Properties of a Native Cation Channel Activated by Ca\textsuperscript{2+} Store Depletion in Vascular Smooth Muscle Cells*

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Depletion of intracellular Ca\textsuperscript{2+} stores activates capacitative Ca\textsuperscript{2+} influx in smooth muscle cells, but the native store-operated channels that mediate such influx remain unidentified. Recently we demonstrated that calcium influx factor produced by yeast and human platelets with depleted Ca\textsuperscript{2+} stores activates small conductance cation channels in excised membrane patches from vascular smooth muscle cells (SMC). Here we characterize these channels in intact cells and present evidence that they belong to the class of store-operated channels, which are activated upon passive depletion of Ca\textsuperscript{2+} stores. Application of thapsigargin (TG), an inhibitor of sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase, to individual SMC activated single 3-pS cation channels in cell-attached membrane patches. Channels remained active when inside-out membrane patches were excised from the cells. Excision of membrane patches from resting SMC did not by itself activate the channels. Loading SMC with BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), which slowly depletes Ca\textsuperscript{2+} stores without a rise in intracellular Ca\textsuperscript{2+}, activated the same 3-pS channels in cell-attached membrane patches as well as whole cell nonselective cation currents in SMC. TG- and BAPTA-activated 3-pS channels were cation-selective but poorly discriminated among Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, Ba\textsuperscript{2+}, Na\textsuperscript{+}, K\textsuperscript{+}, and Cs\textsuperscript{+}. Open channel probability did not change at negative membrane potentials but increased significantly at high positive potentials. Activation of 3-pS channels did not depend on intracellular Ca\textsuperscript{2+} concentration. Neither TG nor a variety of second messengers (including Ca\textsuperscript{2+}, InsP\textsubscript{3}, InsP\textsubscript{4}, GTP\textgamma{}S, cyclic AMP, cyclic GMP, ATP, and ADP) activated 3-pS channels in inside-out membrane patches. Thus, 3-pS nonselective cation channels are present and activated by TG or BAPTA-induced depletion of intracellular Ca\textsuperscript{2+} stores in intact SMC. These native store-operated cation channels can account for capacitative Ca\textsuperscript{2+} influx in SMC and can play an important role in regulation of vascular tone.

Depletion of intracellular Ca\textsuperscript{2+} stores is known to activate store-operated (or capacitative) Ca\textsuperscript{2+} influx in a variety of nonexcitable cells (for review, see Refs. 1–5). The idea of capacitative Ca\textsuperscript{2+} entry (CCE)\textsuperscript{1} was initially proposed for smooth muscle cells (SMC) (6). Over the past years it has been shown that CCE and contraction can be activated in SMC by passive depletion of intracellular Ca\textsuperscript{2+} stores even without activation of receptor-dependent cascades (for review, see Ref. 7), although the nature of the ion channels responsible for CCE in vascular SMC remains obscure. In some nonexcitable cells, highly Ca\textsuperscript{2+}-selective calcium release-activated calcium (CRAC) channels (4, 8) and certain members of the diverse family of TRP channels (9, 10) are thought to be responsible for CCE. However, to date the existence of neither of those has been established in SMC. Importantly, depletion of Ca\textsuperscript{2+} stores was shown to trigger not only Ca\textsuperscript{2+}, but also Na\textsuperscript{+} influx in arterial myocytes (11), which implies that store-operated channels in SMC are poorly selective for cations. In freshly isolated mouse anococcygeus SMC there are strong indications that CCE results from activation of a whole cell nonselective cation current (12, 13), although in rat aortic SMC line A7r5 no currents were detected which could be associated with CCE (14, 15). It is totally unclear if the same or different store-operated channels mediate CCE in SMC from different preparations.

Here for the first time we characterize 3-pS cation channels that are activated by Ca\textsuperscript{2+} store depletion in intact SMC from mouse and rabbit aorta. These channels, contrary to highly Ca\textsuperscript{2+}-selective CRAC channels, are poorly selective for mono- and divalent cations, and under physiological conditions they will allow both Ca\textsuperscript{2+} and Na\textsuperscript{+} to enter SMC. Recently we found that these channels can also be activated in excised membrane patches by calcium influx factor (CIF) partially purified from human platelets or yeast with depleted Ca\textsuperscript{2+} stores (16). Taken together, these data strongly support the idea that the native 3-pS channels, which we found in SMC, belong to the class of store-operated ion channels. Preliminary data have been reported in abstract form (17, 18).

EXPERIMENTAL PROCEDURES

SMC Preparation

Four different preparations of aortic SMC were used in our experiments, and the 3-pS channel described in this paper was found to be the same in acutely dispersed and cultured SMC from mouse and rabbit aorta. Most of the experiments on characterization of the single channels and whole cell currents were done on mSMC in short term culture because they provided the most reliable model for studies of single channels, whole cell currents, and intracellular Ca\textsuperscript{2+}.

Mouse SMC (mSMC)

Mouse SMC were isolated from thoracic aorta of C57BL6 mice (15–20 g). Two animals were anesthetized by inhalation of halothane and

1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; [Ca\textsuperscript{2+}], intracellular Ca\textsuperscript{2+} concentration. TRP, transient receptor potential; CIF, calcium influx factor; NMDG, N-methyl-D-glucamine; InsP3, inositol trisphosphate; InsP4, inositol tetrakisphosphate.

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1 The abbreviations used are: CCE, capacitative calcium entry; SMC, smooth muscle cells; CRAC, calcium release-activated calcium; mSMC, mouse aortic SMC; DM, dissociation medium; rSMC, rabbit aortic SMC; N\textsubscript{P}, open channel probability; TG, thapsigargin; BAPTA, 7113; E-mail: vbolotina@med-med1.bu.edu.

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killed by cervical dislocation. The thoracic aortas were rapidly removed, cleaned of connective tissues, and cut into small pieces.

