (140 mM) in the pipette, bath application of caffeine (10 $\text{mM}$) transiently activated a whole cell current in both mSMC ($n = 13$) and rSMC ($n = 63$). Figure 5A shows a typical whole cell current in rSMC.

**Fig. 5.** Main characteristics of whole cell $\text{Cl}_{\text{Ca}}$ current induced by caffeine. A–C: changes in whole cell current are shown during application of caffeine (10 mM) to rSMC. Ramp depolarizations (from $-100$ to $+50$ mV, 750 ms) are applied every 1 s. Holding potential is $-30$ mV. I-V relationships are shown on right during corresponding ramps before and during caffeine application. Ni$^{2+}$ (5 mM) and TEA (10 mM) are present in bath solution in B, niflumic acid (100 $\mu$M) and TEA (10 mM) are present in C. D: summary data showing density of peak $\text{Cl}_{\text{Ca}}$ current activated by caffeine in control and in presence of TEA (10 mM), Ni$^{2+}$ (5 mM) + TEA (10 mM), nifedipine (5 $\mu$M) + TEA (10 mM), and niflumic acid (niflu. a.; 100 $\mu$M) + TEA (10 mM). Inward currents at $-80$ mV are shown by downward bars, and outward currents at $+50$ mV are shown by upward bars. Numbers show how many experiments are summarized for each treatment. *$P < 0.05$ were considered to be significant.
recorded at 230 mV with voltage ramps applied every 1 s. The caffeine-induced current showed some outward rectification and had a reversal potential of \( \pm 22.4 \pm 2.3 \) mV (\( n = 29 \)), which is close to the calculated equilibrium potential for \( \text{Cl}^- \) under our experimental conditions (\( E_{\text{Cl}} = -30 \) mV). When \( \text{Cl}^- \) concentration of the bath solution was reduced from 149 to 49 mM by equimolar substitution with glutamate, the reversal potential of the caffeine-induced current shifted in the positive direction by 18.7 \( \pm 5.1 \) mV (\( n = 3 \)), consistent with \( \text{Cl}^- \) selectivity of the current. The caffeine-induced current was insensitive to TEA (10 mM, Fig. 5, B and D; \( n = 35 \)), Ni\(^{2+} \) (5 mM, Fig. 5, B and D; \( n = 9 \)), and nifedipine (5 \( \mu \)M, Figs. 5D and 8C; \( n = 6 \)) but was completely inhibited by niflumic acid (100 \( \mu \)M, Fig. 5, C and D; \( n = 8 \)). On the basis of all these data, the caffeine-induced whole cell current was identified as \( \text{Cl}^- \) current under basal conditions. There was no difference in \( \text{Cl}^- \) current density between mSMC and rSMC. At 260 mV, inward current densities were \(-10.2 \pm 0.9 \) (\( n = 12 \)) and \(-10.1 \pm 0.9 \) (\( n = 18 \)) pA/pF, respectively.

Noise analysis. To characterize the single channels that underlie the whole cell current activated by caffeine, we performed noise analysis of the whole cell current. Figure 6A shows an example of the whole cell current recorded in rSMC at -60 mV during application of caffeine. Figure 6B shows an example of the distribution of current variance during the rising phase of the current at -60 mV fitted with the equation \( \sigma^2(i) = il - I^2/N + B \), where \( l \) is a mean whole cell current and \( i \) is an estimated single-channel current. Figure 6C summarizes the dependence of estimated single-channel current on membrane potential in different rSMC (\( n = 8 \)). Single-channel conductance (obtained from the slope of the I-V plot) was estimated to be \( \gamma = 2.0 \) pS, which was similar to the conductance of

![Fig. 6. Noise analysis of whole cell Cl\(_2\) current activated by caffeine. A: an example of whole cell Cl\(_2\) current recorded at -60 mV during application of caffeine (10 mM) in rSMC. B: representative plot of current variance (\( \sigma^2 \)) vs. mean current at -60 mV in same rSMC. Each point represents current variance during sequential 25-ms intervals recorded during rising phase of caffeine-induced Cl\(_2\) current. C: I-V relationship of estimated single Cl\(_2\)-channel current obtained from a polynomial fit on B. Each point represents mean \( \pm \) SE from 3–8 different experiments (shown by no. under each point). Single-channel \( \gamma \) is calculated from slope of I-V relationship.](http://ajpheart.physiology.org/)
the single Cl\textsubscript{Ca} channels recorded in inside-out membrane patches (Fig. 3, A and B). The reversal potential of the estimated single-channel current was around 25 mV, which is very close to the reversal potential of the whole cell Cl\textsubscript{Ca}\textsuperscript{2+} currents. These data are consistent with single Cl\textsubscript{Ca} channels of 2 pS being responsible for caffeine-induced whole cell currents in rSMC.

