Aging increases capacitance and spontaneous transient outward current amplitude of smooth muscle cells from murine superior epigastric arteries

Sebastien Hayoz, Vanessa Bradley, Erika M. Boerman, Zahra Nourian, Steven S. Segal, and William F. Jackson

1Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan; 2College of Veterinary Medicine, Michigan State University, East Lansing, Michigan; 3Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, Missouri; and 4Dalton Cardiovascular Research Center, Columbia, Missouri

Submitted 18 June 2013; accepted in final form 28 March 2014

Hayoz S, Bradley V, Boerman EM, Nourian Z, Segal SS, Jackson MF. Aging increases capacitance and spontaneous transient outward currents of smooth muscle cells from murine superior epigastric arteries. Am J Physiol Heart Circ Physiol 306: H1512–H1524, 2014. First published April 4, 2014; doi:10.1152/ajpheart.00492.2013. —Large conductance Ca$^{2+}$-activated K$^+$ channels (BK$_{Ca}$) contribute to negative feedback regulation of smooth muscle cell (SMC) tone. However, the effects of aging on BK$_{Ca}$ function are unclear. We tested the hypothesis that aging alters SMC BK$_{Ca}$ function in superior epigastric arteries (SEAs) by using perforated patch recording of enzymatically isolated SMCs from 3- to 4-mo-old male C57BL/6 mice (Young) and 24- to 26-mo-old male C57BL/6 mice (Old). SMC capacitance from Young (15.7 ± 0.4 pF; n = 110) was less than Old (17.9 ± 0.5 pF; n = 104) (P < 0.05). SMCs displayed spontaneous transient outward currents (STOCs) at membrane potentials more positive than −30 mV; depolarization increased STOC amplitude and frequency (P < 0.05; n = 19–24). STOC frequency in Young (2.2 ± 0.6 Hz) was less than Old (4.2 ± 0.7 Hz) at −10 mV (P < 0.05; n = 27–30), with no difference in amplitude (1.0 ± 0.1 vs. 0.9 ± 0.1 pA/pF, respectively). At +30 mV, STOC amplitude in Young (3.2 ± 0.3 pA/pF) was less than Old (5.0 ± 0.5 pA/pF; P < 0.05, n = 61–67) with no difference in frequency (3.9 ± 0.4 vs. 3.2 ± 0.3 Hz, respectively). BK$_{Ca}$ blockers (1 μM paxilline, 100 nM iberiotoxin, 1 mM tetraethylammonium) or a ryanodine receptor antagonist (100 μM tetracaine) inhibited STOCs (n ≥ 6; P < 0.05 each). Western blots revealed increased expression of BK$_{Ca}$ α-subunit protein in Old. Pressure myography revealed no effect of age on SEA maximal diameter, myogenic tone, or paxilline-induced constriction (n = 10–12; P > 0.05). Enhanced functional expression of SMC BK$_{Ca}$, dependent STOCs in Old may represent an adaptation of resistance arteries to maintain functional integrity.

aging; resistance arteries; potassium channels; vascular smooth muscle; myogenic tone

LARGE CONDUCTANCE CALCIUM -activated K$^+$ channels (BK$_{Ca}$) contribute to the negative feedback regulation of vascular smooth muscle tone (37). BK$_{Ca}$ are activated by membrane depolarization and increases in subsarcolemmal Ca$^{2+}$ (36, 37), and limit smooth muscle contraction elicited by vasoconstrictors or increases in transmural pressure by attenuating depolarization (20, 37). However, the impact of aging on the function and expression of these ion channels in vascular smooth muscle cells (SMCs) of resistance microvessels remains unclear.

Three different patterns of aging-related changes in expression and function of BK$_{Ca}$ have appeared in the literature. Studies of SMCs from rat coronary arteries have consistently shown a decrease in both the expression and function of BK$_{Ca}$ with aging (1, 31, 38, 46). In contrast, despite an age-associated decrease in the expression of BK$_{Ca}$ α-subunits, BK$_{Ca}$ function, as measured by pressure myography, appeared to be enhanced in arterioles from soleus muscle or unchanged in arterioles from gastrocnemius muscle in aged rats (26). Finally, in rat cerebral arteries, BK$_{Ca}$ expression and function were unaffected by aging (39). Collectively, these data suggest that there are regional differences in the effect of aging on BK$_{Ca}$ expression and function.

In skeletal muscle, blood flow is controlled by changes in tone of both feed arteries and arteriolar networks that they supply (45). However, as shown in resistance networks of the mouse and hamster cremaster muscles, the regulation of BK$_{Ca}$ in arterioles differs from that in feed arteries (49, 50), and it is unknown how aging affects BK$_{Ca}$ function in SMCs of skeletal muscle feed arteries. A recent study of endothelial tubes freshly isolated from murine superior epigastric arteries (SEAs) suggests that aging increased the function of endothelial K$_{Ca}$ (4). Therefore, in the present study, we tested the hypothesis that aging would alter BK$_{Ca}$ expression and/or function in SMCs of SEAs, feed arteries that supply skeletal muscles of the anterior abdominal wall, by using patch clamp electrophysiology on freshly isolated SMCs and pressure myography of isolated SEAs. Our results from SEA SMCs of male C57BL/6 mice indicate that 1) aging increases mean whole-cell current and membrane conductance due to increased membrane capacitance, 2) SMCs display BK$_{Ca}$ and ryanodine receptor (RyR)-dependent spontaneous transient outward currents (STOCs), 3) aging increases the amplitude of STOCs at positive membrane potentials while shifting the voltage-frequency relationship to more negative potentials, 4) aging is associated with an increase in the expression of the BK$_{Ca}$ α-subunit protein, and 5) irrespective of age, the activity of BK$_{Ca}$ appears solely as STOCs. Our data show further that although BK$_{Ca}$ contribute negative-feedback regulation of myogenic tone in SEAs, aging has little effect on their integrated function.