Acutely Dissociated mSMC—Acutely dissociated mSMC were obtained as described previously (19). Briefly, pieces of mouse aorta in 2 ml of Dulbecco’s modified Eagle’s medium containing 4 mg/ml collagenase, 1.5 mg/ml elastase, and 0.5 mg/ml trypsin inhibitor for 1 h at 37 °C. During incubation, tissue was gently triturated every 15 min. Enzymatic digestion was terminated by the addition of 5 ml of fresh Dulbecco’s modified Eagle’s medium (free of enzymes) supplemented with 10% fetal bovine serum (FBS). The cell suspension was centrifuged, supernatant discarded, and the pellet was resuspended in 2 ml of Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 units/ml streptomycin, and 10 mg/ml heparin. A segment of thoracic aorta was rapidly removed, cleaned of connective tissues, cut into small pieces, and rinsed in DM.

Acutely Dissociated rSMC—Acutely dissociated rSMC were obtained by incubation of the pieces of rabbit aorta in DM with 50 units/ml trypsin inhibitor for up to 4 h. After experiments, 20 μl of the cell suspension was placed in a 35-mm polystyrene tissue culture dish or a 0.15-mm glass-bottom chamber, and SMC were allowed to adhere to the bottom before the beginning of the experiment.

Mouse SMC in Short Term Primary Culture—Mouse SMC cultures were prepared in the following way. SMC were acutely dissociated by incubation of pieces of mouse aorta in 2 ml of Dulbecco’s modified Eagle’s medium containing 4 mg/ml collagenase, 1.5 mg/ml elastase, and 0.5 mg/ml trypsin inhibitor for 1 h at 37 °C. During incubation, tissue was gently triturated every 15 min. Enzymatic digestion was terminated by the addition of 5 ml of fresh Dulbecco’s modified Eagle’s medium (free of enzymes) supplemented with 10% fetal bovine serum (FBS). The cell suspension was centrifuged, supernatant discarded, and the pellet was resuspended in 2 ml of Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum, 100 units/ml penicillin G, and 10 mg/ml streptomycin. The cell suspension was placed on coverslips and kept in 35-mm Petri dishes at 37 °C in 5% CO2 for 3–7 days. Under these conditions, mSMC attached to the coverslips but did not divide, had a bipolar morphology, and stained positively for α-actin.

Rabbit Aortic SMC (rSMC)

Male New Zealand White rabbits (2–2.5 kg) were exsanguinated after injection of 30 mg/kg sodium pentobarbital and 150 units/kg heparin. A segment of thoracic aorta was rapidly removed, cleaned of connective tissues, cut into small pieces, and rinsed in DM.

Inward and outward currents are shown as downward and upward deflections, respectively, from the baseline (labeled 0 on the figures). The amplitude of single channel currents was analyzed using all point histograms (see Fig. 2B) or amplitude histograms obtained from the event list (see Fig. 2C). Both methods gave the same single channel current amplitude for the first two current levels (Fig. 2, C and E), but we found the second method to be better for resolving infrequent channel openings in resting SMC (see Fig. 2B) or when more than two single channel current levels were observed at the peak of channel activity (see Fig. 2C). The open channel probability (NP) was analyzed and plotted over time to illustrate the time course of channel activity. The total apparent number of channels (N) in individual patches was estimated after their activation and was based on the maximum number of single channel current levels observed simultaneously at +100 mV. In each membrane patch n varied from 3 to 5 (average N = 4). Because prolonged full activation of all of the channels has not been achieved in some experiments, we cannot exclude the possibility that the actual number of the channels in each patch could be slightly higher than the apparent one. Standard bath solution contained (in mM) 140 NaCl, 2.8 KCl, 2 MgCl2, 5.5 glucose, 10 HEPES (pH 7.4). Standard pipette solution contained (in mM) 140 NaCl, 10 TEA, 0.2 EGTA, 10 HEPES (pH 7.4). In some experiments NaCl in the pipettes was replaced by KCl, CsCl, CaCl2, BaCl2, SrCl2, sodium glutamate, or NMDG-Cl (as specified in Table 1). The liquid junction potential was compensated. In some experiments Ca2+ (1 or 10 mM) was added to 140 mM NaCl-containing pipette or bath solutions. The pipette solutions also contained 100 μM niflumic acid and 100 nM iberiotoxin to prevent activation of ClCa channels (the most abundant channels in aortic SMC), to ensure that they did not “contaminate” the recording of TG- and BAPTA-activated channels. Importantly, in control experiments 100–200 μM niflumic acid did not affect TG-induced Ca2+ influx in mouse aortic SMC.

Because of the extremely small amplitude of single channel currents we were able to record and analyze them only at membrane potentials between ±60 and ±100 mV. For that reason it was impossible to determine experimentally and compare the exact reversal potential of single channel currents under different ion conditions and to provide the standard calculations of channel selectivity for different cations based on the shifts of the reversal potential of single channel current. Because of these technical limitations we only determined the relative cation conductivity of single channels under different ionic conditions (Table 1), which was estimated from the slope conductance of inward or outward current in the presence of different cations. The slope conductance was calculated from the single channel currents at a minimum of three different membrane potentials.

