Characterization and function of Ca^{2+}-activated K^+ channels in arteriolar muscle cells

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Jackson, William F., and Kevin L. Blair. Characterization and function of Ca^{2+}-activated K^+ channels in arteriolar muscle cells. *Am. J. Physiol.* 274 (*Heart Circ. Physiol.* 43): H27–H34, 1998.—We examined the functional role of large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channels in the hamster cremasteric microcirculation by intravital videomicroscopy and characterized the single-channel properties of these channels in inside-out patches of membrane from enzymatically isolated cremasteric arteriolar muscle cells. In second-order (39 ± 1 μm, $n = 8$) and third-order (19 ± 2 μm, $n = 8$) cremasteric arterioles with substantial resting tone, superfusion with the K_{Ca} channel antagonists tetraethylammonium (TEA, 1 mM) or iberiotoxin (IBTX, 100 nM) had no significant effect on resting diameters ($P > 0.05$). However, TEA potentiated O_2-induced arteriolar constriction in vivo, and IBTX enhanced norepinephrine-induced contraction of cremasteric arteriolar muscle cells in vitro. Patch-clamp studies revealed unitary K^+-selective and IBTX-sensitive currents with a single-channel conductance of 240 ± 2 pS between −60 and 60 mV ($n = 7$ patches) in a symmetrical 140 mM K^+ gradient. The free Ca^{2+} concentration ($[Ca^{2+}]$) for half-maximal channel activation was 44 ± 3, 20 ± 1, 6 ± 0.4, and 3 ± 0.5 μM at membrane potentials of −60, −30, +30, and +60 mV, respectively ($n = 5$), with a Hill coefficient of 1.9 ± 0.2. Channel activity increased *e*-fold for a 16 ± 1 mV ($n = 6$) depolarization. The plot of log$[Ca^{2+}]$ vs. voltage for half-maximal activation ($V_{½}$) was linear ($r^2 = 0.9843$, $n = 6$); the change in $V_{½}$ for a 10-fold change in $[Ca^{2+}]$ was 84 ± 5 mV, and the $[Ca^{2+}]$ for half-maximal activation at 0 mV (Ca_0; the Ca^{2+} set point) was 9 μM. Thus, in vivo, K_{Ca} channels are silent in cremasteric arterioles at rest but can be recruited during vasoconstriction. We propose that the high Ca_0 is responsible for the apparent lack of activity of these channels in resting cremasteric arterioles, and we suggest that this may result from expression of unique K_{Ca} channels in the microcirculation.

microcirculation; vasoconstriction; iberiotoxin; tetraethylammonium; skeletal muscle; vascular smooth muscle; calcium ions; oxygen; patch clamp; norepinephrine; hamster; cremaster muscle

CALCIUM-ACTIVATED potassium (K_{Ca}) channels are one of the dominant ion channels found in the plasma membrane of vascular smooth muscle (25). These K^+ channels are activated both by elevated concentrations of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and by membrane depolarization (25). Recent studies have provided evidence that large-conductance K_{Ca} channels regulate membrane potential and resting diameter of small arteries that develop myogenic tone (4, 22–25). Furthermore, recent studies by Nelson and colleagues have suggested that Ca^{2+} sparks, representing focal, subsarcolemmal Ca^{2+} transients with peak amplitudes of ~300 nM, are sufficient to activate K_{Ca} channels at physiological membrane potentials in muscle cells isolated from small cerebral arteries (23). The Ca^{2+} sensitivity of K_{Ca} channels implied from this study, suggesting activation of the channels when $[Ca^{2+}]_i = 100–300$ nM, is consistent with the Ca^{2+} sensitivity for K_{Ca} channels in mesenteric arteries reported by Benham et al. (3). However, other studies have found that K_{Ca} channels require micromolar concentrations of Ca^{2+} to be active at physiological membrane potentials (29; see also 6). Thus there may be regional or species-dependent differences in the properties of K_{Ca} channels that may affect their contribution to the regulation of vascular function in different vascular beds. Furthermore, the single-channel properties and functional role played by K_{Ca} channels in muscle cells in the walls of arterioles in the microcirculation are not known. Therefore, we examined the contribution of K_{Ca} channels to the regulation of resting vascular tone of arterioles in hamster cremasteric muscles in vivo and then characterized the single-channel properties of K_{Ca} channels in inside-out patches of membrane from vascular smooth muscle cells isolated from the same arterioles. Our studies indicate that K_{Ca} channels do not appear to regulate the resting diameter of cremasteric arterioles in vivo, although they may limit vasoconstriction. This functional profile may reflect a relatively high Ca^{2+} set point displayed by K_{Ca} channels in the microcirculation, suggesting that a distinct K_{Ca} channel subtype may be expressed in cremasteric arteriolar muscle cells.

METHODS

Preparation of cremaster muscles for intravital microscopy. All experiments involving animal use were approved by the Western Michigan University Institutional Animal Care and Use Committee and conform with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act. Male golden Syrian hamsters (100–170 g) were anesthetized with pentobarbital sodium (75 mg/kg ip). Cremaster muscles (2) were prepared for intravital video microscopy by standard methods as described previously (10–12). The muscles were exteriorized and pinned out over a Sylgard pedestal and superfused at 5–10 ml/min with a warm, pH 7.4, bicarbonate-buffered physiological salt solution [PSS; composition (in mM): 132 NaCl, 4.7 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 0.03 EDTA, and 20 $NaHCO_3$] that was equilibrated with a gas mixture containing 5% CO_2-95% N_2. The superfusion system allowed the superfusate to be rapidly changed without affecting pH, gas composition, or temperature (34°C) of the superfusion solution. Preparations were viewed with a Leitz Laborlux 12 FS microscope equipped with a 25× long-working distance objective (Zeiss UD 40, NA 0.65). The microscope was coupled to a Sony color video camera, and arterioles in the preparation were observed at a final magnification of ×800 measured at the face of a Sony color video monitor. Continuous records of arteriolar internal diameters

were measured with a video caliper system that was accurate to ±1 μm (15).