METHODS

Solutions. Physiological salt solution (PSS) contained (in mM) 140 NaCl, 5 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose [pH 7.4, 295 mosmol (kg/H$_2$O)]. Ca$^{2+}$-free physiological salt solution (Ca$^{2+}$-free PSS) contained (in mM) 140 NaCl, 5 KCl, 1 MgCl$_2$, 10 HEPES, and 10 glucose [pH 7.4, 295 mosmol (kg/H$_2$O)]. Dissection solution consisted of Ca$^{2+}$-free PSS containing 10 μM sodium nitroprusside, 10 μM diltiazem, and 1% bovine serum albumin (BSA; Affymetrix: Solutions.
USB, Cleveland, OH). Pipette solution contained (in mM) 100 K-aspartate, 43 KCl, 1 MgCl2, 10 HEPES, 1 EGTA, and 10 glucose [pH 7, 295 mosmol (kg/H2O)]. Stock solution of amphotericin B was made daily at a concentration of 3 mg/50 µl DMSO, and 2 to 3 µl of this stock solution were added to 1 ml of pipette solution to allow whole-cell current recording in the perforated-patch configuration (see Whole-cell current recording).

Animal care and use and microdissection. All experiments were approved by, and conducted in accordance with the guidelines of, the Institutional Animal Care and Use Committees at Michigan State University and the University of Missouri and were performed in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals (2011). Mice were housed on a 12:12-h light/dark cycle at ~23°C with fresh water and food available ad libitum. Male C57BL/6 mice (20–30 g; National Institute on Aging colonies at Charles River Laboratories, Wilmington, MA) were studied at 3 to 4 mo (Young) and 24 to 26 mo (Old). For patch clamp and pressure myography experiments, mice were euthanized by CO2 asphyxiation followed by cervical dislocation. After the abdomen was shaved, a ventral midline incision was made in the skin from the sternum to the pubis to expose the entire abdominal wall and the proximal ends of the SEAs. The abdominal wall containing SEAs was removed and placed in 4°C dissection solution. The inclusion of sodium nitroprusside and diltiazem in this solution helped to maintain SMCs in a relaxed state during dissection and subsequent enzymatic isolation; their actions are reversible and without subsequent effect on ionic currents and vascular reactivity (23). Individual SEAs were hand-dissected while viewing through a stereomicroscope as described (49), cleaned of connective tissue and fat, and cut into segments 400–800 µm long.

For RT-PCR measurements and Western blots, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and SEAs were isolated as described above. The anesthetized mice were euthanized by intracardiac injection of an overdose of pentobarbital followed by cervical dislocation.

Enzymatic isolation of SMCs for patch clamp recording. SMCs were enzymatically isolated as described (23). Identical protocols were used to isolate SMCs from SEAs removed from Young and Old. SEA segments were pooled into 1 ml of dissociation solution (see above) with which dissociated cells were suspended. SMCs were dissociated using 1.5 mg/ml collagenase, 93 Units/ml elastase (EMD Millipore: Calbiochem, San Diego, CA), and 1 mg/ml presynaptic and postsynaptic effects. Whole-cell current recording. Whole-cell current recording was interfaced to a personal computer running pCLAMP version 10.2 software (all from Molecular Devices, Sunnyvale, CA). Cell capacitance was determined by application of 10 mV depolarizing pulses from a holding potential of −60 mV using the membrane test utility in pClamp 10.2. For determination of current (I-voltage (V) relationships, cells were held at −60 mV and then stepped to test potentials from −90 to +60 mV in 10-mV increments for 400 ms at each potential. Currents elicited by these voltage steps were filtered at 5 kHz, sampled at 5 kHz, and digitized. The currents recorded during the last 200 ms of the steps were averaged, normalized to cell capacitance, and used for comparison of I-V relationships and assessment of experimental interventions. Blocker-sensitive current densities (in pA/pF) were obtained by subtraction of current densities in the presence of a blocker from their respective control current densities.

Vessel cannulation and pressure myography. Isolated SEAs were cannulated at room temperature with glass micropipettes and tied in place with 11-0 nylon suture. The vessels were tested for leaks by pressurization to 80 cmH2O with no flow through the lumen while immersed in Dissection Solution to obtain maximal diameter and adjust their length to approximate that in situ. If the vessels were leak-free, then transmural pressure was reduced to 20 cmH2O and they were superfused with Ca2+-containing PSS, warmed to 37°C and internal diameter measured (9, 22, 49, 50). Only leak-free vessels were studied; no other selection criteria were applied. After a 30-min equilibration period, pressure was increased to 80 cmH2O. Vessels were allowed to develop myogenic tone, and the responses to phenylephrine (10 µM) and papillamine (1 µM) were assessed. At the end of experiments, vessels were superfused with Ca2+-free PSS and cooled to room temperature to define maximal passive diameters.