Whole Cell Currents—Currents were recorded using conventional whole cell configuration. Bath solution contained (in mM) 130 NaCl, 2.8 KCl, 1 MgCl2, 10 HEPES, 10 TEA, 100 μM niflumic acid (pH 7.4), and 0.1 μM Ca2+ (buffered with EGTA). The pipette solution contained (in mM) 60 aspartic acid, 40 CsCl, 4.5 NaCl, 10 HEPES, and 10 BAPTA-Cs4 (pH 7.2 with CsOH). Pipettes had resistance of 2–5 megohms when filled with pipette solution. Development of the whole cell inward current...
The whole mouse aorta (panel A) and changes in intracellular Ca\(^{2+}\) in single isolated mSMC (panels B and C). Panel A, isometric tension in the ring of mouse thoracic aorta during the application of 4 \(\mu\)M T from the absence of extracellular Ca\(^{2+}\) and after 2.5 mM Ca\(^{2+}\) and 2 mM Ni\(^{2+}\) were added. Panel B, representative recording of [Ca\(^{2+}\)]\(_i\) in mSMC (short-term culture). 2 \(\mu\)M TG was applied (open horizontal bar above the trace) in the absence of extracellular Ca\(^{2+}\) followed by 2 mM Ca\(^{2+}\) (closed bar) and 4 mM Ni\(^{2+}\) (hatched bar). Panel C, summary data from 16 experiments similar to that shown in panel B. The bars represent Ca\(^{2+}\) levels before (1) and after the addition of TG (2), Ca\(^{2+}\) (3), and Ni\(^{2+}\) (4).}

**RESULTS**

**Thapsigargin-induced Contraction and Intracellular Ca\(^{2+}\) Rise in Aortic SMC—**5 \(\mu\)M TG applied to the intact mouse aorta (Fig. 1A) caused a substantial contraction (610 ± 22 mg), but only after the addition of 2 mM extracellular Ca\(^{2+}\), which is consistent with TG-induced activation of Ca\(^{2+}\)-influx. In individual SMC, TG applied in the absence of extracellular Ca\(^{2+}\) (Fig. 1B) caused a small increase in [Ca\(^{2+}\)]\(_i\), which can be explained by passive Ca\(^{2+}\) release from intracellular stores leading to their depletion. Subsequent addition of 5 mM Ca\(^{2+}\) caused a sustained rise in [Ca\(^{2+}\)]\(_i\), which reflects TG-induced activation of Ca\(^{2+}\)-influx (summary data are shown in Fig. 1C). Both Ca\(^{2+}\)-influx-dependent [Ca\(^{2+}\)]\(_i\) rise and contraction were inhibited by 2–5 mM nickel, (Fig. 1).

**Small Conductance Channels Activated by Thapsigargin in Aortic SMC—**To define the nature of the ion channels that are responsible for TG-induced Ca\(^{2+}\)-influx, single channel currents were recorded in cell-attached membrane patches in mouse (mSMC) and rabbit (rSMC) aortic SMC. Extracellular application of 2 \(\mu\)M TG activated small conductance channels in cell-attached membrane patches in mSMC and rSMC which were acutely dispersed or cultured. Fig. 2 shows a typical example (n = 31 out of 52) of single channel activity and current amplitude in cell-attached membrane patches in mSMC (short-term culture) before and after TG application with original traces of single channel outward currents (measured at +100 mV applied with respect to the inside of membrane) at different times of the experiment. Only rare single channel openings could be detected in the resting cells. However, 40–180 s after the application of TG, three to five single channel current levels were observed. Although the amplitude of single channel currents activated by TG was very small, it could be analyzed using either all point histograms (Fig. 2E) or event list histograms (Fig. 2C), which gave similar values of single channel current amplitude.

TG-activated single channel currents was observed in control mSMC (n = 10) and in mSMC with intracellular Ca\(^{2+}\) buffered with 20 \(\mu\)M BAPTA/AM for 20 min, (n = 21). Importantly, in about 30% of SMC, BAPTA loading itself caused activation of the same channels as will be described below. TG-activated small single channel currents were observed in the absence or presence of 100 mM iiberotoxin and 100 \(\mu\)M niflumic acid, inhibitors of K\(_{Ca}\) and Cl\(_{Ca}\) channels, respectively, but no channel openings were recorded in cell-attached membrane patches when 5 mM Ni\(^{2+}\) was present in the pipette (n = 9).

It is important to emphasize that the same small conductance channels were activated by TG in acutely dispersed mSMC and rSMC as well as in rSMC in primary culture. As an example, Fig. 3A shows TG-activated inward single channel currents and their amplitude histogram in a cell-attached membrane patch in SMC acutely dispersed from rabbit aorta (n = 5), which were indistinguishable from those found in mSMC (Fig. 2). Detailed characteristics of TG-activated channels in mSMC and rSMC are presented below.

**Main Characteristics of TG-activated Channels in Inside-out Membrane Patches—**After TG-induced activation in cell-attached patches, single channels remained active for 5–15 min even when the membrane patches were excised from SMC in Ca\(^{2+}\)-free solution (Fig. 2D, n = 4 out of 4). 2 mM LaCl\(_3\) applied to the inside of membrane patches inhibited single channel currents (n = 4). Importantly, excision of membrane patches from resting mSMC (not treated with TG or BAPTA/AM) in the presence or absence of 1 \(\mu\)M or 1 mM CaCl\(_2\) did not by itself activate the channels (n = 54).

