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Evidence for Involvement of Both IKCa and SKCa Channels in Hyperpolarizing Responses of the Rat Middle Cerebral Artery

Alister J. McNeish, PhD; Shaun L. Sandow, PhD; Craig B. Neylon, PhD; Mark X. Chen, PhD; Kim A. Dora, PhD; Christopher J. Garland, PhD

Background and Purpose—Endothelium-derived hyperpolarizing factor responses in the rat middle cerebral artery are blocked by inhibiting IKCa channels alone, contrasting with peripheral vessels where block of both IKCa and SKCa is required. As the contribution of IKCa and SKCa to endothelium-dependent hyperpolarization differs in peripheral arteries, depending on the level of arterial constriction, we investigated the possibility that SKCa might contribute to equivalent hyperpolarization in cerebral arteries under certain conditions.

Methods—Rat middle cerebral arteries (≈175 μm) were mounted in a wire myograph. The effect of KCa channel blockers on endothelium-dependent responses to the protease-activated receptor 2 agonist, SLIGRL (20 μmol/L), were then assessed as simultaneous changes in tension and membrane potential. These data were correlated with the distribution of arterial KCa channels revealed with immunohistochemistry.

Results—SLIGRL hyperpolarized and relaxed cerebral arteries undergoing variable levels of stretch-induced tone. The relaxation was unaffected by specific inhibitors of IKCa (TRAM-34, 1 μmol/L) or SKCa (apamin, 50 nmol/L) alone or in combination. In contrast, the associated smooth-muscle depolarization was inhibited, but only with these blockers in combination. Blocking nitric oxide synthase (NOS) or guanylyl cyclase evoked smooth-muscle depolarization and constriction, with both hyperpolarization and relaxation to SLIGRL being abolished by TRAM-34 alone, whereas apamin had no effect. Immunolabeling showed SKCa and IKCa within the endothelium.

Conclusions—In the absence of NO, IKCa underpins endothelium-dependent hyperpolarization and relaxation in cerebral arteries. However, when NOS is active IKCa contributes to hyperpolarization, whatever the extent of background contraction. These changes may have relevance in vascular disease states where NO release is compromised and when the levels of SKCa expression may be altered. (Stroke. 2006;37:000-000.)

Key Words: EDHF ■ endothelium ■ nitric oxide ■ pharmacology ■ potassium channels

The cerebrovascular circulation has distinct characteristics compared with many other peripheral vessels. For example, blockade of endothelial cell IKCa channels alone is sufficient to block endothelium-derived hyperpolarizing factor (EDHF)–mediated relaxation and hyperpolarization in rat middle cerebral arteries.1,2 This is a dramatic contrast to other peripheral vessels, where in general EDHF–mediated responses are abolished by combined inhibition of both SKCa and IKCa in the endothelium.3

In arteries where blockade of EDHF–mediated responses require the simultaneous block of SKCa and IKCa, such as porcine coronary and rat mesenteric arteries, functional, electrophysiological and immunohistochemical data demonstrate that these channels are clearly expressed only within the endothelium.4–6 Therefore, the critical role of IKCa channels in cerebral vessels may reflect a differential expression of KCa subtypes. In middle cerebral arteries, for example, SKCa subunits may be absent, have a low expression level, or perhaps be masked by high IKCa expression levels. Alternatively, the cellular location of the KCa subtypes may differ between the endothelium and the smooth muscle. However, there is currently little evidence on the relative expression and cellular distribution of SKCa and IKCa in rat middle cerebral arteries, although functional data does suggest that IKCa are localized to endothelial cells.3

The apparent dominant role of IKCa in EDHF responses in the middle cerebral artery may reflect experimental parameters. IKCa and SKCa mediate individual components of endothelium-dependent hyperpolarization in rat mesenteric arteries. SKCa underpin smooth-muscle hyperpolarization, whereas IKCa reverse agonist-induced depolarization-repolarization.7 Thus, the extent of vasoconstrictor stimuli influences the relative contribution of IKCa and SKCa for subsequent changes in membrane potential, so that when the smooth-muscle cells

