Cyclic Nucleotide-gated Channels Mediate Membrane Depolarization following Activation of Store-operated Calcium Entry in Endothelial Cells*

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Calculated agonists induce membrane depolarization in endothelial cells through an unknown mechanism. Present studies tested the hypothesis that pulmonary artery endothelial cells express a cyclic nucleotide-gated (CNG) cation channel activated by store-operated calcium entry to produce membrane depolarization. In the whole-cell configuration, voltage-clamped cells revealed a large non-inactivating, outwardly rectifying cationic current in the absence of extra- or intracellular Ca$^{2+}$ that was reduced upon replenishment of Ca$^{2+}$. The inward current was non-selective for K$^+$, Na$^+$, Cs$^+$, and Rb$^+$ and was not inhibited by high tetraethylammonium concentrations. cAMP and cGMP stimulated the current and changed the cation permeability to favor Na$^+$. Moreover, 8-bromo-cAMP stimulated the current in voltage-clamped cells in the perforated patch mode. The cationic current was inhibited by the CNG channel blocker LY83,583, and reverse transcriptase-polymerase chain reaction cloning identified expression of a CNG channel resembling that seen in olfactory neurons. Activation of store-operated calcium entry using thapsigargin increased a current through the CNG channel. Stimulation of the current paralleled pulmonary artery endothelial cell membrane depolarization, and both the current and membrane depolarization were abolished using LY83,583. Taken together, these data demonstrate activation of store-operated calcium entry stimulates a CNG channel producing membrane depolarization. Such membrane depolarization may contribute to slow feedback inhibition of store-operated calcium entry.

Endothelial cells form a semi-permeable barrier that compartmentalizes circulating blood elements from underlying tissue. Neuro-humoral mediators target endothelium to regulate both production of vasoactive autacoids important for control of blood pressure and cell shape important for control of fluid balance, migration, and angiogenesis (1). G$\gamma$-coupled agonists accomplish these diverse functions partly through generation of inositol 1,4,5-trisphosphate which, upon binding its internal receptor, depletes intracellular calcium stores and activates a membrane calcium entry channel (2–7). This so-called store-operated calcium entry activates endothelial nitric-oxide synthase (8–10), inhibits adenylly cyclase (11), and activates myosin light chain kinase (12, 13) sufficient to produce vasodilation and/or focal intercellular gaps necessary to initiate a localized inflammatory response (1).

Although endothelial cells are non-excitable, membrane potential is a critical determinant of the magnitude of both store-operated calcium entry (7) and permeability (14, 15) responses. Whereas hyperpolarization promotes calcium entry and permeability, depolarization reduces calcium entry and permeability. Activation of store-operated calcium entry by G$\gamma$-coupled agonists including bradykinin or Ca$^{2+}$-ATPase inhibitors like thapsigargin cause an initial hyperpolarization attributed to activation of maxi- or intermediate K$^+$ channels (16). This hyperpolarization is transient but further promotes Ca$^{2+}$ entry by increasing the electrochemical driving force. A large, sustained depolarization occurs subsequently that reduces calcium entry. Mechanism(s) underlying this sustained depolarization are unknown with the exception that it is caused by neither K$^{\text{IR}}$ nor K$^{\text{Ca}}$ channel activity and is La$^{3+}$-sensitive, suggesting a Ca$^{2+}$ dependence.

Either Ca$^{2+}$ or Na$^+$ entry could produce membrane depolarization, and although several cationic conductances have been described in endothelial cells, putative channels mediating Ca$^{2+}$ or Na$^+$ entry are poorly understood (1, 17). Cycling nucleotide-gated (CNG) channel cation channels are permeable to both Ca$^{2+}$ and Na$^+$ and mediate membrane depolarization in neurons (18–20). The membrane-depolarizing effect of CNG channels is well described in retina where physiologically high concentrations of cGMP constitutively activate the “dark” current (21, 22) and in olfactory neurons where increases in cAMP or cGMP promote odorant perception (23–29). More recently, CNG channels have been cloned from diverse tissues, including several brain regions, heart, kidney, testis, liver, and skeletal muscle (30–36). However, in most of these cases a clear link between the CNG channel and a physiological function is unknown. Non-excitable endothelial cells have been shown to express CNG1 channels, and whereas their activation by calcium- elevating agents would be predicted to cause membrane depolarization, a functional role for CNG channels in endothelium has not been established (37). Thus, studies were undertaken to determine whether endothelial cells express an endogenous CNG channel that mediates membrane depolarization following activation of store-operated calcium entry.