Single channel currents in inside-out membrane patches from TG-activated mSMC and rSMC (acutely dispersed or cultured) had identical properties, and Fig. 4 shows a typical example of single channel currents recorded in inside-out mem-
brane patches with amplitude histograms at different membrane potentials. Current-voltage (IV) relationships of single channels under different ionic conditions in inside-out membrane patches from rSMC and mSMC are shown in Fig. 3, B and C, respectively (acutely dissociated cells) and Fig. 5, A and B (cultured cells). The slope conductance in symmetrical 140 mM NaCl was 3.3 ± 0.1 pS (n = 4) in acutely dissociated and 3.4 ± 0.2 pS (n = 6) in cultured mSMC, which was similar to acutely dissociated and cultured rSMC (3.3 ± 0.1 pS, n = 8 and 3.2 ± 0.1 pS, n = 9, respectively). When Na⁺ in the pipette was replaced by 100 mM NMDG-Cl, inward single channel currents (at −100 mV) disappeared, although outward currents recorded at +100 mV in the same membrane patches did not change (n = 3, Fig. 5A). Replacement of Cl⁻ with glutamate in the pipette did not affect single channel currents at both positive and negative membrane potentials (3.4 ± 0.6 pS, n = 3, Fig. 5A), confirming that the 3-pS channel is cation-selective. Fig. 5A shows that the conductance of 3-pS channels did not change significantly when Ca²⁺ was added to Na⁺-containing pipette solution at concentration of 1 mM (3.3 ± 0.7 pS, n = 16) or 10 mM Ca²⁺ (2.9 ± 0.8 pS, n = 3), which showed that, contrary to Ca²⁺-selective channels, the 3-pS channel does not prefer Ca²⁺ over Na⁺ when both cations are present. When Na⁺ in the pipette was replaced by K⁺ or Cs⁺, the single channel inward current had similar slope conductance of 3.4 ± 0.1 pS (n = 3) and 3.4 ± 0.2 pS (n = 4), respectively, which was similar to that in Na⁺. To test if 3-pS channels along with monovalent cations also conduct divalent cations, Na⁺ in the pipette and/or in the bath was replaced by Ca²⁺, Sr²⁺, or Ba²⁺ (Fig. 5, A and B). When 90 mM Ca²⁺ was used in the pipette and 90 mM Ba²⁺ was used in the bath (with no Na⁺ present on

**Fig. 2.** TG-activated single channel currents in cultured mSMC. Panel A, NPo in the cell-attached membrane patch before and after the application of 2 μM TG (open bar) to intact mSMC (in short term culture) loaded with 20 μM BAPTA/AM for 20 min. A similar TG-induced activation of small conductance channels was observed in 31 out of 52 cells. Below, the original traces of single channel outward currents at +100 mV applied to the membrane (equivalent to −100 mV applied to the pipette) are shown at different times of the experiment. The closed state of the channel is marked by 0, and open states are labeled as 1 and 2 at the beginning of the original traces. Panels B and C, amplitude histograms (from the event list) of a single channel current before (panel B) and after (panel C) the application of TG (from the experiment shown in panel A). The numbers above each peak represent the amplitude of corresponding current level. Panel D, example of single channel currents recorded in a cell-attached membrane patch in SMC pretreated with TG (TG) and after the inside-out patch was excised (I/O) into Ca²⁺-free solution containing 5 mM EGTA (0 Ca⁺). Panel E, all-point amplitude histogram from the same single channel current recording as in panel C. The amplitudes of different current levels are shown above each peak.

**Fig. 3.** TG-activated single channel currents in fresh SMC. Panel A, original traces of single channel inward currents recorded cell-attached membrane patch (at −100 mV) before (control) and 4 min after the application of 2 μM TG to intact acutely dissociated rabbit SMC. The closed and open states of the channel are marked by 0 and 1, respectively (openings are downward deflections). All point amplitude histograms of single channel current after the application of TG are shown on the right. Panel B, current-voltage relationship of TG-activated single channels in inside-out membrane patches from acutely dissociated rabbit SMC in symmetrical 140 mM NaCl. Summary data are from eight experiments with S.E. bars shown where they exceed the size of the symbol. Panel C, same as in panel B but for TG-activated channels from acutely dissociated mSMC. 140 mM CsCl was in the pipette and 140 mM NaCl in the bath. Summary data are from four experiments with S.E. bars shown where they exceed the size of the symbol.
brane patch (under symmetrical 140 mM NaCl conditions) at the indi-
ple of TG-activated single channel currents recorded in inside-out mem-
membrane patches at different potentials. This is a typical exam-

brane patch is from cultured rabbit SMC.

0.1 pS, and the outward (Ba^{2+}) current was 1.4 pS with a conduc-
tivity to different cations. Minor changes in conductivity of
solutions which is required for estimation of exact channel
conductance of inward (Ca^{2+}) was 2.7 ± 0.1 pS, and the outward (Ba^{2+}) current was 3.5 ± 0.1 pS (Fig. 5B, n = 8). When 90 mM Ca^{2+} or Sr^{2+} was in the pipette and 140 mM Na^{+} was in the bath, the slope conductance of inward current was 3.0 ± 0.6 pS (for Ca^{2+}, n = 3) and 2.7 ± 0.1 pS (for Sr^{2+}, n = 9), with no apparent change in slope conductance of outward (Na^{+}) current. Working at the limits of resolution of single channel currents, we were not able to determine the exact reversal potential of single channel current in mixed solutions which is required for estimation of exact channel selectivity to different cations. Minor changes in conductivity of the channel in the presence of different cations (summarized in Table I) would suggest that these TG-activated 3-pS channels
are cation-selective but discriminate poorly between different
mono- and divalent cations, and thus under physiological con-
itions would allow both Ca^{2+} and Na^{+} to enter SMC.

In inside-out membrane patches, the NP_{o} of single channels in both mSMC (Fig. 5C) and rSMC (Fig. 5D) was similar and did not change much at negative membrane potentials (NP_{o} = 0.2 at -60 mV) but significantly increased at high positive mem-
brane potentials. Although only one level of single channel currents was usually seen at -100 mV, activation of three to five levels (average four, n = 28) could be detected at +100 mV (Fig. 4). Thus, in the physiological range of membrane potentials the P_{o} of each individual channel activated by TG in SMC could be as low as 0.05.

Thus, the same channels were activated by TG in acutely dissociated or cultured SMC from mouse and rabbit aorta, which appeared to be nonselective cation channels with about a 3-pS conductance.

Importantly, application of 2–5 μM TG (n = 12), 1 μM Ca^{2+} (n = 12) or 1 mM Ca^{2+} (n = 42), 20 μM InsP_{3} (n = 11), 10 μM InsP_{5} (n = 5), 200 μM GTPγS (n = 5), 100 μM cyclic GMP (n = 5), 100 μM cyclic AMP (n = 5), 1.7 mM ATP (n = 6), or 100 μM ADP (n = 5) to inside-out membrane patches excised from the resting mSMC did not activate 3-pS channels.