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are depolarized and constricted by α stimulation (with phenylephrine), block of endothelium-dependent hyperpolarization and relaxation requires inhibition of endothelial SKCa and IKCa. What is not known is whether a similar profile operates during the spontaneous smooth-muscle depolarization and contraction of myogenically active arteries under physiological pressures, such as those in the cerebral circulation.5 For example, in rat posterior cerebral artery mounted at 10 mm Hg, smooth-muscle cell resting membrane potential is ≈ -67 mV, whereas after developing myogenic tone at 60 mm Hg the membrane potential is ≈ -38 mV.9 Inhibition of nitric oxide synthase (NOS) in cerebral arteries will often constrict and depolarize the smooth muscle further.2,10-12 The observation that EDHF in rat middle cerebral artery is solely dependent on IKCa, might reflect some physiological masking of SKCa, function rather than a lack of expression of these channels.

The aim of the present study was to assess smooth-muscle relaxation and hyperpolarization evoked by stimulating the endothelium with SLIGRL, under variable levels of stretch to mimic variable intraluminal pressures, to reveal possible input from SKCa, and define any modulation by endothelium-derived NO in the rat middle cerebral artery. Furthermore, using immunohistochemistry we investigated the expression and distribution of KCa subtypes in this artery to correlate with the functional data. Additionally, electron microscopy was used to examine the anatomy and coupling characteristics in the middle cerebral artery.

Materials and Methods
Whole brain from male Wistar rats (200 to 300 g; Charles River) was removed and stored immediately in ice-cold Krebs solution. Segments of the middle cerebral artery (~2 mm long) were dissected and stored in ice-cold Krebs for use within 30 minutes. The same size vessels were used in all experimental groups after dissection by the same person.

Experimental Protocols for Isometric Tension and Membrane Potential Recordings
Segments of middle cerebral artery (internal diameter ~150 μm) were mounted in a Mulvany-Halpern myograph (model 400A, Danish Myotechnology) in Krebs solution containing (mmol/L): NaCl, 118.0; NaCO3, 24; KCl, 3.6; MgSO4, 1.2; glucose, 11.0; CaCl2, 2.5; gassed with 95% O2 and 5% CO2 and maintained at 37°C. After equilibration for 20 minutes vessels were tensioned to 1 mN (approximates wall tension at 60 mm Hg). In some experiments vessel tension was increased to 4 mN (approximates wall tension at 60 mm Hg). In some experiments, blood vessels were used in all experimental groups after dissection by the same person.

Electronic Microscopy
Anesthetized rats were perfusion-fixed using standard procedures.15 Briefly, animals were perfused via the left ventricle with a clearing solution of 0.1% bovine serum albumin (Sigma; St Louis, Mo; A3059) or normal donkey serum (Sigma; St Louis, Mo; D9663), 30 mmol/L NONOate or 0.1% NaNO3 and 10 U/mL heparin, and then perfuse-fixed with 1% paraformaldehyde, 3% glutaraldehyde in 0.1 mol/L sodium cacodylate, with 35 mmol/L betaine (Sigma, B2629), pH 7.4. Segments of the middle cerebral artery were removed and processed for electron microscopy as previously described.15 Transverse sections (~100 nm thick) were cut and myoendothelial gap junctions and surrounding endothelial cell and smooth-muscle cell regions photographed at ×10,000 to ×40,000 using a transmission electron microscope.

Immunohistochemistry
After perfusion fixation (as above), segments of middle cerebral artery were dissected into PBS, cut along the lateral plane and pinned out flat with either the intima or adventitia uppermost. Whole mount tissues were subsequently processed using standard immunohistochemical procedures.16,17 Briefly, after washing in PBS and incubation (1 hour) in blocking buffer (1% normal donkey serum, or bovine serum albumin and 0.1% Tween-20), vessels were incubated in primary antibody overnight at room temperature. These were: SK2 (dilution, 1:100; Alomone, APC-028), SK3 (1:100; Alomone, APC-025), SK4 (rIK1; 1:100; Alomone, APC-064), SK4 (hIK1; 1:400; M20164) and SK4 (rIK1; 1:1500; IK38/68). Endothelial and smooth-muscle cell layers were identified by taking successive optical sections from the intimal or adventitial and by using the antibody to a-actinin (1:40; Sigma, A5288) and von Willebrand factor (1:300; Sigma, F3520). Tissue was subsequently washed in PBS and incubated in rabbit Alexa 633 (1:100; Molecular Probes; Paisley, UK; A21071) or rabbit FITC (1:40; Sigma; St Louis, Mo; F6005) for 2 hours at room temperature. Preparations were mounted in buffered glycerol and images collected on a confocal microscope. Data for each vessel was from 3 to 6 animals.