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1 The abbreviations used are: CNG, cyclic nucleotide-gated; PAECs, pulmonary artery endothelial cells; TEA, tetraethylammonium; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; IBMX, isobutylmethylxanthine; 8-Br-cAMP, 8-bromo-cAMP; sGC, soluble gua-nylyl cyclase.
MATERIALS AND METHODS
Isolation and Culture of Endothelial Cells—Male Harlan Sprague-Dawley rats (CD strain, 350–400 g; Charles River) were euthanized by an intraperitoneal injection of 50 mg of pentobarbital sodium (Nembutal, Abbott). After sternotomy, the heart and lungs were removed en bloc, and the pulmonary arterial segment between the heart and lung hilus was dissected, split, and fixed onto a 35-mm plastic dish. Pulmonary artery endothelial cells (PAECs) were obtained from the intima by gentle scraping with a plastic cell lifter and were seeded onto a 100-mm Petri dish containing 10 ml of seeding medium (1:1 Dulbecco’s modified Eagle’s medium/Ham’s F-12, 10% fetal bovine serum). Cells were verified as endothelial by positive factor VIII staining and uptake of 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate-labeled acetylated low density lipoprotein. When the primary culture reached confluence, cells were passaged by trypsin digestion into 75-cm² culture flasks (Corning), and standard tissue culture techniques were followed until the cells were used for experiments. Cells were studied between passages 6 and 20. Confluent rat PAECs were enzyme-dispersed, seeded onto 35-mm plastic culture dishes, and then allowed to re-attach for at least 24 h before patch clamp experiments were performed. Whole-cell and perforated patch clamp recordings were obtained from single (electrically isolated) rat PAECs, exhibiting a flat, polyhedral morphology. These cells were chosen for study because their morphology was consistent with rat PAECs from a confluent monolayer (38).

Patch Clamp Electrophysiology and Data Analysis—Conventional whole-cell and nystatin-perforated voltage-clamp configurations were performed to measure transmembrane currents in single rat PAEC by the standard giga-seal patch clamp technique. Perforated patch technique (39) was applied to avoid disturbing the intracellular milieu of the cell, in particular resting cytosolic Ca²⁺. Conventional whole-cell recordings were used to dialyze the cell with our artificial “intracellular” solutions. For nystatin-perforated patch recording, the pipette was filled with nystatin containing intracellular solution and gentle suction applied to achieve giga-ohm resistance. The access resistance gradually decreased within 5 min after the giga-ohm seal was formed, and then the transmembrane current was recorded in the voltage-clamp mode when a steady value was achieved.

Recording pipettes were manufactured from glass capillary tubes (Warner Instrument Corp., Hamden, CT), pulled by a two-stage puller (PC-10, Narishige Co., Ltd., Tokyo, Japan) and heat-polished before use. Pipette resistance was in the range of 2–5 megohms when filled with our intracellular solution. All experiments were performed at room temperature (22–25 °C). An EPC-9 patch clamps amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) was used to acquire data with Pulse/PulseFit software (HEKA) and filtered at 2.9 kHz.

Solutions and Reagents—Patch clamp electrophysiological experiments were performed using two solutions, asymmetrical and symmetrical extra- and intracellular (pipette) solutions. Asymmetrical solutions contained (in mM) the following: for extracellular, 120 glutamic acid, 20 HEPES, and 1N-phenylanthranilic acid and the pH was adjusted to 7.4 with tetraethylammonium (TEA) hydroxide; for intracellular, 145 potassium glutamate, 10 HEPES, 6 MgCl₂ (pH 7.2, titrated with KOH). Symmetrical solutions contained (in mM) the following: for extracellular, 100 potassium methanesulfonate (KCH₃SO₃), 20 HEPES, and 1 N-phenylanthranilic acid and the pH was adjusted to 7.4 with tetraethylammonium (TEA) hydroxide; for intracellular, 145 potassium glutamate, 10 HEPES, 1 MgCl₂, 5 EGTA (pH 7.2 titrated with KOH). The osmolality in all

Fig. 1. Monovalent cation current in whole-cell patches from PAECs. A, current traces obtained in symmetrical K⁺ solutions using the whole-cell patch clamp configuration. Pulses of 200-ms duration were applied every 3 s from −100 to +100 mV in 20-mV steps; holding potential was 0 mV. Data show a large, sustained (non-inactivating) outward current. B, current-voltage relationships obtained in the presence (open circles) and absence (closed circles) of TEA. TEA did not alter the cationic current. Data were normalized to membrane capacitance to yield current density and plotted as mean ± S.E. against voltage (p = ns). pF, picofarad.
Fig. 2. Monovalent cation conductance of PAECs. Current-voltage relationships recorded 3 min after the whole-cell configuration were established using K⁺, Cs⁺, and Na⁺ as charge carriers in 50 mM symmetrical solutions. Solutions did not contain Ca²⁺. In the absence of Ca²⁺, cation permeability favored K⁺. Data were normalized to membrane capacitance to yield current density and plotted as mean ± S.E. against voltage. pF, picofarad.