Protocol for in vivo experiments. Only the central regions of preparations were observed in order to obviate cut-edge effects (27). Arterioles were selected for observation on the basis of their visibility and the presence of resting tone, and only one arteriole was studied per animal. Resting tone was evaluated by observing the dilation of vessels to topical application of 3–10 μl of 1 mM acetyl-β-methylcholine chloride (methacholine). All vessels reported in this study were either on the surface of the muscle or only one skeletal muscle cell layer below the surface of the cremaster. In a typical experiment, arteriolar diameter was measured and the response to methacholine was determined in normal PSS. After recovery from the methacholine, the superfusate was rapidly switched to one containing either iberiotoxin (100 nM) or tetraethylammonium chloride (TEA; 1 mM), which have been shown to selectively inhibit K_{Ca} channels in other vascular muscle cells (25). The diameter of the arteriole was then measured continuously for up to 30 min after exposure to a blocker.

In some experiments, oxygen-induced arteriolar constriction was assessed before and during exposure to 1 mM TEA by equilibrating the superfusate with 21% O_2-5% CO_2-74% N_2 as described previously (10–12).

Preparation of single arteriolar muscle cells. Single arteriolar muscle cells were enzymatically isolated from second- or third-order cremasteric arterioles as described previously (14) with only minor modifications. Hamsters were euthanized with pentobarbital sodium (>150 mg/kg), and cremaster muscles were removed and placed in 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered PSS (4°C) containing 100 μM Ca^{2+}, 10 μM sodium nitroprusside, and 1 mg/ml bovine serum albumin (BSA, see *Solutions*). Single microvessels were then hand-dissected from these tissues and transferred to 1–2 ml of dissociation solution (see *Solutions*) containing 10 μM sodium nitroprusside for 10 min at room temperature. This solution was then replaced by 1 ml of fresh room temperature solution containing 1.5 mg/ml papain and 1 mg/ml dithioerythritol. The solution was warmed to 37°C and incubated for 35 min with occasional agitation. The papain-containing solution was then replaced with 1 ml of 37°C solution containing 1.5 mg/ml collagenase, 1 mg/ml elastase, and 1 mg/ml soybean trypsin inhibitor. This solution was incubated at 37°C for 12–15 min, after which time the enzyme-containing solution was replaced by 2 ml of room temperature dissociation solution. The vessel fragments were allowed to settle to the bottom of the tube, and most of this wash solution was removed. For single-channel studies, 1–3 ml of room temperature Kraftbrühe (KB) solution (17) was then rapidly added to the tube to dissociate the vessel. If further dissociation was required, the solution in the tube was gently triturated using a 1-ml Eppendorf-style pipette. The cell-containing solution was then placed in six 35-mm plastic culture dishes along with sufficient additional KB solution to make 2 ml and was stored at 4°C for up to 4 h. For contraction studies, cells were isolated as described previously (14).

Single-channel studies. Culture dishes containing arteriolar muscle cells were placed on the stage of a Leitz DMIL inverted microscope equipped with contrast enhancement optics and allowed to warm to room temperature for 10 min. They were then superfused with 20–30 ml of 100 μM Ca^{2+} MOPS-buffered PSS, followed by 10–20 ml of 2 mM Ca^{2+} MOPS-buffered PSS. The cells were then allowed to settle and attach to the bottom of the dishes for 10–20 min. Solutions were delivered by gravity at 1–1.5 ml/min and siphoned through a U-shaped capillary tube positioned to maintain bath volume between 1.5 and 2 ml.

Single-channel experiments were carried out in the inside-out configuration (8). Pipettes were pulled from borosilicate glass tubes (no. 6175, A-M Systems, Everett, WA) and fire polished and showed tip resistances of 4–7 MΩ when filled with pipette solution (see *Solutions*). Seals of 5–20 GΩ were established by application of light suction (8), and inside-out patches were formed by rapidly withdrawing the pipette from the cell.

All experiments were conducted at room temperature (20°C). Currents were acquired using an Axopatch 200A amplifier controlled by a Digidata 1200 data-acquisition system that was interfaced to a 80486 DX-2 66 personal computer running pCLAMP version 6 software (all from Axon Instruments, Foster City, CA). Currents were filtered at 1 kHz with the Axopatch 200 internal four-pole bessel low-pass filter and digitized at 5 kHz. Single-channel records were collected for 15–60 s or until 1,500–2,000 events were recorded per channel in the patch. At Ca^{2+} concentrations ≤3 μM, currents were recorded for 5 min. Only data from patches with three or fewer channels were analyzed and reported.

Single-cell contraction studies. Contraction of single cells was assessed as described previously (14). Cells were placed in a 1-ml chamber mounted on the stage of an inverted microscope and allowed to settle, and the chamber was perfused with bath solution (see *Solutions*). After a 10-min equilibration period, a superfusion pipette (10- to 50-μm tip ID) filled with a norepinephrine-containing solution (1 μM) was positioned adjacent to a cell with a micromanipulator, and fluid was ejected from the pipette onto the cells by pressurizing the back of the pipette with a water manometer. The response of the cell was then monitored through the eyepiece of the microscope at ×100 magnification (×10, NA 0.2 objective). A positive response was defined as a >30% shortening of a cell within 15 s of continuous exposure to norepinephrine as described previously (14). At least 30 cells from several different fields were tested in each aliquot, and the final response was calculated as the percentage of cells responding (14). Three such trials were performed for each isolate of cells: two controls in normal bath solution and one in the presence of 100 nM iberiotoxin. The order of treatment was randomized and the results from the two control trials were pooled for final display of the data.