Regulation of Cl\textsubscript{Ca} currents by \textsuperscript{2+}Ca release from intracellular stores. Simultaneous recording of [Ca\textsuperscript{2+}] and whole cell currents in rSMC and mSMC showed that Cl\textsuperscript{−} current is transiently activated simultaneously with caffeine-induced [Ca\textsuperscript{2+}], rise (Fig. 7A). Figure 7B shows the time course of the current (measured at −30 mV) and [Ca\textsuperscript{2+}] (from Fig. 7A), both normalized to their peak amplitude and plotted on the same graph. The rise in [Ca\textsuperscript{2+}] and activation of Cl\textsuperscript{−} current peaked in ~1–2 s, but the decline of Cl\textsubscript{Ca} current was significantly faster than that of [Ca\textsuperscript{2+}]. The declining phase of Cl\textsubscript{Ca} current in mSMC had a duration of τ\textsubscript{off} = 2.6 ± 0.8 s compared with 5.9 ± 2.7 s for that of [Ca\textsuperscript{2+}] (n = 13, P < 0.001). Bath application of the \textsuperscript{2+}Ca ionophore ionomycin (1–10 \textmu M) activated the same Cl\textsuperscript{−} current as that activated by caffeine in mSMC (n = 3) and rSMC (n = 5). When the current had already been activated by ionomycin, caffeine caused no additional effect (n = 3). The same Cl\textsuperscript{−} current was also activated by dialysis of mSMC (n = 3) and rSMC (n = 2) with 1–2 \textmu M Ca\textsuperscript{2+} applied through the pipette in regular whole cell configuration.

Caffeine applied to the cells with intervals ≥5 min repeatedly induced a transient [Ca\textsuperscript{2+}]\textsubscript{i} rise (Fig. 8) and activation of Cl\textsubscript{Ca} current in the presence of extracellular...
Fig. 10. Effect of SNAP on [Ca\(^{2+}\)] and whole cell Cl\(_{\text{Ca}}\) current. A representative experiment with simultaneous recording of [Ca\(^{2+}\)] and whole cell Cl\(_{\text{Ca}}\) current in mSMC during repeated application of caffeine (10 mM) under control conditions and after addition of SNAP (1 mM). Top: changes in [Ca\(^{2+}\)] over course of whole experiment. Bottom: simultaneous recording of [Ca\(^{2+}\)] and Cl\(_{\text{Ca}}\) current is shown during corresponding periods. Current was recorded at −30 mV with ramp depolarizations (from −100 to +60 mV, 150 ms) applied every 1 s.

Fig. 11. NO does not affect single Cl\(_{\text{Ca}}\) channels in excised membrane patches. Representative experiments showing NP\(_0\) for Cl\(_{\text{Ca}}\) channels before and after inside-out membrane patches were excised in 400 nM Ca\(^{2+}\) under control conditions (A) and in presence of NO (1 µM) applied just before patch was excised (B). Original traces of single-channel currents at −100 mV applied to membrane are shown at bottom of each panel at specified time. Inward currents are shown as downward deflections; zero current level is shown by dotted line. Note that there are >2 channels in each membrane patch, resulting in several levels of single-channel currents. Pipette contained 140 mM NMDG.
lar Ca\(^{2+}\) (Fig. 8A and Fig. 9). In the absence of extracellular Ca\(^{2+}\), caffeine was still able to increase [Ca\(^{2+}\)] and activate Cl\(_{\text{Ca}}\) current, but its effect gradually disappeared after a few applications (Fig. 8B). Activation of Cl\(^{-}\) current by caffeine was abolished when the rise in [Ca\(^{2+}\)] was prevented by pretreatment of rSMC (n = 25) with a membrane-permeant Ca\(^{2+}\) chelator, BPAT-AM (20 µM for 20 min, Fig. 8D).