Controls for antibody specificity included omission of the primary antibody, substitution of the primary antibody for an unrelated rabbit IgG (1:100; Chemicon; Temecula, Calif; PF64), incubation in fluorophore alone and preincubation with a 10-fold excess of peptide (when available) to which the antibody was raised. Additionally, the specificity of the antibodies used has been previously demonstrated using Western blotting and immunostaining of transfected cells.15-19 (supplemental Figure I, available online at http://stroke.ahajournals.org).

Solutions and Drugs
Exogenous K+ was added as an isotonic physiological salt solution in which all the NaCl was replaced with an equivalent amount of KCl. Concentrations of K+ used are expressed as final bath concentration, unless specifically stated. Glibenclamide, l-NNAME, ouabain, and papaverine HCl were all obtained from Sigma. Aminophylline and iberotoxin, from Latoxan. ODQ (1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one) from Tocris. SLIGRL from Auspep. DEA NONOate from Alexis. TRAM-34 was a generous gift from H. Wulff (University of California, Davis). All stock solutions were prepared in distilled water except ODQ and TRAM-34 (10 mmol/L), which were dissolved in dimethylsulfoxide (DMSO).

Statistical Analysis
Results are expressed as the mean±SE mean of n animals. Tension values are in mN (always per 2 mm segment) and EM as mV. Vasodilatation is expressed as percentage reduction of the total vascular tone (myogenic tone plus vasoconstrictor response) assessed by relaxation to papaverine (150 μmol/L). Graphs were drawn and comparisons made using 1-way ANOVA with Bonferroni post-test (Prism, Graphpad). P≤0.05 was considered significant.
Results

Isometric Tension and Smooth-Muscle Cell Membrane Potential Recordings

Rat middle cerebral arteries spontaneously developed myogenic tone equivalent to 14.0±5.9% (n=6) of the maximum arterial constriction (6.7±0.5 mN, n=7), at which the resting membrane potential was −48.0±1.2 mV (n=5). Addition of the protease-activated receptor 2 agonist SLIGRL (20 μmol/L) evoked relaxation and smooth-muscle cell hyperpolarization (89.0±10.7% and −18.2±2.7 mV, respectively, n=11). Both responses were completely unaffected by either apamin (50 nmol/L; SKCa blocker), or TRAM-34 (1 μmol/L; IKCa blocker; Figures 1 and 3). However, the combined presence of apamin and TRAM-34 virtually abolished the SLIGRL-induced (EDHF-mediated) hyperpolarization without modifying relaxation because the NO component of the SLIGRL response was still able to elicit maximal relaxation (Figures 1 and 3). The level of myogenic tone was unaffected by apamin and TRAM-34. A similar profile was recorded in vessels where tension was increased to 4 mN (70% of the maximum tension), where relaxation and hyperpolarization evoked by SLIGRL was 77.0±1.3% and 20.2±3.1 mV, respectively (n=9; Figure 3). The relaxation was still unaffected by either apamin, TRAM-34 or the combined application of these drugs, whereas in contrast hyperpolarization to SLIGRL was attenuated by TRAM-34 and apamin in combination (10.5±1.8 mV, n=9; Figures 2 and 3).

In the presence of l-NAME, SLIGRL (20 μmol/L) evoked EDHF-mediated relaxation and hyperpolarization (Figure 3). Alone, l-NAME (100 μmol/L) evoked smooth-muscle depolarization (12.8±0.7 mV, n=4) and associated constriction (2.8±0.4 mN, n=4). Removal of the endothelium similarly depolarized (to −35.6±3.4 mV, n=3) and constricted (1.7±0.2 mN, n=7) arteries. In these arteries, l-NAME did not cause further constriction (0.1±0.0 mN, n=5), and SLIGRL failed to evoke relaxation. In endothelium-intact vessels, relaxation and hyperpolarization to SLIGRL in the presence of l-NAME were completely abolished by TRAM-34 alone (Figure 3), and apamin had no effect.