Fig. 3. Extra- and intracellular divalent cations regulate the monovalent cation current in PAECs. A, current-voltage relationships recorded 3 min after the whole-cell configuration was established in the absence (closed circles) and presence (open circles) of extra- and intracellular Ca²⁺ in symmetrical K⁺ solution (100 mM). Extracellular Ca²⁺ reduced the K⁺ current magnitude (p < 0.05). B, current-voltage relationships recorded 3 min after the whole-cell configuration was established in the absence (closed circles) and presence (open circles) of intracellular Mg²⁺ in symmetrical K⁺ solution (50 mM). Intracellular Mg²⁺ reduced the outward K⁺ current magnitude (p < 0.05). Data were normalized to membrane capacitance to yield current density and plotted as mean ± S.E. against voltage. pF, picofarad.
solutions was adjusted with succrose to 290–300 mOsm. The above asymmetrical extra- and intracellular solutions were used for both whole-cell and perforated patch recordings; however, in perforated patch recordings the intracellular solution was also supplemented with nystatin (100 μg/ml).

LYS3,583 (6-(phenylamino)-5,8quinolinedione) (Research Biochemicals International, Natick, MA) was prepared in ethanol. Working solutions were made fresh each use with final ethanol concentrations of less than 1% (v/v). Thapsigargin (Sigma) was prepared in Me2SO. Dilutions were made with final Me2SO concentrations of less than 0.1% (v/v). These concentrations of ethanol and Me2SO did not alter the electrophysiologic characteristics of endothelial cells. Stock solutions of cAMP and 8-Br-cAMP were dissolved fresh in the extracellular solution. N-Phenylnitrilotriacetic acid was purchased from Fluka (Switzerland). Unless otherwise stated, all chemicals were purchased from Sigma.

Reverse Transcriptase-PCR Cloning of Ca2+-inhibited, cAMP-activated K+ conducting Channel Gene Product—Total RNA was isolated from a confluent, early passage 75-cm2 flask of rat PAECs by the RNasy Total RNA (Qiagen, Inc., Chatsworth, CA) method. Approximately 1 μg of RNA was reverse-transcribed with or without 200 units of Superscript II (Life Technologies, Inc.) reverse transcriptase for 1 h at 42 °C. The first strand cDNA synthesis reactions were primed with an adapter primer (Life Technologies, Inc.) with the following sequence: 5’-GGT CAC CCG TCT CTT TGA CCG TAC ATC TG-3’ (antisense) 5’-TTG ACA GCA TCA ATC TG-3’. PCR was performed with consequent reverse transcriptase products for 30 cycles (Profile: 94 °C, 30 s; 55 °C, 45 s; 72 °C, 1 min and 45 s; final extension 72 °C, 10 min) with the following primer set: (sense) 5’-TGA GGT CTT TGA CCG TAC CTC-3’ and (antisense) 5’-GAG ACC ACC AAT CAA GAA G-3’. The PCR profile was exactly as described above. PCR products were ligated directly into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent Escherichia coli. Plasmids were isolated from positive clones (verified by PCR analysis) using the Qiaprep spin miniprep method (Qiagen, Inc.) and submitted to the Biopolymer Laboratory at the University of South Alabama for automated fluorescence sequence analysis (AB373XL DNA stretch sequencer). Sequence accuracy was confirmed by sequencing in both directions using double-stranded plasmids as templates with universal primers. Nucleotide and amino acid alignments were performed with the assistance of BLAST (NCBI) and DNASIS version 2.0 (Hitachi Software) programs.