Although caffeine-induced activation of Cl\(_{\text{Ca}}\) currents did not require influx of extracellular Ca\(^{2+}\) (as seen in Fig. 8B), the activity of L-type Ca\(^{2+}\) channels appeared to be important for repeated activation of Cl\(_{\text{Ca}}\) currents. Indeed, caffeine-induced Cl\(_{\text{Ca}}\) current gradually disappeared when nifedipine (5 µM) was present in Ca\(^{2+}\)-containing bath solution (Fig. 8C; n = 5), similar to what occurred in Ca\(^{2+}\)-free solution. Washout of nifedipine resulted in partial recovery of caffeine-induced current. Treatment of rSMC with BAY K 8644 (1 µM) for 2–3 min before the next application of caffeine increased the amplitude of caffeine-induced Cl\(_{\text{Ca}}\) current (n = 3; not shown). These results indicate that Cl\(_{\text{Ca}}\) currents in aortic SMC are directly activated by Ca\(^{2+}\) release from intracellular stores and may be indirectly regulated by the state of filling of the stores.

Effect of NO on Caffeine-Induced Ca\(^{2+}\) Release and Ca\(^{2+}\)-Activated Cl\(^{-}\) Channels

Inhibition of whole cell Cl\(_{\text{Ca}}\) currents could provide a mechanism of NO-induced hyperpolarization and relaxation of vascular SMC. To determine whether NO inhibits Cl\(_{\text{Ca}}\) currents, we applied it directly to intact cells and excised membrane patches.

Treatment of intact mSMC with the NO donor SNAP (100 µM) or with a membrane-permeant analog of cGMP, CPT-cGMP (50 µM), did not affect whole cell Cl\(_{\text{Ca}}\) current. Figure 9A summarizes the data showing that there is no significant difference in the peak Cl\(_{\text{Ca}}\) current amplitude (at −60 mV) evoked by three consecutive applications of caffeine (with 4- to 5-min intervals to allow refilling of caffeine-sensitive stores) in the absence and presence of 100 µM SNAP. Figure 9B summarizes the changes in Cl\(_{\text{Ca}}\) currents in similar experiments in the absence and presence of CPT-cGMP.

Interestingly, in similar experiments, a higher concentration of SNAP (1 mM) did not significantly affect Cl\(_{\text{Ca}}\) current within 1 min after its application but significantly inhibited caffeine-induced Cl\(_{\text{Ca}}\) current after 5 min of treatment (Fig. 9C). After 5-min pretreatment of rSMC with 1 mM SNAP, caffeine also failed to activate single Cl\(_{\text{Ca}}\) channels in cell-attached patches (n = 5; not shown), although subsequent excision of inside-out membrane patches into Ca\(^{2+}\)-containing solution produced normal activation of Cl\(_{\text{Ca}}\) channels despite the continued presence of SNAP (n = 11).

To test the possible role of intracellular Ca\(^{2+}\) in mediating the effects of a high concentration of SNAP on Cl\(_{\text{Ca}}\) channels, the effect of SNAP on caffeine-induced Ca\(^{2+}\) release was recorded separately or simultaneously with Cl\(_{\text{Ca}}\) currents. SNAPP (100 µM) did not significantly affect the caffeine-induced transient rise in [Ca\(^{2+}\)], even when applied for 5 min (Fig. 9A). In contrast, 1 mM SNAP caused a slowly developing, sustained rise in basal [Ca\(^{2+}\)], and progressive disappearance of the caffeine-induced Ca\(^{2+}\) release (Fig. 10, top). This effect was accompanied by disappearance of caffeine-induced Cl\(_{\text{Ca}}\) currents measured simultaneously with [Ca\(^{2+}\)] (Fig. 10). Importantly, SNAP did not affect the basal whole cell current, and the SNAP-induced sustained [Ca\(^{2+}\)] rise did not activate Cl\(_{\text{Ca}}\) current in the absence of caffeine. The correlation between the time-dependent effects of SNAP (1 mM) on caffeine-induced Cl\(_{\text{Ca}}\) release and Cl\(_{\text{Ca}}\) current (Figs. 9C and 10) points to the possibility that downregulation of Cl\(_{\text{Ca}}\) currents occurs secondary to the effect of SNAP on Ca\(^{2+}\) release from intracellular stores. It is important to point out that there was some difference in the degree of inhibition of Cl\(_{\text{Ca}}\) current and Ca\(^{2+}\) release (Figs. 9 and 10), which could be caused either by additional direct effect of NO on Cl\(_{\text{Ca}}\) channels or by the partial Ca\(^{2+}\)-dependent inactivation of Cl\(_{\text{Ca}}\) channels (34) as a result of the major increase in basal [Ca\(^{2+}\)] observed in the presence of 1 mM SNAP.