Loading SMC with BAPTA Activated 3-pS Nonselective Cation Channels and Whole Cell Currents—BAPTA, a high affinity Ca^{2+} chelator, activates CRAC currents in a variety of nonex-
citable cells and is commonly used to deplete intracellular Ca^{2+} stores without an increase in [Ca^{2+}], or activation of receptor-
dependent pathways (for review, see Refs. 4 and 21). Dialysis of
mSMC with 10 mM BAPTA in the absence of extracellular Ca^{2+} stores slowly activated an inward current (at -50 mV) which usually started after 40–60 s and reached a stable plateau of 39 ± 13 pA (n = 6) within 2–3 min (Fig. 6, A and B). The current was inhibited by 2 mM LaCl_{3}, n = 5). The IV relationship of the BAPTA-induced whole cell current (Fig. 6C) reversed at 2.5 ± 1.4 mV and showed strong outward rectification, especially at high positive potentials (not shown).

When mSMC were loaded with 20 μM BAPTA/AM for 20 min, small conductance channels were activated in 25 out of 72 cells, which were the same as 3-pS channels activated by TG. Fig. 6F shows an example of single channel currents and their ampli-
tude histograms at different membrane potentials in a mem-
brane patch excised from a BAPTA-loaded mSMC. The IV relationship (Fig. 6E) and the increase in NP_{o} at high positive membrane potentials (Fig. 6D) of BAPTA-activated channels were similar to the 3-pS channels activated by TG in mSMC and rSMC (see Fig. 5). In mSMC not loaded with BAPTA, spontaneous activation of 3-pS channels was observed only in 3 out of 26 cells.

Because BAPTA-activated 3-pS channels and whole cell currents were both poorly cation-selective and both showed significant outward rectification at high positive membrane potentials, we determined whether activation of 3-pS channels could indeed produce the whole cell current with characteristics of that experimentally observed in BAPTA-loaded mSMC. The amplitudes of the hypothetical whole cell current were calculated from the IV relationship (Fig. 6D) and NP_{o} (Fig. 6E) of

Fig. 4. TG-activated single channel currents in inside-out membrane patches at different potentials. This is a typical exam-

Fig. 5. Properties of single channels activated by TG in mouse (panels A and C) and rabbit (panels B and D) SMC. Panel A, current-voltage relationship of TG-activated single channels in inside-out membrane patches from cultured mSMC. The ion composition of the pipette solution was (in mM) 140 NaCl (●), 140 NaCl and 1 CaCl_{2} (●), 140 NaCl and 10 CaCl_{2} (●), 90 CaCl_{2} (●), 100 NMDG (●) or 100 sodium glutamate (●) with 10 HEPES (pH 7.4). In all experiments the ion composition of the bath solution was (in mM) 140 NaCl, 1 Ca^{2+}, 1 Mg^{2+}, 2.8 KCl, and 100 HEPS, each point is an average of 3–16 experiments with S.E. bars shown where they exceed the size of the symbol. Panel B, same as in panel A but for single TG-activated channels from cultured rabbit SMC. The ion composition of the pipette solution was (in mM) 140 NaCl (●), 90 CaCl_{2} (●), 90 SrCl_{2} (●), or 140 KCl (●). The bath contained (in mM) 140 NaCl (●), 90 BaCl_{2} (●), or 90 SrCl_{2} (●). 10 mM HEPES (pH 7.4) was present in all solutions. Panel C, voltage dependence of the NP_{o} in inside-out membrane patches from TG-activated cultured mSMC. Summary data are from 18 experiments. Panel D, same as in panel C but for channels from TG-activated cultured rabbit SMC. Summary data are from 6 experiments.
FIG. 6. Single channels and whole cell currents activated by SMC loading with BAPTA. Panel A, representative experiment showing the changes in the amplitude of the whole cell current (at −50 mV) during the dialysis of mSMC with 10 mM BAPTA (open bar) and after the application of 2 mM La3+ (closed bar). Panel B, summary data showing the amplitudes of the whole cell currents in mSMC at the moment of breaking into the cell (0), after 60 and 180 s of SMC dialysis with 10 mM BAPTA, and after application of lanthanum (+La) in six experiments similar to the one shown in panel A. Panel C, summary of six experiments showing the current-voltage relationship (V) of the peak whole cell current activated in SMC by cell dialysis with 10 mM BAPTA. ○, estimated amplitude of the hypothetical whole cell current calculated from the IV relationship (panel E) and NP0 (panel D) of 3-pS single channel currents (for details, see “Results”). Panel D, voltage dependence of NP0 of 3-pS channels in inside-out membrane patches from mSMC activated by loading with 20 μM BAPTA/AM for 20 min. Panel E, summary of 25 experiments showing current voltage (IV) relationship of single channels in inside-out membrane patches (under symmetrical 140 mM NaCl conditions) from mSMC activated by loading with BAPTA/AM. Panel F, representative traces of single channel currents at different membrane potentials in an inside-out membrane patch from mSMC loaded with BAPTA/AM under symmetrical 140 mM NaCl conditions. Single channel openings are shown as upward (at positive membrane potentials) or downward (at negative membrane potentials) deflections. The closed state of the channel is shown by 0; open states are labeled 1, 2, and 3 at the beginning of the original traces. Panels on the right represent all points histograms calculated for each trace.

Discussion

The present study provides the first insight into the nature of single ion channels that are responsible for store-operated Ca2+-influx in vascular SMC. We found Ca2+-conducting nonselective cation channels of very small (3-pS) conductance which are activated after loading mSMC with BAPTA and/or treatment with TG, which are commonly used to passively deplete intracellular Ca2+-stores. The very small amplitude of single channel currents activated by TG and BAPTA in mouse and rabbit aorta SMC might explain why these channels have not been described previously. Indeed, many different types of nonselective cation channels have been described in SMC (15, 22–37), but the single channel properties and mechanism of activation of the 3-pS channel distinguish it from all of the channels found in SMC so far (for review, see Refs. 5, 38, and 39). Our results strongly support the possibility that novel 3-pS channels are store-operated and are responsible for TG-induced Ca2+-influx in individual SMC and contraction of the aorta.