Estimation of Membrane Potential (Em)—Confluent rat PAECs were loaded with 1 μM of the anionic potentially sensitive fluorescent dye, bis(1,3-dibutylbarbituric acid) trimethine oxonol, according to methods previously described (40). Cells were studied with an Olympus IX70 inverted microscope at × 400 using a xenon arc lamp photomultiplier system (Photon Technologies Inc., Monmouth Junction, NJ), and data were acquired and analyzed with PTI Felix software. Cells (3–4) were excited by the xenon arc lamp (490 nm wavelength), and emission of epifluorescence at 520 nm (signal averaged) was measured. Total fluorescence intensity was adjusted by reducing the arc lamp illumination intensity to minimize photobleaching of the dye, although some degree of photobleaching still occurred during the experiment. Data were corrected for the calculated rates of photobleaching for each individual experiment. An estimation of the relationship between fluorescence intensity and change in $E_m$ was performed by exchanging the experimental physiologic salt solution (in mM, 11 1-glucose, 0.6 MgSO4, 1 KH2PO4, 4.7 KCl, 118 NaCl, 25 HEPES, 2 CaCl2, titrated to pH 7.35–7.45 using 11 NaOH) for a high [K]-low [Na+] solution (in mM, 11 1-glucose, 0.6 MgSO4, 1 KH2PO4, 50 KCl, 68 NaCl, 25 HEPES, 2 CaCl2, titrated to pH 7.35–7.45 using 11 NaOH) and recording the resulting increase in fluorescence (depolarization).

Measurement of GMP Content—Confluent PAECs in 12-well plates were treated as indicated in Dulbecco’s modified Eagle’s medium containing 10 mM HEPES. Cyclic GMP in cell extracts was then determined with the assistance of double-stranded plasmids as templates with universal primers. Nucleotide and amino acid alignments were performed with the assistance of reverse transcriptase-PCR (RT-PCR) Cloning of Ca2+-inhibited, cAMP-activated K+ conducting Channel Gene Product—Total RNA was isolated from a confluent, early passage 75-cm2 flask of rat PAECs by the RNasy Total RNA (Qiagen, Inc., Chatsworth, CA) method. Approximately 1 μg of RNA was reverse-transcribed with or without 200 units of Superscript II (Life Technologies, Inc.) reverse transcriptase for 1 h at 42 °C. The first strand cDNA synthesis reactions were primed with an adapter primer (Life Technologies, Inc.) with the following sequence: 5’-GGT CAC CCG TCT CTT TGA CCG TAC ATC TG-3’ (antisense) 5’-TTG ACA GCA TCA ATC TG-3’. PCR was performed with consequent reverse transcriptase products for 30 cycles (Profile: 94 °C, 30 s; 55 °C, 45 s; 72 °C, 1 min and 45 s; final extension 72 °C, 10 min) with the following primer set: (sense) 5’-TGA GGT CTT TGA CCG TAC CTC-3’ and (antisense) 5’-GAG ACC ACC AAT CAA GAA G-3’. The PCR profile was exactly as described above. PCR products were ligated directly into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent Escherichia coli. Plasmids were isolated from positive clones (verified by PCR analysis) using the Qiaprep spin miniprep method (Qiagen, Inc.) and submitted to the Biopolymer Laboratory at the University of South Alabama for automated fluorescence sequence analysis (AB373XL DNA stretch sequencer). Sequence accuracy was confirmed by sequencing in both directions using double-stranded plasmids as templates with universal primers. Nucleotide and amino acid alignments were performed with the assistance of BLAST (NCBI) and DNASIS version 2.0 (Hitachi Software) programs.

RESULTS

K+ Currents in PAECs—Initial experiments were performed using voltage-clamped cells to test for the presence of cationic currents in PAECs. Fig. 1A shows membrane current traces and current-voltage (I-V) relationships recorded using symmetrical K+ methylsulfonate solutions, since CNG channels poorly discriminate between K+ and Na+ (25, 42–46). The holding potential was 0 mV. Current was measured for 200 ms at voltages ranging from −100 to +100 mV. PAECs exhibited a sustained (non-inactivating) current that was outwardly rectifying at positive voltages. As expected in symmetrical solutions, the reversal potential was 0 mV. Inclusion of tetraethylammonium in the patch pipette did not alter the K+ current (Fig. 1B). Similarly, 4-aminopyridine did not inhibit the current (data not shown), indicating the K+ conductance was not due to KATP or KCa channel activity.

Table I

| Current-voltage conditions | cAMP | K+ | Ca2+ | Na+ |
|---------------------------|------|----|------|-----|
| +                         | 1    | 0.51| 1.69 |
| −                         | 1    | 0.45| 0.63 |