To determine whether NO can inhibit single Cl\(_{\text{Ca}}\) channels directly, we excised inside-out membrane patches from mSMC in the absence or presence of authentic NO (1 µM). Figure 11 shows that NO did not prevent normal activation of single Cl\(_{\text{Ca}}\) channels when inside-out membrane patches were excised into 400 nM Ca\(^{2+}\) (n = 12). Excising the membrane patch in the presence of SNAP (1 mM) also did not affect activation of Cl\(_{\text{Ca}}\) channels (n = 5). NO also did not affect the time required for the rundown of Cl\(_{\text{Ca}}\) channels. In 1 µM Ca\(^{2+}\), complete rundown occurred after 393 ± 74 s (n = 9) in the presence and after 405 ± 66 s (n = 6) in the absence of NO (P > 0.5). These results indicate that there is no direct effect of NO on activation or rundown of single Cl\(_{\text{Ca}}\) channels.

It is also important to mention that NO (1 µM) applied to intact mSMC was never observed to activate single Cl\(_{\text{Ca}}\) channels in cell-attached membrane patches (Fig. 11; n = 29). When applied to excised membrane patches, NO never activated single Cl\(_{\text{Ca}}\) channels after they had rundown (n = 5).

DISCUSSION

Single Cl\(_{\text{Ca}}\) Channels in Aortic SMC

A small (1.8 pS) channel was activated in cell-attached membrane patches of SMC from mouse and rabbit aorta by extracellular application of caffeine or by exciting inside-out membrane patches into Ca\(^{2+}\)-containing solution. The Ca\(^{2+}\)-dependence and Cl\(^{-}\)-selectivity of this channel were evident from 1) strict dependence of channel activation on [Ca\(^{2+}\)], 2) insensitivity of inward currents to the complete substitution of cations with NMDG in the pipette, 3) sensitivity to substitution of Cl\(^{-}\) with glutamate, and 4) inhibition with niflumic acid, a Cl\(^{-}\)-channel blocker. Noise analysis of the caffeine-induced whole cell current showed an estimated single-channel conductance (2.0 pS) similar to that of single Cl\(_{\text{Ca}}\) channels (1.8 pS) recorded in...
excised patches. This result strongly suggests that these 1.8-pS channels indeed are responsible for the whole cell Cl\textsubscript{Ca} current in SMC from mouse and rabbit aorta. Single Cl\textsubscript{Ca} channels with similar conductance were reported in the A7r5 fetal rat aortic SMC line (1.8 pS) (31) and in human mesenteric artery (2.8 pS) (19), but those studies were limited because of the fast rundown of Cl\textsubscript{Ca} channels in excised membrane patches.

Whole Cell Cl\textsubscript{Ca} Current in Aortic SMC

Increasing [Ca\textsuperscript{2+}] with caffeine or ionomycin, or by simple dialysis of SMC with 1–2 µM Ca\textsuperscript{2+} in the patch pipette, activated a whole cell Cl\textsubscript{Ca} current that was similar to those described in other SMC (for review, see Ref. 22). The Ca\textsuperscript{2+} dependence and Cl\textsuperscript{−}-selective conductances of this current were evident from 1) simultaneous activation of the current with the rise in [Ca\textsuperscript{2+}], 2) absence of current activation in the presence of BAPTA, 3) the reversal potential approximating E\textsubscript{Cl}, 4) inhibition by niflumic acid but not TEA or Ni\textsuperscript{2+}, and 5) sensitivity to variation in Cl\textsuperscript{−} concentration. The rise in [Ca\textsuperscript{2+}] also activated K\textsubscript{Ca} current in mSMC and rSMC, but its contribution was excluded in our experiments with 140 mM Cs\textsuperscript{+} present in the pipette and 10 mM TEA added to the bath solution. There are some reports showing that caffeine can activate Ca\textsuperscript{2+}-independent nonselective cation currents in nonvascular SMC (11). However, these currents were not present in mSMC and rSMC, because Ni\textsuperscript{2+}, an inhibitor of nonselective cation channels, did not affect caffeine-induced whole cell current.