Solutions. MOPS-buffered PSS contained (in mM) 148 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 0.1 or 2 $CaCl_2$, 0.03 EDTA, 2 MOPS, 5 glucose, and 2 Na pyruvate (pH = 7.4 with NaOH/HCl) at room temperature. Dissociation solution contained (in mM) 137 NaCl, 5.6 KCl, 1 $MgCl_2$, 0.42 Na_2HPO_4, 0.44 NaH_2PO_4, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 4.17 $NaHCO_3$, and 0.1 $CaCl_2$ (pH 7.4). KB solution contained (in mM) 85 KCl, 30 KH_2PO_4, 5 $MgSO_4$, 2 Na_2ATP, 0.2 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 Na pyruvate, 5 succinate, 5 creatine, 20 glucose, and 20 taurine (pH = 7.2) and 10 mg/ml BSA. Bath solution for inside-out patch-clamp studies contained (in mM) 140 KCl, 1 $MgSO_4$, 0.03 Na_2EDTA, 10 NaMOPS, 1 EGTA, 1 *N*-hydroxyethylethylenediaminetriacetic acid, and 1 nitrilotriacetic acid (pH = 7.2 with NaOH). In some experiments 135 mM KCl in this solution was replaced by 135 mM NaCl. $CaCl_2$ was then added from a 1 M stock to yield the appropriate free Ca^{2+} concentrations. This was monitored with a Ca^{2+}-selective macroelectrode (Orion 93200 electrode, Fisher Acumet pH meter) that was calibrated between 10 nM and 1 mM with solutions of known free Ca^{2+} concentrations (32). All Ca^{2+} concentrations reported in the text refer to the concentration of free Ca^{2+}. The pipette

solution for inside-out patch-clamp studies contained (in mM) 100 K gluconate, 40 KCl, 1 $MgCl_2$, 2 MOPS, 1 EGTA, and 0.1 $CaCl_2$ (pH = 7.2 with NaOH). Bath solution for single-cell contraction studies consisted of (in mM) 135 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH = 7.4 with NaOH).

Drugs and chemicals. Iberiotoxin was purchased from Research Biochemicals and BSA from U.S. Biochemicals. All other compounds were purchased from Sigma. Iberiotoxin was dissolved in water to yield a concentration of 100 μM. Aliquots of this solution were then stored at −20°C until used. On the day of an experiment an appropriate amount of this solution was thawed and diluted in the appropriate PSS or pipette solution to yield a final concentration of 100 nM. Solutions of TEA (1 M) were stored frozen and then diluted in the appropriate solution on the day of an experiment. All other solutions were made fresh each day.

Data analysis and statistics. Data are presented as means ± SE or means ± 95% confidence intervals. Curve fitting was performed with SigmaPlot for Windows (Jandel Scientific, San Rafael, CA). All statistical comparisons were performed at the 95% confidence level.

RESULTS

Effects of K_{Ca} channel blockers on resting tone of cremasteric arterioles in vivo. Arterioles in the present study had substantial resting tone (Fig. 1). Topical application of methacholine (3–10 μl of 1 mM solution) caused second-order arterioles to dilate 106 ± 12% from a resting diameter of 39 ± 1 μm (n = 8) and third-order arterioles to increase their diameters by 107 ± 21% from a resting diameter of 19 ± 2 μm (n = 8) (Fig. 1).

Superfusion of cremaster muscles with TEA (1 mM) had no effect on arteriolar diameters in the steady state (Fig. 1). Resting diameters of second-order arterioles were 39 ± 1 μm before exposure to TEA and were 40 ± 2 μm after 6 min of superfusion with this blocker (n = 5, $P < 0.05$) and remained at this level for at least 30 min after exposure to TEA. In third-order arterioles TEA (1 mM) elicited a brief transient constriction in three of the five arterioles studied, such that the mean diameter decreased from 17 ± 2 to 13 ± 2 μm (n = 5, $P < 0.05$) immediately after exposure to TEA. However, within 2 min, diameters returned to control diameters (17 ± 2 μm, $P > 0.05$) and stayed at this level for at least 30 min of exposure to this quaternary ammonium K_{Ca} channel blocker.

Superfusion of cremaster muscles with iberiotoxin (100 nM) also had no effect on diameters of either class of arterioles (Fig. 1). Second-order vessels had diameters of 38 ± 0.3 μm before exposure to this peptide K_{Ca} channel blocker and remained at this diameter (38 ± 1.5 μm) during a 30-min exposure to iberiotoxin (n = 3, $P > 0.05$). Similarly, third-order arterioles had diameters of 24 ± 3 μm before exposure to this peptide and remained at this diameter (24 ± 3 μm) during 30 min of superfusion with the blocker (n = 3, $P > 0.05$).

Effects of K_{Ca} channel blockers on arteriolar muscle cell contraction. To test whether K_{Ca} channels might be involved in the negative feedback regulation of arteriolar muscle cell tone in the presence of a vasoconstrictor, we assessed the effects of 1 mM TEA on oxygen-induced constriction of arterioles in vivo. We found that TEA significantly potentiated the effects of oxygen on these microvessels (Fig. 2*A*). Because of the expense of using iberiotoxin in vivo, we were precluded from assessing its effects on oxygen reactivity. However, we did assess the effects of this peptide K_{Ca} channel blocker on norepinephrine-induced contraction of single arteriolar muscle cells in vitro (Fig. 2*B*). As was observed for TEA and oxygen in vivo, iberiotoxin significantly increased the percentage of single arteriolar muscle cells that contracted when exposed to 1 μM norepinephrine (Fig. 2*B*).