$K^+$ Currents Due to a Non-selective Channel—We next examined the permeability ratio to monovalent cations. Ion replacement studies were performed using Rb+ and Cs+. In these experiments, intracellular K+ methylsulfonate (100 mM) was replaced with either 100 mM Rb+ nitrate or 100 mM Cs+.
methylsulfonate. These replacements resulted in a 22 and 60% decrease in conductance, respectively, indicating the permeability ratio was $K^+ (1) \cdot Rb^+ (0.8) \cdot Cs^+ (0.4)$. Because the anionic carrier for $Rb^+$ was different from $Cs^+$, we performed studies to examine the anionic contribution to the current. Studies were conducted using both 100 mM $K^+$ glutamate and methylsulfonate. The magnitude of the $K^+$ current was slightly associated with its predominant anion. For example, the outward $K^+$ conductance at 100 mV was 12.3 ± 1.04 pA/picofarads when glutamate was the anion and 15.0 ± 2.47 when methylsulfonate was the anion, suggesting the presence of an anionic conductance that contributed to the current. We performed ion replacement studies using symmetrical $K^+$, $Cs^+$, and $Na^+$ in a glutamate solution (Fig. 2). Under these conditions the inward permeability ratio was $K^+ (1) > Na^+ (0.6) > Cs^+ (0.5)$, and the outward permeability ratio was $K^+ (1) > Cs^+ (0.4) > Na^+ (0.1)$. Taken together, these findings suggest the presence of a current conducted through a non-selective cation channel.

Conductance of monovalent cations through some non-selective cation channels is inhibited by $Ca^{2+}$ and $Mg^{2+}$. We tested whether $Ca^{2+}$ and $Mg^{2+}$ regulate the $K^+$ current observed presently. Fig. 3 shows the $K^+$ current magnitude was similarly inhibited by either of the divalent cations tested, irrespective of whether $Ca^{2+}$ or $Mg^{2+}$ were placed in the intra- or extracellular pipette solutions. Thus, the $K^+$-conducting chan-
Regulation of the Cationic Current—To address putative non-selective channel(s) that may mediate the observed cationic current, we investigated the effect of cAMP on the I-V profile, within a physiologically relevant range of voltages. Inclusion of cAMP in the internal solution increased the cation current (Fig. 4A) and altered the ion permeability where Na\(^+\) conductance was favored over K\(^+\) and Cs\(^+\) (Fig. 4B; Table I). This change in ion permeability induced by cAMP resembles the slip-mode conductance observed in tetrodotoxin-sensitive Na\(^+\) channels (47). The outwardly rectifying nature of the I-V plot illustrated in Figs. 1 and 2 and the permeability ratio favoring Na\(^+\) in the presence of cAMP generally resemble the electrophysiological profile previously described in both endogenous CNG channels and overexpressed CNG2 channels (18). Importantly, cyclic nucleotide increased the current within a physiologically relevant range of voltages.

CNG channels are non-selective cation channels recently shown to be expressed in diverse cell types (30–36), although expression in non-excitable endothelial cells is not fully resolved (37). Since cAMP stimulated the cationic current similar to CNG channels of the olfactory neuron, RT-PCR cloning was performed to address whether PAECs express CNG2 channels. Primers were designed to isolate the pore region of CNG channel exhibits features of divalent cation block.

FIG. 6. Activation of store-operated calcium entry stimulates a cationic current. Current-voltage relationships recorded 10 min after the whole-cell configuration was established in the absence (open circles) and presence (closed circles) of 1 \(\mu\)M thapsigargin applied to the patch pipette. The left panel shows a representative trace in control and thapsigargin-treated cells, and the right panel shows the summary data. Thapsigargin stimulated an inward and outward cationic current \((p < 0.05)\). Data were normalized to membrane capacitance to yield current density and plotted as mean ± S.E. against voltage.

FIG. 7. Inhibition of CNG channels reduces the cationic conductance. Current-voltage relationships recorded 10 min after the whole-cell configuration were established in the absence (closed circles) and presence (open circles) of 40 \(\mu\)M LY83,583. PAECs were pretreated with LY83,583 for 15 min before establishing the whole-cell configuration. The left panel shows a representative trace in control and LY83,583-treated cells, and the right panel shows the summary data. LY83,583 reduced the cationic current \((p < 0.05)\). Data were normalized to membrane capacitance to yield current density and plotted as mean ± S.E. against voltage. pF, picofarad.

By using physiological salt solutions, we examined the function of endogenously expressed CNG channels in PAECs. Voltage-clamped cells exhibited a limited degree of run-down over 10 min (data not shown). Inclusion of thapsigargin in the patch pipette increased the current 300% above control values and right-shifted the reversal potential, consistent with stimulation of either a Na\(^+\) or Ca\(^{2+}\) current (Fig. 6). The thapsigargin-stimulated current progressively increased over time until its peak was reached 3 min after establishing a seal; this peak increase in current was stable until completion of the experiment (data not shown).