Ca\textsuperscript{2+}-Dependent Activation and Inactivation of Cl\textsubscript{Ca} Channels

Activation of Cl\textsubscript{Ca} channels was strictly Ca\textsuperscript{2+} dependent, because 1) single channels were activated by excising membrane patch only into Ca\textsuperscript{2+}-containing bath solution with a threshold of [Ca\textsuperscript{2+}] \textasciitilde 100 and 200 nM; 2) simultaneous recording of [Ca\textsuperscript{2+}] and whole cell current showed a strong correlation between the rise in [Ca\textsuperscript{2+}] and activation of Cl\textsubscript{Ca} current; 3) chelation of intracellular free Ca\textsuperscript{2+} with BAPTA prevented activation of Cl\textsubscript{Ca} current; and 4) intracellular dialysis with 1–2 µM Ca\textsuperscript{2+} solution activated Cl\textsubscript{Ca} current. Our data showed that threshold [Ca\textsuperscript{2+}] for activation of single channels was between 100 and 200 nM, consistent with the estimates obtained previously from simultaneous recording of [Ca\textsuperscript{2+}] and whole cell current in other SMC (25, 33). Because of the relatively high [Ca\textsuperscript{2+}] threshold necessary to activate the channel, it is apparent why there was no basal Cl\textsubscript{Ca} channel activity and no corresponding whole cell currents observed in resting SMC that had a basal [Ca\textsuperscript{2+}] <100 nM.

The mechanisms of Cl\textsubscript{Ca} channel inactivation and/or rundown appeared to be more complicated. The faster rate of decline of the Cl\textsubscript{Ca} current compared with that of [Ca\textsuperscript{2+}] after caffeine-induced Ca\textsuperscript{2+} release supports the possibility of Ca\textsuperscript{2+}-dependent inactivation of Cl\textsubscript{Ca} channels recently proposed by Wang and Kotlikoff (34). In excised membrane patches, we found that rundown of single Cl\textsubscript{Ca} channels strongly depends on [Ca\textsuperscript{2+}], because increasing Ca\textsuperscript{2+} concentration significantly accelerated the disappearance of channel activity. Our results with both single channels and whole cell current are consistent with strong Ca\textsuperscript{2+} dependence of the inactivation and/or rundown of Cl\textsubscript{Ca} channels, although there were no effects of R-24571 (inhibitor of Ca\textsuperscript{2+}- calmodulin) or calyculin A (inhibitor of protein phosphatases 1 and 2A) on the Ca\textsuperscript{2+}-dependent rundown. Moreover, some Ca\textsuperscript{2+}-independent rundown was observed that occurred after the channel was excised from the cell even in the absence of Ca\textsuperscript{2+}. At the level of single channels, it was not possible to distinguish the rundown caused by inactivation from that induced by excising the membrane patch. Importantly, our results indicate that one can significantly decrease the rundown of single Cl\textsubscript{Ca} channels in inside-out membrane patches by reducing Ca\textsuperscript{2+} concentration applied to the intracellular surface of the membrane, which might prove helpful for longer recording of single Cl\textsubscript{Ca} channels.

NO Does Not Affect Single Cl\textsubscript{Ca} Channels, But Whole Cell Cl\textsubscript{Ca} Current Can Be Indirectly Inhibited by a High Concentration of SNAP That Affects Intracellular Ca\textsuperscript{2+}

Our data indicate that NO does not inhibit Cl\textsubscript{Ca} channels in aortic SMC directly. Indeed, activation of single Cl\textsubscript{Ca} channels by a physiological concentration of Ca\textsuperscript{2+} (400 nM), which could be attained during the application of agonists, was not affected by authentic NO (1 µM) or SNAP (=1 mM) in inside-out membrane patches. Application of NO (1 µM) to the inside-out membrane patch also did not affect rundown of Cl\textsubscript{Ca} channels. These findings are consistent with the absence of a direct effect of NO on Cl\textsubscript{Ca} channels in excised membrane patches. This is in contrast to K\textsubscript{Ca} channels, which we found to be affected by NO under similar experimental condition (4).

Importantly, our data also suggest that NO does not affect Cl\textsubscript{Ca} channels indirectly via cGMP or other second messengers in intact aortic SMC. Indeed, the membrane-permeant analog of cGMP (CPT-cGMP, 50 µM) did not affect whole cell Cl\textsubscript{Ca} currents evoked by caffeine. These data are inconsistent with the possibility of cGMP-dependent inhibition of Cl\textsubscript{Ca} channels recently proposed in SMC from opossum esophagus (38).