Characterization of K_{Ca} channels in inside-out patches of arteriolar muscle cell membrane. Large-conductance unitary currents were observed in inside-out patches of membrane from hamster cremasteric arteriolar muscle cells that were both Ca^{2+} and voltage dependent (see

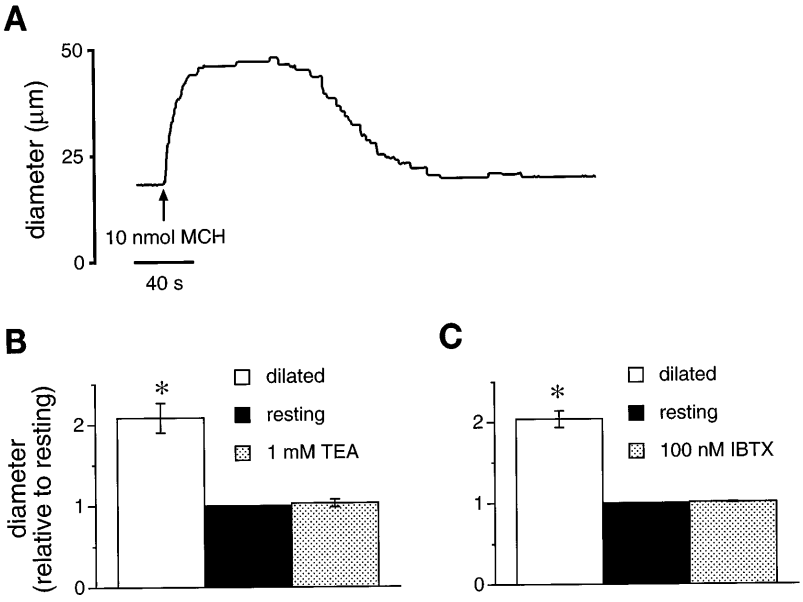

Fig. 1. Lack of effect of tetraethylammonium (TEA) and iberiotoxin (IBTX) on arteriolar diameter in vivo. *A*: digitized diameter record for representative 3rd-order arteriole with resting diameter of 18 μm, demonstrating resting arteriolar tone. At the arrow, a 10-nmol bolus of methacholine (MCH) was added to superfusate, causing arteriole to dilate to 47 μm (161% increase in diameter). The vessel then recovered to near its original diameter. *B* and *C*: data are diameters (±SE) relative to resting state; n = 10 for TEA (*A*) and 6 for IBTX (*B*). Because responses were similar, those for second- and third-order vessels have been pooled together for display purposes. Dilated, maximum diameter observed with application of 3–10 nmol of MCH. * Significantly greater than resting diameter ($P < 0.05$).

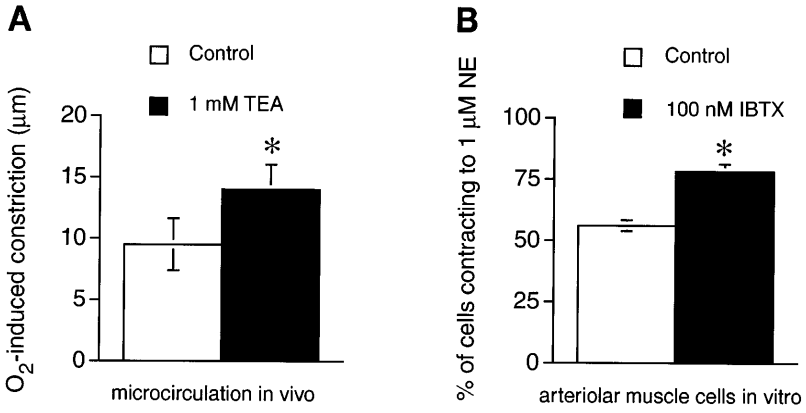

Fig. 2. Ca^{2+}-activated K^+ (K_{Ca}) channels limit vasoconstriction in vivo and in vitro. Data are means ± SE (n = 5) for oxygen-induced constrictions of arterioles in superfused cremaster muscles in vivo (*A*) and for norepinephrine (NE)-induced contraction of single cremasteric arteriolar muscle cells in vitro (*B*). *A*: resting diameters were 19 ± 1 and 19 ± 2 μm for control and TEA-treated vessels, respectively. *B*: cells were isolated from second- and third-order arterioles with diameters ranging from 15 to 50 μm. *Significantly greater than control response ($P < 0.05$).

Figs. 3 and 5). The channels underlying these events appeared to be potassium selective (Fig. 3*B*) and had a single-channel conductance, measured as the slope of the current-voltage relationship between −60 and +60 mV in symmetrical 140 mM K^+, of 240 ± 2 pS (n = 7 patches, Fig. 3*B*). These channels were observed in essentially every patch excised from these cells with ≥2 channels present in every patch.

As a further test of the hypothesis that these large-conductance channels represent K_{Ca} channels described in the other system, we assessed the effects of iberiotoxin, a selective antagonist for K_{Ca} channels (25), on single-channel currents (Fig. 4). To accomplish this we filled the tips of patch pipettes with iberiotoxin-free solution and then back-filled the pipettes with solution containing 100 nM iberiotoxin. This allowed us to initially record channel activity that would then decay with time as iberiotoxin diffused into the tip and interacted with channels in the patch. Inclusion of 100 nM iberiotoxin in the pipette solution completely blocked channel activity after 5 min of recording (Fig. 4). Inhibition of channel activity was also observed when 1 mM TEA was placed in the pipette after a similar protocol (data not shown). No significant run down in channel activity was observed over this time period in control experiments in which these blockers were not added to the pipette solution: channel activity was 96 ± 11% (n = 6) of initial activity after 5 min in experiments in which iberiotoxin was excluded from the pipette solution (Fig. 4).

Ca^{2+} and voltage sensitivity of arteriolar muscle K_{Ca} channels. Figure 5 summarizes the effects of Ca^{2+} and voltage on the activity of K_{Ca} channels in inside-out patches of arteriolar muscle membrane. At negative membrane potentials that spanned the physiological range (−60 to −30 mV), no channel activity was observed during 5-min recordings until the Ca^{2+} concentration bathing the cytosolic side of inside-out patches was increased from 1 to ≥3 μM; at ≤1 μM, no events were detected in 5-min recordings at −60 or −30 in five separate patches. At membrane potentials of −60, −30, +30, and +60 mV, the Ca^{2+} concentrations required for 50% activation (i.e., $NP_o/NP_{omax} = 0.5$, where N is the number of channels in the patch, P_o is the individual channel open probability, NP_o is the channel activity, and NP_{omax} is the maximum NP_o) were 44 ± 3, 20 ± 1, 6 ± 0.4, and 3 ± 0.5 μM, respectively. Hill coefficients were not significantly different among the four membrane potentials examined ($P > 0.05$) and averaged