We next examined whether the cationic current was inhibited by LY83,583, a potent CNG channel blocker (49, 50), by using physiological salt solutions. PAECs were incubated for 15 min in the presence of 40 \(\mu\)M extracellular LY83,583 prior to establishing a seal. Cells incubated with LY83,583 exhibited a 54% lower K\(^+\) conductance at +100 mV than did the untreated cells (Fig. 7). Moreover, LY83,583 inhibited the current induced by both cAMP and thapsigargin, to the level obtained in control experiments, suggesting that cAMP and thapsigargin activate a similar CNG channel (Fig. 8). To confirm this idea further, maximal concentrations of cAMP and thapsigargin were applied together in the patch pipette. The combined application of cAMP and thapsigargin did not produce an additive increase in current (Fig. 8C). Taken together, these results support the
idea that PAECs possess a CNG channel that regulates cationic conductance.

Effect of 8-Br-cAMP on the Cationic Current in Intact PAECs—We performed experiments using a perforated voltage-clamp configuration to test for the presence of the non-selective cationic current. Solutions were designed to examine the outward K+ current, as recently described for CNG channels (51). Under control conditions, e.g. in the presence of 10 mM extracellular Ca2+ in asymmetrical K+ solution, the transmembrane current possessed a current density at +100 mV of 1.76 ± 0.36 pA/picofarads. Application of 1 mM 8-Br-cAMP to the extracellular solution increased the outward current 530% (Fig. 9). These data support the idea that cAMP can stimulate cationic currents in PAECs through a CNG channel.

Activation of Store-operated Ca2+ Entry Causes Membrane Depolarization by Stimulating a CNG Channel—Fig. 10 shows results from experiments conducted to determine contribution of the CNG channel to membrane potential change following thapsigargin challenge. Similar to previous accounts (16), thapsigargin produced a transient hyperpolarization of PAEC membranes followed by a gradual and sustained depolarization. When PAECs were pre-treated (15 min) with the CNG channel blocker, LY83,583, the thapsigargin-induced hyperpolarization was augmented. The LY83,583-treated cells also displayed a gradual repolarization in membrane potential, but the rate of repolarization was less than that observed in cells treated with thapsigargin alone. Thus, these data indicate an LY83,583-sensitive channel contributes to limiting the degree of membrane hyperpolarization in response to thapsigargin.

To confirm that cAMP and cGMP produce membrane depolarization in endothelial cells, PAECs were treated with agents that increase cyclic nucleotides and membrane potential assessed using bisoxonol as described in Fig. 10. Treatment with IBMX (500 μM) for 5 min did not increase cGMP above constitutive levels (constitutive = 0.085 versus IBMX = 0.88 pmol/well) although it did produce an approximate 5-fold increase in cAMP in our previous reports (53). Thus, the 10-mV depolarization induced by IBMX was likely due to increased cAMP. In contrast, atrial natriuretic peptide increased cGMP 10-fold; it similarly produced an approximate 10-mV depolarization, suggesting either increased cAMP or cGMP can initiate a depolarizing current. Neither IBMX nor atrial natriuretic peptide produced an additive increase in the depolarization induced by thapsigargin, consistent with the idea that depolarization evoked by thapsigargin occurs through a cAMP/cGMP-sensitive CNG channel.


cGMP and CNG Channels in PAECs—To confirm that cGMP in addition to cAMP was capable of stimulating CNG channels in PAECs, cGMP was applied by the patch pipette to voltage-clamp cells in the whole-cell configuration. As seen in Fig. 4 using cAMP, Fig. 11A shows that cGMP stimulates an inward cationic conductance at physiologically relevant membrane potentials (e.g. ~40 to ~70 mV) (16). We examined whether thapsigargin produces a time-dependent increase in cGMP in association with its increase in cationic conductance. Fig. 11B demonstrates that thapsigargin doubles cGMP, whereas sodium nitroprusside does not increase cGMP in rat PAECs over the time course germane to changes in membrane potential. The inability of SNP to increase cGMP suggests the cells do not express soluble guanylyl cyclase (sGC). In contrast, stimulation of particulate guanylyl cyclase using atrial natriuretic peptide induced a 10-fold increase in cGMP (data not shown).