Real-time PCR. SMCs used for real-time (RT)-PCR were isolated as described (16). Briefly, freshly dissected SEAs were cannulated with glass micropipettes and blood was flushed from their lumens with dissection solution. Blood-free segments (1 to 2 mm) were then incubated in 1 ml of dissociation solution containing 0.62 mg/ml papain, 1.5 mg/ml collagenase, and 1 mg/ml dithioerythritol and incubated for 30 min at 34°C. Vessel segments were then transferred to enzyme-free dissociation solution at room temperature, and SMCs isolated by gentle trituration of the segments through the heat-polished tip of a micropipette (ID ~100–120 µm). Approximately 500–1000 SMCs were aspirated into the micropipette and transferred to a vial containing 200 µl lysis buffer (RNAaqueous Micro Kit; Ambion, Austin, TX). Total RNA was extracted using the RNAqueous Micro Kit following the manufacturer’s instructions. RNA integrity was verified electrophoretically by SYBR safe gel staining (Invitrogen, Carlsbad, CA) and by OD 260 nm/OD 280 nm absorption ratio >1.95 using a Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE). Reverse transcription was performed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), along with RNase-Free DNase Set (5Prime, Gaithersburg, PA) following the instructions. Taqman primer assays specific to mouse Glucuronidase-β (Gusb; NM_010368.1, Mm00446953_m1), BKCa α-subunit (BKCa; NM_010610.2, Mm00516078_m1), and BKCa β1-subunit (BKβ1; NM_031169.4, Mm00446621_m1) were purchased from Applied Biosystems (Foster City, CA). Duplicate cDNA (2.5 ng) samples were used as templates to perform RT-PCR for 40 cycles on an Applied Biosystems 7900 Fast Real Time PCR System, and the results were averaged. Taqman Fast Advanced Master Mix (Applied Biosystems) was used under the following temperature conditions as set by the manufacturer: 50°C for 2 min, 95°C for 20 s, 95°C for 1 s, and 60°C for 20 s. A minus RT reaction was also performed to ensure no genomic DNA contamination. Linearity and efficiency (E) of amplification for each gene product were verified by creating standard curves, plotting the critical threshold (CT) versus log of the dilution of cDNA. Efficiency-normalized relative abundance (40) was determined for BKα and BKβ using Gusb as the reference transcript and was computed as (E_{Gusb,CT}/E_{target,CT}) as described (49).
Western blot analysis of BKCa α-subunit protein expression. For protein extraction, bilateral SEAs from an individual mouse were dissected, flushed to remove residual blood, and pooled. Each pair of arteries was homogenized using a precooled pestle and mortar in 25 μl radioimmunoprecipitation assay solution plus 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Each homogenate was transferred to a precooled microcentrifuge tube, incubated on ice for 30 min, and then sonicated for 45 s. Finally, cellular debris was removed by centrifugation at 8,000 g for 5 min at 4°C. The supernatant was removed from each sample and used to determine the total protein concentration using the BCA protein Assay kit (Thermo Scientific) and a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Each sample was normalized to the calibrator and a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, IL). Equal amounts of total protein (2 μg) were dissolved in freshly prepared 2X Laemmli buffer containing β-mercaptoethanol (5% vol/vol) and left at room temperature for 20 min.

To evaluate the relative expression of BKCa α-subunits in SEAs from Old vs. Young, protein extracts were separated by SDS-PAGE on 10% TGX Precast Gels (Bio-Rad, Hercules, CA) and then transferred onto polyvinylidene difluoride membranes overnight at 4°C in an electric field of 25 volts. Each gel/blot contained samples from 2 to 3 Young and 2 to 3 Old along with an arbitrary SEA sample from an aged mouse (calibrator) that was run on each gel/blot to account for variability between experiments. The blots were probed with a mouse anti-BKCa antibody (clone L6/60, 1:500; NeuroMab, Davis, CA) and a mouse monoclonal anti-α smooth muscle actin primary antibody (Sigma-Aldrich). The membranes were incubated overnight at 4°C in TBST (in mM) of 137 NaCl, 3 KCl, 20 Tris·HCl, and 0.1% TWEEN 20 containing the primary antibodies. Finally, specific binding was visualized using anti-mouse IgG (whole molecule)-peroxidase conjugated secondary antibody (1:5,000; Sigma-Aldrich). Bound antibodies for α-BKCa were detected using SuperSignal West Dura ECL Chemiluminescent Substrate (34075; Thermo Scientific), and SuperSignal West Pico ECL Chemiluminescent Substrate (34080; Thermo Scientific) was used for α-actin. Images were collected using a ChemiDoc XRS+ System (Bio-Rad) and analyzed by Image Lab software (Bio-Rad). The ratio of band intensities for α-BKCa to α-actin for each sample was normalized to the α-BKCa-to-α-actin intensity ratio for the calibrator included in each experiment.

Drugs and chemicals. All compounds were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. Other than amphoterin (see Solutions, above) and pxaxilline, drugs were dissolved in distilled water as concentrated stock solutions and were frozen at −20°C until used. Paxilline was dissolved in DMSO. On the day of experiments, stock solutions were thawed and then added to PSS to yield the concentrations indicated in the text.

Data analysis and statistics. Summary data are expressed as means ± SE. Statistical significance was determined using Student’s paired and unpaired t-tests, Welch’s corrected t-tests to account for differences in sample variances, or two-way analyses of variance followed by Bonferroni t-tests for post hoc comparisons of means using Prism Software 5.0 (Graphpad Software; San Diego, CA). Prism 5.0 also was used to perform linear and nonlinear regression analyses and to statistically compare fitted lines and curves. The number of observations refers to the number of SMCs or SEA preparations studied from at least 3 animals, with no more than 4 cells studied from any one animal. A value of P < 0.05 was considered statistically significant.