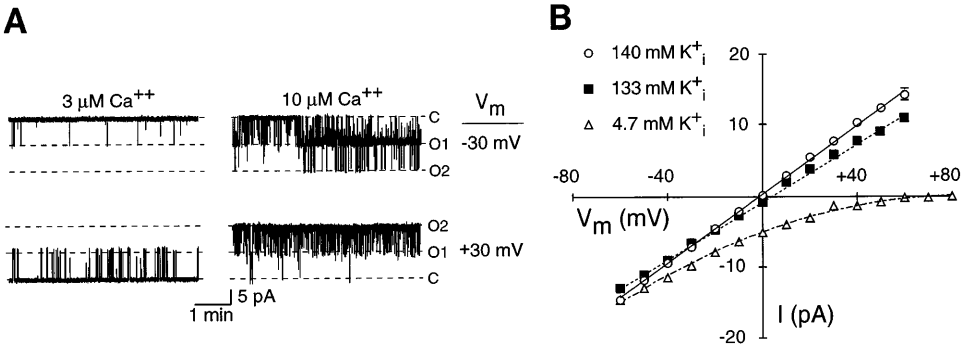

Fig. 3. Voltage- and Ca^{2+}-sensitive K^+ channels in cremasteric arteriolar muscle cell membranes. *A*: digitized current traces of unitary currents from a single membrane patch studied at 2 different membrane potentials (V_m) and 2 different Ca^{2+} concentrations as indicated. Two channels were present in patch. Dashed lines represent state of channels (C, closed; O1, 1 channel open; O2, 2 channels open). *B*: K^+ selectivity of K_{Ca} channels. Data are mean unitary current amplitudes ± SE (n = 4–7; SEs are smaller than symbols) for pipette/bath K^+ gradients [pipette K^+ (K_o) = 140 mM; bath K^+ (K_i) as indicated]. The calculated K^+ equilibrium potentials for K^+ gradients (K_o/K_i) were 0 mV for 140/140 mM K^+, 3.6 mV for 140/133 mM K^+, and 84 mV for 140/4.7 mM K^+. The measured currents in these gradients reversed near their theoretical values. Slope conductances under the 3 conditions were 240 ± 2 pS between −60 and 60 mV for the 140/140 mM K^+ gradient, 202 ± 2 pS between −60 and 60 mV for the 140/133 mM K^+ gradient, and 166 ± 2 pS between −60 and −20 mV for the 140/4.7 mM K^+ gradient.

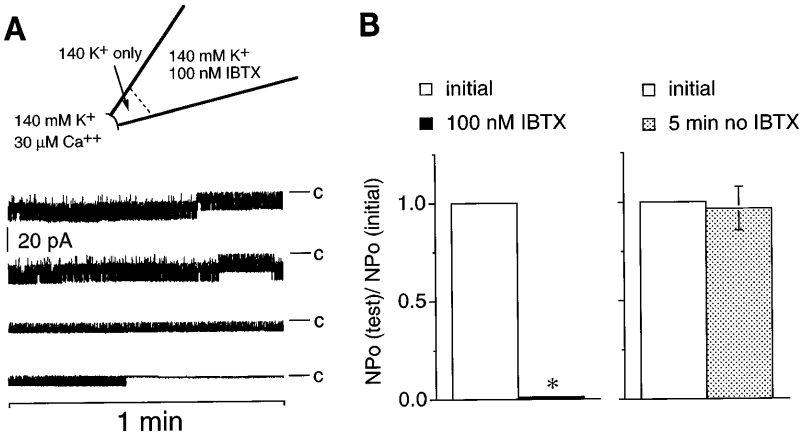

Fig. 4. IBTX inhibits K_{Ca} channels in cremasteric arteriolar muscle cell membranes. *A*: schematic (*top*) of experimental design (see text for more information) and a digitized record from a typical experiment (*bottom*) showing 4 consecutive 1-min sweeps from a patch with 3 channels (C = closed state) held at +30 mV are shown. As IBTX diffused into tip of pipette, channel activity decreased and was abolished by 4 min. *B*: mean data from several patches. Channel activity (NP_o, where N is the number of channels in the patch and P_o is the individual channel open probability) is expressed relative to activity recorded during 1st minute after excision of patch from membrane (initial). Inclusion of 100 nM IBTX in pipette abolished channel activity (*Not significantly different from 0, $P < 0.05$, $n = 5$). When IBTX was not placed in pipette, no run down of channel activity was observed.

1.9 ± 0.2 ($n = 4$), suggesting parallel shifts of the Ca^{2+} response curves induced by changes in voltage.

The data in Fig. 5*A* were fit to the Boltzmann equation, $NP_o/NP_{omax} = 1/[1 + \exp[(V_{½} - V_m)/K]]$, where V_m is the membrane potential, $V_{½}$ is the voltage for half-maximal activation, and K is the logarithmic voltage sensitivity (ΔV required for e-fold increase in activity) (5); the resulting $V_{½}$ values were then plotted against log[Ca^{2+}] as suggested by Carl et al. (5) (Fig. 5*B*), where [Ca^{2+}] is free Ca^{2+} concentration. The voltage sensitivities estimated from the curve fits were similar for all concentrations of Ca^{2+} tested and indicated that channel activity increased e-fold (~2.72 times) for a 16 ± 1 mV ($n = 6$) depolarization. As can be seen in Fig. 5*B*, the semilog plot of Ca^{2+} vs. $V_{½}$ yielded a linear relationship [slope = −84 ± 5 mV/log[Ca^{2+}] (in M), intercept = −423 ± 26 mV, $r^2 = 0.9843$, $P < 0.05$]. From the equation for the line fit through these data we estimated that $\Delta V_{½}$, the change in $V_{½}$ for a 10-fold change in Ca^{2+}, was 84 ± 5 mV and that the Ca^{2+}-axis intercept [Ca_0; the Ca^{2+} set point (5)] was ~9 μM.