DISCUSSION

Neuro-humoral calcium agonists induce membrane depolarization in endothelial cells, although the mechanism of this depolarization is unknown (16). Our present studies were undertaken to determine whether endothelial cells express an endogenous CNG channel that mediates membrane depolarization following activation of store-operated calcium entry. Data collectively support a new working model of events that control membrane depolarization in response to inflammatory calcium agonists, in which activation of store-operated Ca2+ entry stim-

![Graph A](image1.png)

**FIG. 8.** Inhibition of CNG channels prevents cAMP and thapsigargin from activating an inward cationic current. Current-voltage relationships recorded 10 min after the whole-cell configuration was established in the absence (closed circles) and presence (open circles) of 40 μM LY83,583. PAECs were pretreated with LY83,583 for 15 min before establishing the whole-cell configuration. A, inclusion of 400 μM cAMP in the patch pipette stimulated an inward cationic current. The current stimulated by cAMP was abolished by LY83,583 (p < 0.05). B, thapsigargin (1 μM) stimulated an inward cationic current that was also abolished by LY83,583 (p < 0.05). C, inclusion of both cAMP (400 μM) and thapsigargin (1 μM) to the patch pipette did not produce an additive increase in current density. Data were normalized to membrane capacitance to yield current density and plotted as mean ± S.E. against voltage. pF, picofarad.
ulates a depolarizing Na\(^{+}\) and Ca\(^{2+}\) conductance through CNG channels (Fig. 12).

Since functional expression of CNG channels had not been established in endothelial cells, we initially determined whether PAECs possess a non-selective cation conductance attributed to channels regulated by cyclic nucleotides. CNG channels are non-selective to monovalent cations. Electrophysiological measurements in PAECs demonstrated the presence of a non-selective cation current consistent with that seen in endogenously and heterologously expressed CNG channels (18–20). The observed current was non-inactivated and outwardly rectifying at positive voltages. In unstimulated PAECs the current was observed when either K\(^{+}\), Na\(^{+}\), Cs\(^{+}\), or Rb\(^{+}\) were utilized as charge carriers, although K\(^{+}\) permeability was favored over Na\(^{+}\), Cs\(^{+}\), and Rb\(^{+}\). These findings generally support the presence of non-selective cationic currents present in endothelial cells (1, 17).

Four findings support the idea that endogenously expressed CNG channel(s) mediate the non-selective cation current in PAECs. First, inclusion of cAMP in the patch pipette promoted

![Image](http://www.jbc.org/)

**FIG. 9.** 8-Br-cAMP stimulates the cationic current in intact PAECs. Current-voltage relationships of the cationic current was measured in the perforated patch mode using asymmetrical K\(^{+}\) solutions to amplify the outward current. After establishing a seal in the perforated patch mode control measurements (closed circles) were made and 1 mM 8-Br-cAMP (open circles) was applied for 10 min. 8-Br-cAMP increased the current amplitude (p < 0.05). Data were normalized to membrane capacitance to yield current density and plotted as mean ± S.E. against voltage. pF, picofarad.

![Image](http://www.jbc.org/)

**FIG. 10.** LY83,583 potentiates thapsigargin-induced membrane hyperpolarization and decreases the rate of repolarization. Changes in PAEC membrane potential (ΔEm) over time are shown in response to thapsigargin ± 15 min pretreatment with 40 μM LY83,583. Raw data (arbitrary fluorescence units, counts/s) were normalized to the average basal fluorescence intensity measured 100 s prior to thapsigargin administration, and the resulting changes in fluorescence were converted into estimates of ΔEm in mV as described under “Materials and Methods.” Representative trace is shown. Similar results were obtained in five separate experiments.

![Image](http://www.jbc.org/)

**FIG. 11.** Activation of CNG channels in PAECs occurs in response to increased cGMP. **A,** current-voltage relationships recorded 3 min after the whole-cell configuration was established in the absence (closed circles) and presence (open circles) of 400 μM cGMP applied to the patch pipette. 100 mM symmetrical K\(^{+}\) was used as the charge carrier. Data indicate cGMP increased the inward K\(^{+}\) current (p < 0.05). Data were normalized to membrane capacitance to yield current density and plotted as mean ± S.E. against voltage. **B,** confluent monolayers of PAECs were treated with 1 μM thapsigargin for the indicated times or with 100 μM sodium nitroprusside (SNP) for 5 min. Measurements of cGMP indicate thapsigargin but not sodium nitroprusside (SNP) slightly increased cGMP. Similar results were obtained in the presence of 500 μM IBMX (data not shown). pF, picofarad.
Store-operated Calcium Entry and CNG Channels