RESULTS

Aging increases mean whole-cell currents and cell capacitance. SMCs displayed large (>1 nA at +60 mV), outwardly rectified, whole-cell currents (Fig. 1). The majority of cells studied in both Young (79%, n = 102 cells from 42 mice) and Old (84%, n = 93 cells from 39 mice; P > 0.05 vs. Young) displayed noisy currents (Fig. 1A). The remaining cells displayed currents as shown in Fig. 1B. Thus the presence or absence of “noisy” outward currents was independent of age. However, outward currents recorded at positive membrane potentials (0 to +60 mV) in SMCs from Old were significantly larger than those recorded in SMCs from Young (Fig. 1C). The magnitude of inward currents at negative membrane potentials (−90 to −30 mV) also was significantly greater in Old versus Young (Fig. 1D; P < 0.05). Whole-cell conductance, computed from the linear portion of the I–V relationship between −90 and −30 mV, was significantly greater in Old (317 ± 11
STOCs were present in SEA SMCs. We hypothesized that the noisy variation in outward currents observed in recordings from most SMCs (Fig. 1A) was due to the presence of STOCs (5, 36). Therefore, we acquired gap-free recordings (2–5 min, 5-kHz sampling frequency) of whole-cell currents from SMCs clamped at designated membrane potentials between −30 and +30 mV in 10-mV increments, with the order randomized between cells. Figure 2A shows typical recordings of STOCs in SMCs from Young and Old. As expected, the amplitude of STOCs increased with depolarization, and STOC occurrence increased in frequency at more positive membrane potentials in SMCs from both Young and Old (Fig. 2). The amplitude of STOCs was greater in Old such that the slope of the relationship between voltage and STOC amplitude was significantly greater in Old (0.049 ± 0.004 pA·pF·1−1·mV−1) versus Young (0.031 ± 0.001 pA·pF·1−1·mV−1; P < 0.05; Fig. 2B). Aging also was associated with an apparent leftward-shift in the STOC frequency-voltage relationship (Fig. 2C). The variability of responses was such that no significant differences between Young and Old could be detected between parameters estimated from Boltzmann fits to the data from respective age groups (i.e., the 95% confidence intervals for the parameters overlapped), despite detecting a significant difference between the overall fits to the two data sets (P = 0.039). Analysis of the data shown in Fig. 2C by two-way ANOVA confirmed significant effects of age and voltage on STOC frequency (P < 0.05) with no interaction (P > 0.05). However, subsequent comparison of means using Bonferroni-corrected t-tests did not reveal differences in the means at each voltage, likely due to the low statistical power of this multiple comparison method.

A larger sample size of SMCs supported the findings suggested by the data shown in Fig. 2, B and C. At −10 mV (n = 27 cells from 11 mice for Young and n = 31 cells from 14 mice for Old), there was no significant difference in STOC amplitude between Young and Old (Fig. 2D; P > 0.05). However, STOC frequency (Fig. 2E) was significantly higher in Old versus Young (P < 0.05) at this voltage. At +30 mV (n = 67 cells from 28 mice for Young and n = 61 cells from 30 mice for Old), STOC amplitude was greater in SMCs from Old versus Young (Fig. 2D; P < 0.05). However, STOC frequency did not differ between SMCs from Old versus Young at this positive membrane potential (Fig. 2E). In SMCs from Young and Old, STOC amplitude was significantly (P < 0.05) higher at +30 mV than at −10 mV (Fig. 2D). No significant effect of voltage on STOC frequency was detected in this group of cells (P > 0.05; Fig. 2E).

BKCa and RyR underlie STOCs in SEA SMCs. In SMCs from cerebral arteries, STOCs are generated by activation of BKCa following Ca2+ release events (Ca2+ sparks) from a cluster of RyR located near the plasma membrane (36). To test whether this model applied to STOCs recorded in SEA SMCs, we investigated the effect of several known BKCa blockers on STOCs recorded at +30 mV. We found that STOCs in SMCs of both Young and Old were abolished by the indole alkaloid...
paxilline (1 μM; Fig. 3) (27), with little or no recovery after washout (data not shown). This was not a vehicle effect, because 0.1% DMSO alone produced a significant increase in both the amplitude and frequency of STOCs in SMCs from Young (Fig. 3, A, C, and E; P < 0.05). In SMCs from Old, 0.1% DMSO also significantly increased STOC amplitude (Fig. 3D; P < 0.05), with no significant effect on STOC frequency. From these data we conclude that STOC inhibition was due to the actions of paxilline and not to an effect of DMSO.

The scorpion venom peptide iberiotoxin (100 nM) (14) significantly (P < 0.05) inhibited STOC amplitude and frequency in SEA SMCs from both Young and Old (Fig. 4, A and B). The quaternary ammonium compound tetraethylammonium (TEA; 1 mM) (29) also reduced STOC amplitude significantly in Young and Old (Fig. 4C), but only inhibited the frequency of STOCs in SMCs from Young (Fig. 4D). Unlike the irreversible effect of paxilline, STOC activity recovered after washout of either iberiotoxin or TEA (data not shown).

To test the role of RyR in STOCs, we also assessed the effects of the RyR antagonist tetracaine (33, 41, 47, 49, 50) on STOC amplitude and frequency. Tetracaine (100 μM) significantly (P < 0.05) and reversibly inhibited both STOC amplitude and frequency in SMCs from both Young (Fig. 4E) and Old (Fig. 4F). Thus, irrespective of age, STOCs in SEA SMCs likely arise from Ca\(^{2+}\) events through RyR and subsequent activation of BK\(_{\text{Ca}}\).

**BK\(_{\text{Ca}}\) do not contribute to mean whole-cell currents in SEA SMCs.** To examine whether BK\(_{\text{Ca}}\) contribute to the mean current-voltage relationship, we repeated the protocols represented in Figs. 1 and 2 in the presence of the BK\(_{\text{Ca}}\) blockers. Neither paxilline (1 μM) nor iberiotoxin (100 nM) significantly affected the mean current-voltage relationship elicited by 10-mV voltage steps between −90 and +60 mV in SMCs from Young or Old (Fig. 5). However, the “noise” of the currents at positive holding potentials was attenuated (Fig. 5B), consistent with the inhibition of STOCs. As observed in gap-free recordings, the effects of paxilline appeared to be irreversible (data not shown). In contrast, current “noise” returned after washout of iberiotoxin (data not shown). Consistent with the lack of significant effect of paxilline and iberiotoxin on whole-cell currents during voltage-step protocols, we also found that these BK\(_{\text{Ca}}\) antagonists had no significant effect on the baseline currents onto which STOCs were superimposed during gap-