DISCUSSION

K_{Ca} channels have been proposed to play a substantial role in the regulation of membrane potential and hence tone of vascular smooth muscle cells in arteries that display myogenic tone (4, 23, 25). Our studies suggest that, under resting conditions, this may not be the case in the microcirculation of skeletal muscle. We found that two blockers of K_{Ca} channels, TEA and iberiotoxin, at concentrations that inhibited the activity of these channels in patch-clamp experiments in cells from cremasteric arterioles as well as other types of vascular muscle (4, 23, 25), had either no effect (iberiotoxin) or only a transient effect (TEA) on the resting diameters of arterioles in cremaster muscles in vivo. These data suggest that K_{Ca} channels are not very active under native conditions in the cremasteric microcirculation, despite the fact that these arterioles display substantial resting tone. Similar results have been reported in the cerebral (26) and cremasteric (18) microcirculations of rats. These observations are in direct contrast to findings in small, myogenically active arteries studied in vitro, where inhibition of K_{Ca} channels by either TEA or iberiotoxin depolarized and constricted these vessels (4, 23, 25). Our patch-clamp studies provide an initial explanation for these observations. We propose that the K_{Ca} channels found in cremasteric arteriolar muscle cells have a high Ca^{2+} set point under resting conditions such that they are not active at normal resting membrane potentials.

In inside-out patches of cremasteric arteriolar muscle membrane, we found that internal Ca^{2+} concentrations ≥3 μM were required to observe unitary K_{Ca} channel

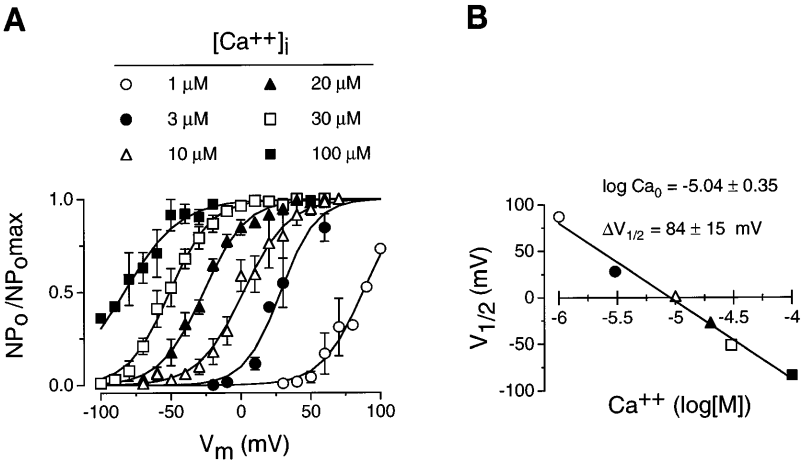

Fig. 5. Voltage and Ca^{2+} sensitivity of K_{Ca} channels in cremasteric arteriolar muscle cell membranes. *A*: voltage activation curves for indicated intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). Data are means ± SE of channel activities (NP_o; $n = 5–10$) expressed relative to maximum channel activity (NP_{omax}) observed. Solid lines, best fit curves to Boltzmann equation, $NP_o/NP_{omax} = 1/[1 + \exp[(V_{½} - V_m)/K]]$, where $V_{½}$ is the V_m required for half-maximal activation of the channels and K is the logarithmic voltage sensitivity (ΔV required for e-fold increase in activity). *B*: estimation of change in $V_{½}$ for a 10-fold change in [Ca^{2+}] ($\Delta V_{½}$) and estimation of Ca^{2+} axis-intercept (Ca_0; Ca^{2+} set point) for cremasteric arteriolar muscle K_{Ca} channels. $V_{½}$ values were estimated from data in *A*. Analysis of variance indicated a significant regression ($P < 0.05$). From regression analysis, we then estimated $\Delta V_{½}$ (equal to absolute value of slope of the line), logCa_0, and their 95% confidence intervals as shown. See text for more information.

current between −60 and −30 mV, voltages that span the normal range of membrane potential observed in cremasteric arteriolar muscle cells in vivo (30, 31) and in vitro (14). Consistent with these findings, we previously observed that iberiotoxin had no effect on macroscopic K^+ currents elicited between −90 and 0 mV in cremasteric arteriolar muscle cells studied using the whole cell perforated-patch technique (14) or using conventional whole cell methods with cells dialyzed with pipette solutions containing Ca^{2+} concentrations as high as 300 nM (13).

The lack of activity of K_{Ca} channels in arteriolar smooth muscle cells at negative membrane potentials could be caused by a low voltage sensitivity, a low Ca^{2+} sensitivity, or a high Ca^{2+} set point (5). We found that a 16-mV depolarization was required to produce an *e*-fold increase in channel activity in cremasteric arteriolar muscle cells. This voltage sensitivity is well within the range of values estimated in other smooth muscle cells [*e*-fold increase per 10–20 mV (5, 25)]. Thus arteriolar muscle cells do not appear to display a low voltage sensitivity.

We assessed the Ca^{2+} sensitivity of K_{Ca} channels in inside-out patches of cremasteric arteriolar muscle cell membranes by estimating $\Delta V_{‰}$, as suggested by Carl et al. (5). We obtained a value of 84 ± 5 mV from our analysis. This value is also similar to $\Delta V_{‰}$ values observed in other smooth muscles (5) and suggests that the Ca^{2+} sensitivity of these channels is not low. The average Hill coefficient that we measured (1.9 ± 0.2) also was similar to values reported in earlier studies of other smooth muscles (1, 3, 5, 9, 20), further supporting this conclusion.

On the other hand, the Ca_0 value [the Ca^{2+} set point (5)] was found to be 9 μM. Previous studies of K_{Ca} channels in myocytes isolated from guinea pig mesenteric arteries, rat pulmonary arteries, and rabbit portal veins have found Ca_0 to be 0.5 μM (3), 1.5 μM (1), and 1 μM (9), respectively, values consistent with the Ca_0 estimated in a variety of smooth muscles (5, 20). Our estimate of the Ca^{2+} set point is 6- to 18-fold higher than that observed in myocytes from larger vessels supplying other tissues. This implies that subsarcolemmal Ca^{2+} concentrations must be 6- to 18-fold greater in arteriolar muscle cells to initiate channel activity compared with muscle cells in larger vessels, which is consistent with our observations that channel activity is not observed at physiological membrane potentials for Ca^{2+} concentrations < 3 μM and that blockade of K_{Ca} channels in vivo has little effect on resting arteriolar diameter.