Characteristics of the 3-pS Channel in SMC—Even though the amplitude of single channel currents did not exceed 0.4 pA even at ± 100 mM, the low noise patch-clamp system allowed us to resolve clearly single channel openings and to perform some analysis of their amplitude and open channel probability. TG- and BAPTA-activated channels appeared to be nonselective cation channels that conducted and poorly discriminated between monovalent (Na+, K+, Cs+) and divalent (Ca2+, Sr2+, Ba2+) cations. Their single channel conductance was close to 3 pS for a variety of cations tested. As a result of activation of such nonselective cation channels both Ca2+ and Na+ are expected to enter SMC causing not only a rise in intracellular Ca2+, but also significant influx of Na+. Indeed, depletion of Ca2+ stores has recently been shown to activate both Ca2+ and Na+ influx in arterial myocytes (11). Influx of Na+ into SMC could result in membrane depolarization that could potentially trigger activation of voltage-dependent Ca2+-selective channels that might contribute to additional Ca2+ influx and SMC contraction. Single channel recording during SMC activation with TG provides some indirect evidence of TG-induced SMC depolarization. Indeed, rare openings of single channels in
resting SMC result in outward currents of about 0.22 pA at +100 mV applied in respect to the cytoplasmic side of cell-attached membrane patches (Fig. 2, A and B). Simple calculations show that this amplitude is about what one would expect from a 3.4-pS channel if the resting membrane potential is around ~40 mV (indeed we found the resting membrane potential of BAPTA-loaded SMC to be ~39 ± 2 mV). After robust activation of the channels by TG, their current amplitude increased to about 0.31 pA (Fig. 2, A and C) which is what could be expected from a 3.4-pS channel if the membrane potential of SMC at that point is about ~10 mV. Thus, the increase in the amplitude of single channel outward currents in cell-attached membrane patches is consistent with significant TG-induced depolarization of SMC. Importantly, 3-pS channels were found to be present and open rarely in resting SMC. The activation of the channels was observed within 1–3 min after the application of TG (Fig. 2), which is within the timeframe of the TG-induced onset of Ca²⁺ influx in SMC and other cells (20, 40). The NPo did not change significantly within the physiological range of membrane potentials, but it increased dramatically at high positive potentials (Fig. 5, C and D). This increase of NPo was very typical for 3-pS channels in both cell-attached and inside-out membrane patches, but the mechanism underlying this phenomenon is unclear.

3-pS Nonselective Cation Channels Could Underlie BAPTA-activated Whole Cell Current—A critical “trademark” feature of store-operated channels is that they can be activated independently of intracellular Ca²⁺ rise and major signaling cascades. Such conditions are achieved by cell dialysis with 10–20 mM BAPTA. Indeed, BAPTA provides a fast and strong buffering of intracellular Ca²⁺, preventing Ca²⁺ stores from refilling and promoting their passive depletion. Extracellular Ca²⁺ must also be eliminated to prevent saturation of BAPTA by Ca²⁺ entering the cell. Activation of the whole cell current under these conditions is thought to be one of the strongest pieces of evidence for the existence of store-operated channels in different types of cells (4).

Dialysis of mSMC with 10 mM BAPTA in our experiments activated a poorly selective whole cell current with a reversal potential around 0 mV (please note that Cl⁻ equilibrium potential under our experimental conditions was ~31 mV). The time course of the development of this current was similar to that observed for ICRAC in nonexcitable cells, but unlike ICRAC, the current showed strong outward, rather than inward rectification, under physiological ionic conditions (when both Ca²⁺ and Na⁺ were present in the bath). Poor cation selectivity and pronounced outward rectification strongly resemble that of the 3-pS channels, which were also activated in mSMC as a result of BAPTA/AM loading (Fig. 6). Moreover, the I/V relationship of the inward and outward whole cell currents simulated from single 3-pS channel currents (using their NPo and I/V relationship) is similar to the BAPTA-activated whole cell current recorded in mSMC.

Thus, the BAPTA-activated current is likely to result from activation of the 3-pS nonselective cation channels found in the same mSMC. We estimated that about 5,000 channels must be present in mSMC to account for the BAPTA-activated whole cell current. Indeed, at 50 mV the average whole cell inward current (I) of about 40 pA develops after 3 min in BAPTA-loaded mSMC (Fig. 6B). Assuming that the single channel current at ~50 mV is γ = 0.16 pA, and each channel is opened with P_o = 0.05, the minimum number of the channels which could produce the whole cell current of 40 pA will be n = I/γP_o = 5,000. If the channels are homogeneously distributed in the plasma membrane, and an average mSMC has a capacitance of 20 picofarads and surface of about 2,000 μm², then two to three channels are expected to be present in every 1 μm² of plasma membrane. This is in close agreement with an average of four channels which we observed in 1–2-μm² membrane patches. Thus, 3-pS channels are likely to be responsible for the whole cell current activated during mSMC cell dialysis with BAPTA.