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**Fig. 3.** Blockade of large conductance Ca\(^{2+}\)-activated K\(^+\) channels (BK\(_{\text{Ca}}\)) with paxilline abolishes STOCs in SEA SMCs. A and B: representative gap-free recordings of STOCs in a SMC from Young held at +30 mV. A: exposure to 0.1% DMSO (vehicle for paxilline) increased STOC amplitude and frequency as shown. B: subsequent exposure to paxilline (1 μM) in 0.1% DMSO abolished STOCs. C–F: data are mean ± SE amplitude (C and D) and frequency (E and F) of STOCs recorded in SMCs from Young (C and E; n = 6 cells from 3 mice) or Old (D and F; n = 6 cells from 3 mice) before application of DMSO (Control), after application of 0.1% DMSO, and after application of 1 μM paxilline in 0.1% DMSO. *P < 0.05 compared with Control.
free recordings. Thus, at +30 mV, baseline current before paxilline was 1.8 ± 0.2 vs. 1.7 ± 0.1 pA/pF in the presence of paxilline (n = 5, P > 0.05) in SMCs from Old. In SMCs from Young, baseline current before paxilline was 1.7 ± 0.2 pA/pF vs. 1.5 ± 0.1 pA/pF in the presence of paxilline (n = 6, P > 0.05). Similarly, baseline current at +30 mV was 1.8 ± 0.2 pA/pF before iberiotoxin vs. 1.7 ± 0.2 pA/pF in the presence of iberiotoxin (n = 10, P > 0.05) in SMCs from Old. In SMCs from Young, baseline current was 1.9 ± 0.2 pA/pF before iberiotoxin vs. 1.8 ± 0.3 pA/pF in the presence of iberiotoxin (n = 8, P > 0.05).

*KV channels contribute to mean whole-cell currents in SEA SMCs. Contrary to the lack of effect of paxilline or iberiotoxin, TEA (1 mM) significantly (P < 0.05) attenuated whole-cell currents in SMCs from both Young and Old at positive holding potentials (Fig. 6, A and B). The TEA-sensitive currents recorded in SMCs from Young and Old were not significantly different (Fig. 6C). This effect of TEA (1 mM) on whole-cell currents was also observed in SMCs pretreated with paxilline (1 μM) (Fig. 6, D and E), suggesting that TEA (1 mM) blocked K^+ channels in addition to BKCa. Indeed, the difference in effect between TEA compared with paxilline or iberiotoxin could be explained by blockade of voltage-dependent K^+ (KV) channels by TEA, which inhibits some KV channels at 1 mM (32). Consistent with its effects on whole-cell currents recorded during voltage step protocols, TEA (1 mM) significantly reduced baseline currents observed during gap-free recordings: at +30 mV, TEA reduced the baseline current from 1.7 ± 0.3 pA/pF to 1.4 ± 0.2 pA/pF in SMCs from Old (n = 12, P < 0.05) and from 1.6 ± 0.2 pA/pF to 1.1 ± 0.2 pA/pF in SMCs from Young (n = 15, P < 0.05).

To assess the contribution of KV channels to whole-cell currents in SEA SMCs, we examined the effect of the KV channel inhibitor 4-aminopyridine (4-AP; 3 mM) (32). We found that 4-AP significantly (P < 0.05) inhibited outward currents at positive potentials in SMCs from both Young and Old (Fig. 7, A and B). The magnitudes of the 4-AP-sensitive currents were not different between cells from respective age groups (Fig. 7C). Addition of TEA (1 mM), in the presence of 4-AP (3 mM) produced significant (P < 0.05) additional block of outward currents in SMCs from both Young and Old (Fig. 7, A and B). The total current sensitive to the combination of these blockers was not different between age groups (Fig. 7D) and their cumulative effect suggest that they are each inhibiting distinct ion channels. Washout of 4-AP led to recovery of currents in SMCs from both Young and Old (data not shown). Application of 3 mM 4-AP also significantly (P < 0.05) inhibited outward currents in SMCs pretreated with 1 μM paxilline (data not shown; n = 7 cells from 3 Young and n = 6 cells from 3 Old), further confirming that 4-AP acts on channels other than BKCa.

The effects of tetracaine on whole-cell currents also were assessed because this local anesthetic not only blocks RyR but also has been shown to block KV channels in dorsal root ganglion neurons (28). We found, at the same concentration used to inhibit STOCs (Fig. 4, E and F), that tetracaine (100 μM) significantly (P < 0.05) inhibited whole-cell currents in SMCs from both age groups (Fig. 8, A and B), and this effect was reversible upon washout (data not shown). The tetracaine-sensitive currents detected in SMCs from both age groups were not significantly different (Fig. 8C), indicating a lack of effect of aging on the ion channels inhibited by tetracaine. Consistent
with its effect on whole-cell currents recorded during voltage step protocols, tetracaine (100 μM) significantly reduced baseline currents observed during gap-free recordings: at +30 mV, tetracaine (100 μM) reduced the baseline current from 2.5 ± 0.2 pA/pF to 2.0 ± 0.1 pA/pF in SMCs from Young (n = 5, P < 0.05) and from 4.0 ± 0.6 pA/pF to 3.3 ± 0.7 pA/pF in SMCs from Old (n = 8, P < 0.05).

Lack of effect of polyethylene glycol-conjugated catalase on whole-cell currents and STOCs. Aging has been shown to increase the function of KCa channels expressed in endothelial cells isolated from SEAs, an age-related effect that was eliminated by exposure of the cells to polyethylene glycol-conjugated catalase (PEG-catalase) (4). Therefore, we also assessed the effects of PEG-catalase (500 U/ml) on SMC whole-cell currents between −90 and +60 mV, as well as STOC amplitude and frequency assessed at +30 mV. We found that PEG-catalase had no significant effect on whole-cell currents or the properties of STOCs in SMCs isolated from Young or Old (Fig. 9). Consistent with the current densities shown in Fig. 9, A and B, baseline currents during gap-free recordings also were not significantly affected by PEG-catalase: control = 4.4 ± 1.4 pA/pF vs. PEG-catalase = 4.9 ± 1.3 pA/pF in Young (n = 8, P > 0.05) and control = 1.9 ± 0.4 pA/pF vs. PEG-catalase = 2.1 ± 0.4 pA/pF in Old (n = 10, P > 0.05).