The mechanisms responsible for the high Ca^{2+} set point that we observed remain to be established. We do not think that our method for isolation of the cells, which included the use of sodium nitroprusside, somehow elevated the Ca_0 in these cells. We have observed similar behavior in hamster cells isolated using diltiazem instead of sodium nitroprusside (Jackson, unpublished observations) and in rat cremasteric arteriolar muscle cells isolated in the absence of any dilator (W. F. Jackson and N. J. Rusch, unpublished observations). Thus it is unlikely that our data can be explained by simple methodological differences.

We think that it is more likely that cremasteric arteriolar muscle cells express K_{Ca} channels that inherently have an elevated Ca_0. Recent molecular studies suggest that variations in expression of both the α- and β-subunits, which are thought to make up these channels, can influence the response of the channels to changes in Ca^{2+} (19). For example, expression of the β-subunit along with the α-subunit for the human vascular smooth muscle K_{Ca} channels reduces the Ca_0 value for unitary currents from 30 μM (α-subunit alone) to 5 μM (α- + β-subunits) (estimated from data in Ref. 19). Tissue-specific expression of spliced variants of these two subunits could explain the differences in Ca_0 values that have been reported. It is also possible that the high Ca_0 results from stable covalent modification of the channel proteins (i.e., stable phosphorylation). This should prove to be fertile ground for future investigations.

We propose that this high Ca^{2+} set point is responsible for the apparent lack of activity of K_{Ca} channels that we observed in resting arterioles. $[Ca^{2+}]_i$ in rat cremasteric arteriolar muscle cells in vitro measured in cannulated pressurized vessels with substantial myogenic tone has been estimated to be <100 nM (21), although this value may underestimate the near-membrane Ca^{2+} concentration to which the channels are exposed (7, 23). Focal release of Ca^{2+} from the sarcoplasmic reticulum (Ca^{2+} "sparks") have been suggested to elevate local Ca^{2+} concentrations to ~300 nM (23). Even if the peak concentration of Ca^{2+} sparks has been underestimated (7), it seems unlikely that concentrations substantially greater than 1 μM would be routinely observed near the membrane of the arteriolar muscle cells except during conditions when Ca^{2+} release from the sarcoplasmic reticulum is increased or influx of Ca^{2+} through Ca^{2+} channels is augmented. However, a final conclusion will require the simultaneous measurement of membrane potential, near-membrane Ca^{2+} concentrations, and channel activity in the living microcirculation.

We did observe transient effects of TEA on third-order arterioles in vivo. This may be interpreted as evidence in support of the hypothesis that there is some K_{Ca} channel activity in the microcirculation that contributes to resting arteriolar tone in this tissue. However, the lack of effect of iberiotoxin argues against this conclusion. Instead, we suggest that the diameter responses observed in the presence of TEA may have resulted from some other effect, such as nonselective blockade of another K^+ channel type. In support of this hypothesis, Kleppisch and Nelson (16) have shown that 1 mM TEA is not selective for K_{Ca} channels but may also inhibit ATP-sensitive K^+ channels in some arterial muscle cells. Thus TEA may not be a completely selective blocker of K_{Ca} channels, which may explain its transient effects observed in vivo.

The apparent lack of activity of K_{Ca} channels in vivo and whole cell experiments in vitro (13, 14) does not mean that these channels do not play a role in the

regulation of arteriolar tone in the microcirculation. On the contrary, we found that iberiotoxin (100 nM) in vitro and TEA (1 mM) in vivo significantly potentiated norepinephrine-induced contraction of single muscle cells and oxygen-induced arteriolar constriction, respectively. Therefore K_{Ca} channels may participate in the negative feedback regulation of arteriolar muscle cell contraction during activation by vasoconstrictors. This most likely occurs because of an increase in Ca^{2+} influx and release and membrane depolarization produced during vasoconstriction. In addition, recent studies have provided evidence that these channels may be activated by vasodilators that act through the adenosine 3′,5′-cyclic monophosphate or guanosine 3′,5′-cyclic monophosphate second messenger systems (26, 28). Thus, although not active under resting conditions, K_{Ca} channels may be recruited and participate in the regulation of arteriolar tone during vasoconstriction and may be activated to induce vasodilation. Hence, these channels may still play a role in the local, neural, and hormonal regulation of blood flow in the microcirculation.

In summary, we propose that the K_{Ca} channels expressed in cremasteric arteriolar muscle cells have a high Ca^{2+} set point that renders them silent under resting conditions even in arterioles with substantial resting tone. Our observations are in conflict with a number of in vitro experiments on cannulated arteries that display myogenic tone (see Ref. 25), and this points to the necessity of examining the physiological function and characteristics of ion channels in the microcirculation. Future studies should be directed toward examining the molecular expression of K_{Ca} channels in arterioles to determine if the high Ca^{2+} set point that we observed reflects expression of unique channel subunits or some other modification of the channels.