Na⁺ influx, consistent with prior reports of CNG channel regulation in neurons (25). In the presence of cAMP, ion permeability favored Na⁺ over other monovalent cations and reflected the ion permeability ratio of olfactory CNG channels similarly activated by cAMP (18). Second, cyclic nucleotides stimulated the cationic current of voltage-clamped cells in the whole-cell configuration, and 8-Br-cAMP directly activated the current of voltage-clamped cells in the perforated patch configuration. Third, monovalent cationic conductances were reduced by intra- and extracellular divalent cations, demonstrating cationic block (52). This feature of CNG channels was found paradoxical in that Na⁺ influx accounts for 80% of cationic conductance in physiological solutions (20). Ca²⁺ influx contributes to 15% of the total current even though it is at 100-fold lower extracellular concentration, suggesting the channel actually conducts Ca²⁺ with preference over Na⁺. Indeed, homomeric CNG channels are selectively permeable to Ca²⁺ (53). Fourth, pretreatment with the CNG channel inhibitor LY83,583 (49, 50) nearly abolished the observed current. These data collectively support the idea that CNG channels contribute to the non-selective cation permeability in endothelial cells.

cAMP stimulation of an inward Na⁺ current is consistent with CNG channels of the olfactory neuron. Whereas retinal CNG channels exhibit exquisite sensitivity to cGMP, olfactory CNG channels possess similar sensitivities to cAMP and cGMP (25, 27, 28). We therefore tested for expression of CNG2 in PAECs by specifically amplifying the pore region of a CNG2 channel gene product. The pore region was amplified since it is not only responsive to cyclic nucleotide binding but also contributes to ion selectivity. Indeed, mammalian K⁺-selective channels including K_SUST and human ether-a-go-go-related gene channels possess high homology with CNG channels in certain regions, including S2, S5, S6, and COOH-terminal segments, but differ in the pore region (54). In contrast, Shaker K⁺ channels possess high homology with CNG channels within the pore region, with the exception that they contain two residues, tyrosine and glycine, not found in CNG channels. Deletion of these two residues converts the K⁺-selective channel into a non-selective channel (55). Thus, amplification of the CNG2 pore region in endothelial cells supports the idea that CNG channels mediate the non-selective cation conductance and resolves that other K⁺-selective channels do not contribute to our present observations.

By having established that CNG channels mediate a non-selective cation conductance in endothelial cells, we next determined their contribution to membrane depolarization following activation of store-operated calcium entry. Thapsigargin activated the CNG channel in voltage-clamped cells, and in membrane potential studies it induced a sustained depolarization reliant upon CNG channel function. Although our studies did not directly establish the cation responsible for the depolarizing current, either Na⁺ or Ca²⁺ could mediate this response. Na⁺ carries the depolarizing current in response to odorant stimuli in neurons, although both endogenous and heterologously expressed CNG channels preferentially conduct Ca²⁺ (20, 53). The data together provide the first evidence that membrane depolarization following activation of store-operated calcium entry occurs via cationic conductance through a CNG channel.

Olfactory CNG channels may be directly activated by cAMP, cGMP, or nitric oxide. Prior work from our laboratory indicated activation of store-operated calcium entry inhibits type 6 adenyl cyclase in PAECs and decreases cAMP 30–50% (11, 56, 57). Thus, cAMP may contribute to the constitutive activity of CNG channels but does not regulate membrane depolarization following thapsigargin. Thapsigargin stimulates the production of nitric oxide in endothelial cells, which has been reported to activate sGC and increase cGMP (9, 10, 58). Our studies demonstrated that thapsigargin modestly increased cGMP in cultured PAECs, an increase that was perhaps limited because sGC can be down-regulated in culture (59). Indeed, sGC is expressed in endothelial cells in vivo suggesting calcium agonists produce membrane depolarization through elevations in cGMP. Endothelial cells express endothelial nitric-oxide synthase, and thapsigargin stimulates nitric oxide production in vivo and in vitro (60, 61). The recently described direct stimulation of olfactory CNG channels by nitric oxide supports the idea that in addition to regulation by cGMP, nitric oxide may be coupled to membrane potential through its direct stimulation of endogenous CNG channels (62, 63). Thus, whereas activation of store-operated calcium entry produces an initial hyperpolarization through activation of Kᵦ_channels, the subsequent depolarization occurs in response to cGMP and nitric oxide stimulation of CNG channel function.

In summary, our present studies support a new working model of events that control the PAEC membrane potential response to Ca²⁺ agonists (Fig. 12). Activation of store-operated Ca²⁺ entry increases cGMP (and nitric oxide) that stimulates Na⁺ and Ca²⁺ conductance through a CNG channel, causing membrane depolarization. The physiological significance of our observations remains speculative. However, the effect of membrane depolarization on store-operated Ca²⁺ entry is unequivocal; depolarization reduces Ca²⁺ entry in endothelial cells (40). Therefore, it is likely this depolarization contributes to slow feedback regulation of Ca²⁺ entry in endothelial cells, which has wide ranging significance from control of nitric oxide production (64, 65) to control of endothelial cell shape (1, 14).

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