Expression of BKCa subunits in SEA SMCs. Real-time PCR was used to assess the effects of aging on the expression of BKCa α-and β1-subunit mRNA levels in SMCs isolated from SEAs. We found no significant difference (P > 0.05) in the relative abundance of transcripts for these subunits between SMCs from Young and Old (Fig. 10). However, Western blots of whole-vessel lysates revealed significantly greater expression of BKCa α-subunit protein in SEAs isolated from Old versus Young (Fig. 11).

BKCa contribute to the negative feedback regulation of myogenic tone in SEA. To examine the functional role of BKCa in the regulation of myogenic tone in SEAs, we assessed the effects of paxilline (1 μM) on the diameter of cannulated, pressurized arteries. We found that SEAs from Young and Old had similar maximal diameters (Fig. 12, A-C). When pressurized to 80 cmH2O at 37°C, SEAs developed myogenic tone that was not different between age groups (Fig. 12, A, B, and D). Vasconstriction induced by paxilline (1 μM) also was not significantly different in SEAs from Young versus Old (Fig. 12, A, B, and E). For reference, paxilline-induced constriction was similar in magnitude to that produced by exposure of SEAs to the α1-adrenergic receptor agonist phenylephrine (10 μM), with no difference between Young and Old in the phenylephrine-induced response (respectively: 20 ± 2% constriction, n = 10 vs. 18 ± 2% constriction, n = 12; P > 0.05). These data indicate that BKCa contribute negative feedback to the regulation of myogenic tone in murine SEAs and that aging has no significant effect on this integrated function in an intact resistance artery.

DISCUSSION

The major new findings from the present study are: 1) SMCs from a murine skeletal muscle feed artery display BKCa- and RyR-dependent STOCs, 2) aging increased the effect of membrane depolarization on STOC amplitude, 3) aging shifted the effect of membrane depolarization on STOC frequency to more negative membrane potentials, 4) aging was associated with an increase in expression of BKCa α-subunit protein, and 5) aging had no significant effect on the integrated function of SEAs in terms of development of myogenic tone or the contribution of BKCa to the regulation of myogenic tone. We also found that aging was associated with an increase in whole-cell capacitance of SEA SMCs, extending observations from mouse mesenteric arteries (11) to a skeletal muscle feed artery.

SMCs isolated from murine SEAs displayed large, outwardly rectified whole-cell currents at positive membrane potentials (Fig. 1), typical of vascular SMCs (20, 23, 37). The magnitude of the outwardly rectified currents recorded between 0 and +60 mV was significantly greater in cells isolated from Old versus Young mice, as were the small inward currents recorded between −90 and −30 mV, consistent with an increase in SMC membrane conductance. However, this age-associated increase in whole-cell current and membrane conductance was eliminated when the data were normalized to...
whole-cell capacitance, because cell capacitance was also significantly greater in Old. We interpret this increase in capacitance to indicate an increase in membrane area. Our findings suggest that the functional expression of ion channels responsible for the whole-cell currents is similar per unit area of membrane in SMCs from Young and Old. We speculate that the increase in whole-cell capacitance in SMCs from aged mice results from an increase in SMC size, as has been previously reported in SMCs isolated from mouse mesenteric arteries (11), and suggested in studies of rat mesenteric arteries (8). Thus vascular SMC hypertrophy may be a common response to aging of the arterial system. When taken in light of sustained vasomotor tone and maximal diameters of SEAs with aging, such adaptations in SMCs may serve to maintain functional integrity of the intact vessel. The mechanisms responsible for the age-related increase in SMC membrane capacitance reported here and elsewhere (11) remain to be defined.

**Freshly isolated SMCs from SEAs display STOCs.** At membrane potentials more positive than −30 mV, STOCs were observed in SMCs from both Young and Old and were superimposed on more steady, voltage-dependent outward currents (Figs. 1 and 5). Membrane potential of SMCs in pressurized skeletal muscle resistance arteries is ∼−30 mV (13), suggesting that STOCs at this membrane potential are physiologically relevant. These spontaneous events were abolished by paxilline (1 μM; Figs. 3 and 5) and inhibited by iberiotoxin (100 nM; Fig. 4) and TEA (1 mM; Fig. 4), established blockers of BK<sub>Ca</sub> (7, 14, 27). Thus STOCs in SMCs of the SEA are carried by currents through BK<sub>Ca</sub>, consistent with earlier reports (10, 36, 51). STOCs also were inhibited by the RyR blocker tetracaine (100 μM; Fig. 4). These data support the interpretation that RyR also are involved in the generation of STOCs in SMCs of cerebral arteries have shown that depolarization increases the frequency of Ca<sup>2+</sup> sparks, which may be triggered by voltage-dependent Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels (24). Our data are consistent with this behavior.

Aging increased the effects of depolarization on STOC amplitude (Fig. 2B). At −30 mV, STOC amplitudes were similar in SMCs from young and old mice. However, at +30 mV, STOC amplitude was significantly increased in cells from Old vs. Young (Fig. 2, B and D). With the exception of the data shown in Figs. 3, D and E, and Fig. 9, we observed this age-related increase in STOC amplitude (Figs. 2 and 4). We suggest that the variability and small sample sizes (n = 6 cells from 3 animals per group in Fig. 3 and 8–10 cells from 3 to 4 animals in Fig. 9) explain why this pattern was not observed in the data sets shown in Figs. 3 and 9, where STOC amplitude appeared lower in cells from aged animals. The difference in STOC amplitude shown in Figs. 2 and 4 between age groups might be due, in part, to the increased expression of BK<sub>Ca</sub> α-subunit protein that we observed (Fig. 11). However, this cannot be the sole explanation of the difference, because an increase in density of BK<sub>Ca</sub> expressed per unit membrane

**Fig. 6.** TEA inhibits whole-cell current densities of SEA SMCs irrespective of paxilline. Data are mean current densities ± SE (in pA/pF) in the absence (Control) or presence of TEA (1 mM), as indicated. A and B: TEA inhibits whole-cell current density at positive membrane potentials in SMCs from Young (A; n = 32 cells from 14 mice) or Old (B; n = 30 cells from 14 mice). C: TEA-sensitive currents in SMCs from Young were not different from those recorded in SMCs from Old. D and E: TEA inhibits whole-cell current densities in the presence of paxilline in SMCs from Young (D; n = 7 from 4 mice) and Old (E; n = 8 from 3 mice), respectively. *P < 0.05 compared with Control.
BKCa expression, the voltage- and Ca\(^{2+}\) likely arise. In rat coronary SMCs, although aging decreased

Control.