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REFERENCES

1. **Albarwani, S., B. E. Robertson, P. C. G. Nye, and R. Z. Kozlowski.** Biophysical properties of Ca^{2+}- and Mg-ATP-activated K^+ channels in pulmonary arterial smooth muscle cells isolated from the rat. *Pflügers Arch*. 428: 446–454, 1994.
2. **Baez, S.** An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc. Res*. 5: 384–394, 1973.
3. **Benham, C. D., T. B. Bolton, R. J. Lang, and T. Takewaki.** Calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea-pig mesenteric artery. *J. Physiol. (Lond.)* 371: 45–67, 1986.
4. **Brayden, J. E., and M. T. Nelson.** Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* 256: 532–535, 1992.
5. **Carl, A., H. K. Lee, and K. M. Sanders.** Regulation of ion channels in smooth muscles by calcium. *Am. J. Physiol*. 271 (*Cell Physiol*. 40): C9–C34, 1996.
6. **Fay, F. S.** Calcium sparks in vascular smooth muscle: relaxation regulators. *Science* 270: 588–589, 1995.
7. **Guerrero, A., J. J. Singer, and F. S. Fay.** Simultaneous measurement of Ca^{2+} release and influx into smooth muscle cells in response to caffeine. A novel approach for calculating the fraction of current carried by calcium. *J. Gen. Physiol*. 104: 395–422, 1994.
8. **Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth.** Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch*. 391: 85–100, 1981.
9. **Inoue, R., K. Kitamura, and H. Kuriyama.** Two Ca-dependent K-channels classified by the application of tetraethylammonium distribute to smooth muscle membranes of the rabbit portal vein. *Pflügers Arch*. 405: 173–179, 1985.
10. **Jackson, W. F.** Prostaglandins do not mediate arteriolar oxygen reactivity. *Am. J. Physiol*. 250 (*Heart Circ. Physiol*. 19): H1102–H1108, 1986.
11. **Jackson, W. F.** Arteriolar tone is determined by activity of ATP-sensitive potassium channels. *Am. J. Physiol*. 265 (*Heart Circ. Physiol*. 34): H1797–H1803, 1993.
12. **Jackson, W. F.** Regional differences in mechanism of action of oxygen on hamster arterioles. *Am. J. Physiol*. 265 (*Heart Circ. Physiol*. 34): H599–H603, 1993.
13. **Jackson, W. F., K. L. Blair, and N. J. Rusch.** Large-conductance, Ca^{2+}-activated K^+ channels in arteriolar muscle cells have low Ca^{2+}-sensitivity (Abstract). *Microcirculation* 3: 92, 1996.
14. **Jackson, W. F., J. M. Huebner, and N. J. Rusch.** Enzymatic isolation and characterization of single vascular smooth muscle cells from cremasteric arterioles. *Microcirculation* 4: 35–50, 1977.
15. **Jackson, W. F., and J. R. Searle.** An affordable video caliper (Abstract). *FASEB J*. 3: A1390, 1989.
16. **Kleppisch, T., and M. T. Nelson.** ATP-sensitive K^+ currents in cerebral arterial smooth muscle: pharmacological and hormonal modulation. *Am. J. Physiol*. 269 (*Heart Circ. Physiol*. 38): H1634–H1640, 1995.
17. **Klöckner, U., and G. Isenberg.** Endothelin depolarizes myocytes from porcine coronary and human mesenteric arteries through a Ca-activated chloride current. *Pflügers Arch*. 418: 168–175, 1991.
18. **Loeb, A. L., I. Gödény, and D. E. Longnecker.** Functional evidence for inward-rectifier potassium channels in rat cremaster muscle arterioles in vivo (Abstract). *Microcirculation* 4: 160, 1997.
19. **McCobb, D. P., N. L. Fowler, T. Featherstone, C. J. Lingle, M. Saito, J. E. Krause, and L. Salkoff.** A human calcium-activated potassium channel gene expressed in vascular smooth muscle. *Am. J. Physiol*. 269 (*Heart Circ. Physiol*. 38): H767–H777, 1995.
20. **McManus, O. B.** Calcium-activated potassium channels: regulation by calcium. *J. Bioenerg. Biomembr*. 23: 537–560, 1991.
21. **Meininger, G. A., D. C. Zawieja, J. C. Falcone, M. A. Hill, and J. P. Davey.** Calcium measurement in isolated arterioles during myogenic and agonist stimulation. *Am. J. Physiol*. 261 (*Heart Circ. Physiol*. 30): H950–H959, 1991.
22. **Nelson, M. T.** Regulation of arterial tone by potassium channels. *Jpn. J. Pharmacol*. 58, *Suppl*. 2: 238P–242P, 1992.
23. **Nelson, M. T., H. Cheng, M. Rubart, L. F. Santana, A. D. Bonev, H. J. Knot, and W. J. Lederer.** Relaxation of arterial smooth muscle by calcium sparks. *Science* 270: 633–637, 1995.
24. **Nelson, M. T., J. B. Patlak, J. F. Worley, and N. B. Standen.** Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol*. 259 (*Cell Physiol*. 28): C3–C18, 1990.
25. **Nelson, M. T., and J. M. Quayle.** Physiological roles and properties of potassium channels in arterial smooth muscle. *Am. J. Physiol*. 268 (*Cell Physiol*. 37): C799–C822, 1995.
26. **Paterno, R., F. M. Faraci, and D. D. Heistad.** Role of Ca^{2+}-dependent K^+ channels in cerebral vasodilatation induced

by increases in cyclic GMP and cyclic AMP in the rat. *Stroke* 27: 1603–1607, 1996.
27. **Proctor, K. G., and D. W. Busija.** Relationships among arteriolar, regional, and whole organ blood flow in cremaster muscle. *Am. J. Physiol.* 249 (*Heart Circ. Physiol.* 18): H34–H41, 1985.
28. **Robertson, B. E., R. Schubert, J. Hescheler, and M. T. Nelson.** cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am. J. Physiol.* 265 (*Cell Physiol.* 34): C299–C303, 1993.
29. **Stehno-Bittel, L., and M. Sturek.** Spontaneous sarcoplasmic reticulum calcium release and extrusion from bovine, not porcine, coronary artery smooth muscle. *J. Physiol. (Lond.)* 451: 49–78, 1992.
30. **Stekiel, W. J., S. J. Contney, and J. H. Lombard.** Sympathetic neural control of vascular muscle in reduced renal mass hypertension. *Hypertension* 17: 1185–1191, 1991.
31. **Stekiel, W. J., S. J. Contney, and N. J. Rusch.** Altered β-receptor control of in situ membrane potential in hypertensive rats. *Hypertension* 21: 1005–1009, 1993.
32. **Tsien, R. Y., and T. J. Rink.** Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochim. Biophys. Acta* 599: 623–638, 1980.