Although NO in vascular SMC does not inhibit the activity of Cl\textsubscript{Ca} channels directly or through cGMP, it is attractive to speculate that it could still suppress activation of Cl\textsubscript{Ca} currents indirectly by affecting the basal level of [Ca\textsuperscript{2+}] or Ca\textsuperscript{2+} release from intracellular stores. Indeed, progressive disappearance of caffeine-induced Cl\textsubscript{Ca} current followed the disappearance of caffeine-induced Ca\textsuperscript{2+} release in SMC treated with a high concentration of SNAP (1 mM). The effect of SNAP starts right after application, with a progressive rise in basal [Ca\textsuperscript{2+}]. The time-dependent reduction in caffeine-
induced Ca\textsuperscript{2+} release can explain the reduction in corresponding Cl\textsubscript{Ca} currents. On the other hand, the rise in basal [Ca\textsuperscript{2+}] can cause some Ca\textsuperscript{2+}-dependent inactivation of Cl\textsubscript{Ca} channels described by Wang and Kotlikoff (34). This additional inactivation could be the reason why Cl\textsubscript{Ca} current was inhibited more than corresponding Ca\textsuperscript{2+} release.

The effect of NO on Ca\textsuperscript{2+} release from caffeine-sensitive stores has been extensively studied in cardiac and skeletal muscle during the last few years, but the results are still controversial. Some authors found NO-induced activation of ryanodine (and caffeine-sensitive) ion channels that release Ca\textsuperscript{2+} from the stores (29, 35), whereas others showed that NO can inhibit these channels and suppress Ca\textsuperscript{2+} release from the stores (18, 23, 36). Our experiments were not designed to resolve this controversy, but theoretically could be explained by NO-induced activation of Ca\textsuperscript{2+}-release channels in SMC that causes a gradual, time-dependent depletion of the stores and a rise in basal [Ca\textsuperscript{2+}]. It is important to emphasize, however, that very high concentrations of NO donors were necessary to affect caffeine-sensitive Ca\textsuperscript{2+} release channels, Ca\textsuperscript{2+} stores, and, in our experiments, activation of Cl\textsubscript{Ca} currents, which raises a question as to the physiological significance of such effects of NO donors. Other mechanisms can affect the filling state of caffeine-sensitive stores and secondary activation of Cl\textsubscript{Ca} channels in SMC. For example, L-type Ca\textsuperscript{2+} channels in airway smooth muscle provide an important pathway by which caffeine-sensitive stores are refilled (16, 17). Although Ca\textsuperscript{2+} influx itself is not necessary for Cl\textsubscript{Ca}-channel activation in aortic SMC, we found that Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels is very important for repetitive activation of Cl\textsubscript{Ca} currents with caffeine. The progressive disappearance of caffeine-induced Ca\textsuperscript{2+} release and Cl\textsubscript{Ca} currents during application of SNAP (1 mM) could partially result from the inhibition of L-type Ca\textsuperscript{2+} channels by NO. Indeed, inhibition of whole cell L-type Ca\textsuperscript{2+} current by NO donors has been reported in SMC (3, 7, 15, 25) and theoretically can indirectly affect activation of Cl\textsubscript{Ca} channels. Thus our studies demonstrated that NO does not affect Cl\textsubscript{Ca} channels directly or via cGMP, but Cl\textsubscript{Ca} current in SMC could be suppressed indirectly via different effects of NO on intracellular free and stored Ca\textsuperscript{2+}.

The authors thank Dr. S. Sims and Dr. S. Zakharov for helpful comments and Dr. T. Adachi for measurement of NO concentration.

This study was supported by the National Heart, Lung, and Blood Institute (Grants HL-54150-02, HL-31607-14, HL-55993-03), the American Heart Association (Grant 9417730), and the Naito Foundation Subsidy for Inter-Institute Research.

Address for reprint requests and other correspondence: V. M. Botolina, Vascular Biology Unit, Boston Medical Center, R 408, 88 E. Newton St., Boston, MA 02118-2393 (E-mail: vbotolina@med. med.Lu.edu).

Received 17 December 1998; accepted in final form 28 May 1999.

REFERENCES