C

4-AP (3 mM) inhibited outward currents at positive membrane potentials in SEA SMCs. However, the inhibition of currents through K\(_V\) channels other than BKCa by TEA, and in addition to RyR with tetracaine, indicates limited utility of these two blockers in functional experiments in SEAs. For this

4-AP-sensitive current 4-AP + TEA-sensitive current

Capacitance should have been accompanied by a parallel shift upward in the voltage-STOC amplitude relationship, and this predicted shift was not observed. The increased slope of the voltage-STOC amplitude relationship in Fig. 2B could result from a steeper voltage-dependence of BKCa expressed in SMCs from Old, or from an age-related increase in the voltage-dependent amplitude of Ca\(^{2+}\) sparks from which the STOCs likely arise. In rat coronary SMCs, although aging decreased BKCa expression, the voltage- and Ca\(^{2+}\)-dependent activation of these channels appeared unchanged (38). The effects of aging on subsarcolemmal Ca\(^{2+}\) signals that affect BKCa activity is not known. Studies in which Ca\(^{2+}\) sparks and STOCs are recorded simultaneously in SEA SMCs from Young and Old are needed to distinguish between age-related changes in the voltage-dependence of BKCa and/or Ca\(^{2+}\) signaling in SEA SMCs.

Aging also tended to shift the voltage-STOC frequency relationship to the left, such that the peak effects of voltage on STOC frequency occurred at more negative membrane potentials in SMCs from Old versus Young mice (Fig. 2C). This conclusion is supported by our observation that STOC frequency was higher in Old compared with Young at \(-10\) mV in a large sample of SMCs (Fig. 2E). The voltage-dependence of Ca\(^{2+}\) sparks (and hence STOCs) may be related to the voltage-dependence of Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (24). Aging is associated with an increase in the current density through these channels in mouse mesenteric artery SMCs (11). Thus the leftward shift in the effects of membrane potential on STOC frequency may be related to altered Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels and their subsequent effect on Ca\(^{2+}\) sparks. However, an increase in Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels might be predicted to increase myogenic tone in the aged. That we did not observe a difference in myogenic tone in the present study suggests that either the effect of an increase in Ca\(^{2+}\) entry is exactly balanced by an increase in STOCs or that additional compensatory adaptations were present. It is also possible that aging affects the coupling of Ca\(^{2+}\) sparks to BKCa. Again, further studies will be required to investigate these relationships.

In contrast with the lack of effects of paxilline and iberiotoxin on mean whole-cell currents (Fig. 5), TEA (1 mM) and tetracaine (100 \(\mu\)M) significantly attenuated the steady, outwardly rectified currents recorded from SEA SMCs (Figs. 6–8) and reduced the baseline current in gap-free recordings. These inhibitory effects suggest that in SEA SMCs, TEA and tetracaine block K\(_V\) channels, in addition to blocking BKCa and RyR, respectively. Our observations are consistent with findings in neurons that TEA (1 mM) (32) and tetracaine (100 \(\mu\)M) (28) can inhibit some K\(_V\) channels. In testing the effect of the known K\(_V\) channel blocker 4-AP (3 mM), we found that it too significantly attenuated outwardly rectified, whole-cell currents (Fig. 7). These data support our conclusion that K\(_V\) channels carry the outward currents in SMCs of SEAs. However, the currents inhibited by TEA appeared distinct from those inhibited by 4-AP because their effects were additive (Fig. 7, A and B). These data suggest the presence of multiple K\(_V\) channel isoforms in SEA SMCs. Further studies will be required to define the molecular identity of the currents inhibited by TEA and tetracaine in these cells. Nevertheless, the inhibition of currents through K\(^{+}\) channels other than BKCa by TEA, and in addition to RyR with tetracaine, indicates limited utility of these two blockers in functional experiments in SEAs. For this
expression of BKCa α-subunit protein with age differs from results reported for coronary arteries (1, 31, 38, 46) and arterioles from rat soleus and gastrocnemius muscles (26), where decreased protein expression was observed, and from rat cerebral arteries where aging had no effect on BKCa α-subunit protein expression (39). These differences in the effect of aging on protein expression in different vascular beds further indicate that there are important regional differences in the effect of and adaptations to aging in the circulation.

Pressure myography of intact SEAs. Aging had no significant effect on intact SEAs studied by pressure-myography (Fig. 12). These results are consistent with the lack of effect of aging on first-order arterioles from in rat gracilis muscle (34). Also consistent with studies in aged rats (34), we found that vasoconstriction induced by the α-adrenergic receptor agonist phenylephrine was similar (18–20%; see RESULTS) between SEAs from Young versus Old mice. However, in contrast with studies in microvessels of the rat, where aging was associated with decreased myogenic tone (26, 34), we found that myogenic tone was not different between SEAs from Young and Old (Fig. 12D). This difference could mean that there are species (mouse vs. rat), regional (abdominal muscles vs. limb muscles), or vessel order (feed arteres vs. first-order arterioles)-dependent differences in the vascular adaptation to aging.