Native 3-pS Channels Are Not Regulated by Intracellular Ca²⁺—Application of TG to intact SMC causes a pronounced increase in [Ca²⁺]_i, and some of the channels could be activated by TG-induced intracellular Ca²⁺ rise rather than by depletion of Ca²⁺ stores. Several lines of evidence obtained in our experiments exclude this possibility. First, 3-pS channels were not activated directly by Ca²⁺ when inside-out membrane patches were excised into Ca²⁺-containing solutions. Two different concentrations of Ca²⁺ in the bath were used in these experiments (1 μM and 1 mM) to ensure that the possibility of a bell-shaped dependence on Ca²⁺ concentration was not overlooked. For comparison, in our recent studies (19), we demonstrated that excision of membrane patches from the same cells into Ca²⁺-containing solution activated Ca²⁺-dependent Cl⁻ channels (in the absence of niflumic acid). These results show that contrary to ClCa⁻ channels, 3-pS cation channels are not activated by Ca²⁺. Second, TG-induced activation of the same 3-pS cation channels was observed in both control SMC and SMC in which [Ca²⁺]_i was buffered with BAPTA/AM. There is always a concern that loading the cells with BAPTA/AM may not be enough to buffer Ca²⁺ completely in certain regions of subplasmalemmal space with restricted diffusion. This problem is impossible to rule out entirely. However, we showed recently (19) that identical loading of mouse aortic SMC with BAPTA/AM completely prevents the caffeine-induced global intracellular Ca²⁺ rise as well as activation of ClCa⁻ channels, which are known to be highly sensitive and reliable sensors of Ca²⁺ rise occurring beneath the plasma membrane. Thus, it is very unlikely that TG causes a significant Ca²⁺ rise in BAPTA/AM-loaded mSMC which could affect 3-pS channels. Third, depletion of intracellular stores with BAPTA (in the absence of extracellular Ca²⁺) does not increase intracellular Ca²⁺, although it does activate single 3-pS nonselective cation channels and whole cell currents. Thus, it seems unlikely that Ca²⁺ could be a natural activator of 3-pS channels.

3-pS Nonselective Cation Channels Are Likely to Be Native Store-operated Channels in SMC—The results of our studies provide several lines of evidence that in intact SMC 3-pS channels are activated by depletion of intracellular Ca²⁺ stores rather than by other mechanisms. First, we found 3-pS channels to be activated by TG and BAPTA, which are known to cause passive depletion of Ca²⁺ stores and activation of store-operated channels and whole cell currents in a variety of nonexcitable cells. Second, activation of 3-pS channels and corresponding whole cell currents do not require intracellular Ca²⁺ rise or activation of InsP₃, or other receptor-dependent cascades and could be achieved by simple loading of SMC with the Ca²⁺ chelator BAPTA. Third, a variety of second messengers that are known to be involved in Ca²⁺ homeostasis of SMC (Ca²⁺, InsP₃, InsP₄, GTPyS, cAMP, cGMP, ATP, ADP) did not activate single 3-pS channels in inside-out membrane patches, excluding their role as physiological activators of these channels.

Recently, we found that 3-pS nonselective cation channels in inside-out membrane patches from SMC are activated by CIf partially purified or bioassayed from yeast or human platelets with depleted Ca²⁺ stores (16). This CIf is thought to be one of the possible messengers produced by endoplasmic reticulum upon depletion of Ca²⁺ stores which can activate plasma membrane channels and cause CCE. These data strongly support

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the idea that 3-pS nonselective cation channels (which we showed here to be activated in intact SMC upon depletion of their stores) indeed belong to the class of store-operated channels.

Although their electrophysiological characteristics are significantly different, activation properties of 3-pS cation channels and whole cell currents resemble those of other store-operated channels and currents in nonexcitable cells. The onset of TG-induced activation of 3-pS channels falls within the time frame of passive depletion of Ca\(^{2+}\). stores when sarco-endoplasmic reticulum-dependent back-sequestration of Ca\(^{2+}\) is inhibited. After being activated, 3-pS channels remain active for several minutes even after inside-out patches are excised from SMC, which is similar to the store-operated currents recorded in giant inside-out membrane patches from Xenopus oocytes (41). BAPTA loading of SMC also activates the whole cell nonselective cation current with a time course similar to that of activation of I\(_{\text{CRAC}}\). However, the electrophysiological characteristics of the currents in SMC are different from that known for CRAC channels (for review, see Ref. 4). For example, I\(_{\text{CRAC}}\) in nonexcitable cells is highly Ca\(^{2+}\)-selective and conducts Na\(^+\) only in the absence of Ca\(^{2+}\), whereas BAPTA and TG-activated single channels in SMC are poorly cation-selective, and their Na\(^+\) conductance does not change significantly in the presence or absence of Ca\(^{2+}\). Also, in the presence of Ca\(^{2+}\) on one side of the membrane and Na\(^+\) on the other, the I/V relationship of 3-pS channels is linear with approximated reversal potential around zero mV. In the presence of Na\(^+\) and Ca\(^{2+}\) the whole cell current in SMC reverses at 0 mV and has significant outward rectification, whereas I\(_{\text{CRAC}}\) reverses at high positive potentials (around the Ca\(^{2+}\) equilibrium potential) and has a very pronounced inward rectification. The whole cell currents and single 3-pS channels could be inhibited by millimolar concentrations of nickel or lanthanum (above 2 mM), whereas I\(_{\text{CRAC}}\) is inhibited by lanthanum in the micromolar range. Finally, under the same ionic conditions (in the presence of Na\(^+\) and Ca\(^{2+}\)) the conductance of CRAC channels was estimated to be about 9 fS (42), which is 300 times less than the 3-pS conductance we observed. Thus, selectivity and conductance of 3-pS channels in SMC are clearly different from CRAC channels found in nonexcitable cells, but that does not exclude the possibility that both channels could be similarly regulated by the filling state of intracellular Ca\(^{2+}\) stores.

The poor cation selectivity of TG- and BAPTA-activated channels in SMC resembles that of the TRP1 channel expressed in some mammalian cells (43–45), although its conductance was estimated to be around 16 pS. The possibility that 3-pS nonselective cation channels in SMC, which is similar to the store-operated currents recorded in giant inside-out patches from Xenopus oocytes (41), could be related to the growing family of TRP channels needs further investigation.