We interrogated the physiological role played by BKCa in SEA SMCs by examining the effects of paxilline (1 μM) on the diameter of SEAs using pressure-myography. We found that this BKCa antagonist caused significant constriction (Fig. 12E), similar in magnitude to that produced by the α1-adrenergic agonist phenylephrine (10 μM). These data indicate that BKCa contribute significantly to the negative feedback regulation of myogenic tone in murine SEAs, consistent with data reported in other resistance arteries (7, 10, 36, 49, 51). However, our finding that paxilline-induced constriction of SEAs was not significantly affected by aging (Fig. 12E) suggests that any age-related differences in BKCa-dependent STOCs (Fig. 2) do not translate into altered negative feedback regulation of myogenic tone under the conditions of our experiments. It should be noted that STOC amplitude and frequency at −30 mV, likely the membrane potential of SMCs in a pressurized feed artery exhibiting spontaneous myogenic tone (13), were not different between SMCs from Young and Old (Fig. 2, B and C), consistent with the lack of effect of aging on paxilline-induced constriction.

reason, we did not investigate the actions of TEA or tetracaine in our pressure myography studies (Fig. 12).

The TEA-sensitive, 4-AP-sensitive, and tetracaine-sensitive outward currents were similar in SMCs from Young and Old (Figs. 6, 7, and 8). These data collectively suggest that aging does not affect the overall function of the Kv channels that underlie respective currents. Such lack of effect of aging on Kv channel currents is consistent with findings from pressure-myography studies performed in first-order arterioles from rat gastrocnemius muscle (26), but differ from data obtained in arterioles from rat soleus muscle where Kv channel function appeared greater in vessels from aged animals (26). In light of the present data, these collective findings support the hypothesis that there is regional heterogeneity in the adaptation of resistance arteries to aging.

**mRNA and protein expression of BKCa subunits.** We found that aging had no significant effect on the expression of mRNA for BKCa subunits in SMCs isolated from SEAs (Fig. 10). In contrast, Western blot analysis of lysates of whole SEAs showed that expression of the BKCa pore-forming α-subunit was significantly increased in Old versus Young (Fig. 11). These data support the often cited lack of correlation between message and protein expression (48). Our finding of increased

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**Fig. 9.** Lack of effect of polyethylene glycol-conjugated (PEG)-catalase on whole-cell currents and STOCs in SMCs from SEAs. A and B: mean whole-cell current densities ± SE (n = 6 cells from 3 animals for Young and Old) in the absence (Control) and presence of PEG-catalase (500 U/ml). Two-way ANOVA revealed significant effects of voltage on mean current densities (P < 0.05), but no significant effect of PEG-catalase (P > 0.05) in both Young and Old. C–F: mean amplitude (in pA/pF; C and D) or frequency (in Hz; E and F) ± SE (n = 8 cells from 3 mice for Young and 10 cells from 4 mice Old) in the absence (PSS) and presence of PEG-catalase (500 U/ml). Paired t-tests revealed no significant effect of PEG-catalase on amplitude (P > 0.05) or frequency (P > 0.05) of STOCs in Young (C and E) or Old (D and F).

**Fig. 10.** Relative abundance of transcripts for BKCa subunits in SEA SMCs. Data are mean relative abundances ± SE (n = 7 independent samples from 7 mice) for the α- and β1-subunits of BKCa, in samples of SEA SMCs, as indicated. There was no significant difference (P > 0.05) in relative abundance of either transcript in Young vs. Old. Gusb, glucuronidase-β.
Our observation that paxilline-induced constriction of murine SEA was not significantly affected by aging (Fig. 12E) is consistent with the findings in first-order arterioles from rat gastrocnemius muscle in which iberiotoxin was used to block BKCa (26). Our results differ, however, from data obtained in rat soleus muscle arterioles, where aging was associated with an increase in iberiotoxin-induced constriction (26). This could be related to differences in the muscle fiber type from which the vessels originated (26). The fiber-type composition of mouse abdominal muscles has not been established. However, in rats (17) abdominal muscles are a mixture of type I and type II fibers. We predict a similar fiber-type distribution in murine abdominal muscles based upon complementary fiber type profiles of other skeletal muscles (6). The lack of effect of aging on apparent BKCa function in murine SEAs also contrasts with findings in rat coronary arteries (1, 31), where aging was associated with a decrease in BKCa expression and function. Regional- and/or species-related heterogeneity in response to aging may explain these differences.

**Limitations.** We did not explore endothelial cell function in the present study and cannot exclude a modulatory role for endothelial cells in the observed responses. Thus we cannot exclude an effect of aging on endothelial cell function in the present study, as this was not examined. A recent investigation of endothelial cells isolated from SEAs has shown that the resting membrane potential is more negative in cells isolated from Old compared with Young, a difference that could be eliminated by scavenging hydrogen peroxide with catalase (4). In contrast, we found that PEG-catalase had no effect on whole-cell currents or the properties of STOCs in Young or Old. These data suggest that aging may have selective effects on endothelial cells versus SMCs. The lack of effect of aging on myogenic tone in the present study also suggests that the age-related difference in endothelial cell membrane potential and hydrogen peroxide production previously observed (4) did not translate into altered whole-vessel behavior under the conditions of our experiments. In turn, these findings suggest that additional compensatory adaptations to aging may have occurred.

The Western blot experiments that we performed (Fig. 11) do not define the cell-type in which the increased BKCa...
evaluate BKCa subunit transcript levels.

**ACKNOWLEDGMENTS**

We thank Rebecca Shaw for performing the real-time quantitative PCR to evaluate BKCa subunit transcript levels.

**GRANTS**

This research was supported by National Heart, Lung, and Blood Institute (NHLBI) Grant R01-HL-086483. V. Bradley was supported by NHLBI Research Education Grant R25-HL-103156 and the Michigan State University College of Veterinary Medicine Summer Research Program. E. M. Boerman was supported by SF32 HL-118836. The support of Z. Nourian by Michael A. Hill and NHLBI Grant R01 HL-092241 is gratefully acknowledged.

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**AUTHOR CONTRIBUTIONS**


**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).