**Physiological Relevance of Store-operated Cation Channels in Vascular SMC.—**The existence of store-operated Ca\(^{2+}\) influx in vascular SMC is strongly supported by many studies that demonstrated Ca\(^{2+}\) influx in SMC and contractions in different vessels caused by sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase inhibitors (12, 15, 35, 46–56). Recently, we demonstrated that this pathway in vascular SMC is regulated by nitric oxide, the major endothelium-derived relaxing factor, which indirectly inhibits CCE by enhancing sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase-dependent refilling of Ca\(^{2+}\) stores (20). This implies the physiological importance of CCE in regulation of vascular tone, but the nature of the channels involved in this store-regulated process remains obscure. Indeed, studies in a fetal rat aortic cell line (A7r5) failed to reveal single channels or cation currents activated by TG (14, 15), although in freshly isolated mouse aortic SMC a whole cell nonselective cation current was described which correlated with capacitative Ca\(^{2+}\) influx (12, 13).

Our studies for the first time demonstrate the presence of small conductance (3-pS) nonselective cation channels in intact SMC from mouse and rabbit aorta which are activated by depletion of intracellular Ca\(^{2+}\) stores (with BAPTA or TG). Importantly, we found and described these channels in both acutely dispersed and cultured SMC. These new data, together with demonstration of TG-induced Ca\(^{2+}\) influx in isolated mSMC (Fig. 1), TG-induced Ca\(^{2+}\) influx in intact smooth muscle strips of mouse aorta (20), and TG-induced contraction of the mouse aorta (Fig. 1), strongly suggest that TG-activated 3-pS cation channels could be responsible for store-operated Ca\(^{2+}\) influx and contraction of aortic SMC.

At the end, it is important to mention that although we found 3-pS channels in both freshly dispersed and cultured SMC, it was easier to record and study them in cultured SMC. Also, SMC in culture showed more consistent responses to TG (judged by TG-induced Ca\(^{2+}\) influx), which was observed in 80–90% of cells, whereas such responses in acutely dissociated cells varied from isolation to isolation, and on average TG-induced Ca\(^{2+}\) influx was observed in only about 10% of freshly dissociated SMC. Such differences in the number of cells responding to TG most probably reflect the result of enzymatic shock which is unavoidable during the acute isolation of fresh SMC but can be reduced if SMC are allowed to recover for a few days in short term culture. Culture of the cells does not apparently change the electrophysiological properties of these 3-pS channels. This simple explanation is also supported by the fact that TG-induced Ca\(^{2+}\) influx is present in intact smooth muscle strips of mouse aorta (20) before their exposure to enzymes. Interestingly, the same methods of acute isolation of SMC from mouse and rabbit aorta did not affect SMC responsiveness to caffeine which causes Ca\(^{2+}\) release from the stores and activation of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels (19) in 90% of freshly dispersed cells. The reasons for the relative partial impairment of capacitative Ca\(^{2+}\) influx mechanism immediately after enzymatic treatment of SMC need further investigation.

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**REFERENCES**

1. Putney, J. W., Jr. (1986) *Cell Calcium* 7, 1–12.
2. Berridge, M. J. (1995) *Biochem. J.* 312, 1–11.
3. Clapham, D. E. (1995) *Cell* 80, 259–268.
4. Parekh, A. B., and Penner, R. (1997) *Physiol. Rev.* 77, 901–930.
5. Barritt, G. J. (1999) *Biochem. J.* 337, 153–169.
6. Castells, R., and Droogmans, G. (1994) *J. Physiol. (Lond.)* 371, 263–279.
7. Gibson, A., McFadzean, I., Wallace, P., and Wayman, C. P. (1998) *Trends Pharmacol. Sci.* 19, 266–269.
8. Hoth, M., and Penner, R. (1992) *Nature* 355, 353–356.
9. Friel, D. D. (1996) *Cell* 85, 617–619.
10. Montell, C. (1997) *Mol. Pharmacol.* 52, 755–763.
11. Arnon, A., Hamlyn, J. M., and Blautstein, M. P. (2000) *Am. J. Physiol.* 278, C163–C173.
12. Wayman, C. P., McFadzean, I., Gibson, A., and Tucker, J. F. (1996) *Br. J. Pharmacol.* 117, 566–572.
13. Wayman, C. P., Wallace, H. M., Gibson, A., and McFadzean, I. (1999) *Eur. J. Pharmacol.* 376, 325–329.
14. Iwasawa, K., Nakajima, T., Hazama, H., Goto, A., Shin, W. S., Toyο-oka, T., and Omata, M. (1997)* J. Physiol. (Lond.)* 503, 257–262.
15. Iwamuro, Y., Miwa, S., Zhang, X. F., Minowa, T., Enoki, T., Okamoto, Y., Hasegawa, M., Furutani, H., Okazawa, M., Ishikawa, M., Hashimoto, N., and Masaki, T. (1999) *Br. J. Pharmacol.* 126, 1107–1114.
16. Trepakov, E. S., Cautera, P., Marchase, R. B., Cohen, R. A., and Bolotina, V. M. (2000) *J. Biol. Chem.* 275, 26158–26163.
17. Bolotina, V. M., Weisbrod, R. M., Gericke, M., Taylor, P., and Cohen, R. A. (1997) *Biophys. J.* 72, 236 (abstr.).
18. Trepakov, E. S., Cautera, P., Gericke, M., Marchase, R. B., Cohen, R. A., and Bolotina, V. M. (2000) *Biophys. J.* 78, 193 (abstr.).
19. Hikawa, Y., Gericke, M., and Bolotina, V. M. (1999) *Am. J. Physiol.* 277, H1732–H1744.
20. Cohen, R. A., Weisbrod, R. M., Gericke, M., Yaghoubi, M., Bierl, C., and Bolotina, V. M. (1999) *Circ. Res.* 84, 210–219.
21. Hoth, M., Faasolo, C., and Penner, R. (1993) *Ann. N. Y. Acad. Sci.* 707, 198–209.