The soluble guanylate cyclase inhibitor ODQ (10 μmol/L) had a similar effect to l-NAME in that it depolarized (to −35.5±6 mV, n=3) and constricted (Δtension=3.8±0.7 mN, n=3) arteries. As with the l-NAME effect, SLIGRL-induced relaxation (73.5±6.2%, n=6) was sensitive to DEA-NONOate (300 nmol/L), yet apamin and TRAM-34 were without effect (15.0±5.9%, n=3).

In an attempt to identify the mechanism for activation of SKCa by SLIGRL in the presence of NO, a direct action of the NO donor DEA-NONOate (300 nmol/L) was investigated in arteries at normal tension in the absence and presence of l-NAME. In the absence of l-NAME, 300 nM DEA-NONOate hyperpolarized smooth-muscle cells by −11.6±1.5 mV (n=6), which was unaltered by apamin (−14.4±4 mV, n=4). Similarly, in the presence of l-NAME, DEA-NONOate again stimulated hyperpolarization (−12.2±1.2 mV, n=11), yet apamin and TRAM-34 were without effect (Figure 4). NO can cause smooth-muscle hyperpolarization by activating either KATP or BKCa channels. Glibenclamide (block of KATP) had no significant effect on DEA-NONOate-induced hyperpolarization, whereas hyperpolarization was sensitive to block with iberotoxin (block of BKCa; to −4.3±1.6 mV, n=4; P<0.05). None of these treatments affected the relaxation to DEA-NONOate (Figure 4). Furthermore, ODQ had no significant effect on DEA-NONOate-induced hyperpolarization (−8.4±3.5 mV, n=6), but significantly inhibited relaxation (15.7±9.5%, n=7; P<0.05; Figure 4).

Figure 1. Effect of KCa channel inhibitors on responses to 20 μmol/L SLIGRL in rat middle cerebral arteries at normal tension (1 mN). Traces of simultaneous recording of smooth-muscle membrane potential (top) and tension (bottom) in rat middle cerebral arteries under control conditions (a) and in the presence of TRAM-34 (1 μmol/L; b) or TRAM-34 and apamin (50 nmol/L; c), recorded from the same artery. Dashed lines correspond to −45 mV and 2 mN.

Figure 2. Effect of KCa channel inhibitors on responses to 20 μmol/L SLIGRL in rat middle cerebral arteries at high stretch-induced tension (4 mN). Traces of simultaneous recording of smooth-muscle membrane potential (top) and tension (bottom) under control conditions (a) and in the presence of TRAM-34 (1 μmol/L; b) or TRAM-34 and apamin (50 nmol/L; c), recorded from the same artery. Dashed lines correspond to −50 mV and 2 mN.
Morphology and KCa Expression in the Rat Middle Cerebral Artery

Middle cerebral arteries comprised 3 to 4 smooth-muscle cell layers and contained homo- and heterocellular gap junctions. Adjacent endothelial cells were coupled by large gap junctional plaques, whereas junctions between adjacent smooth-muscle cells or between smooth-muscle and endothelial cells (myoen- dothelial gap junctions) were relatively small (Figure 5). The latter were on a bulbous enlargement (≈1.2 μm across) at the end of a thin stalk (≈100 nm) projecting from an endothelial cell through the internal elastic lamina (Figure 5).

Positive staining for SK2 (KCa2.2), SK3 (KCa2.3) and IK1 (KCa3.1) was observed (Figure 6), with SK2 (mainly perinuclear location) and SK3 (low-level punctate staining on or near the plasmalemma) being restricted to the endothelium and IK1 present on both endothelial and smooth-muscle cells (plasmalemma and cytoplasmic location). The characteristics of IK staining were the same with each of 3 different IK channel antibodies.

Discussion

We demonstrate that SKCa can contribute to endothelium-dependent hyperpolarization in the rat middle cerebral artery, but only when the vessels are able to synthesize NO. Furthermore, the contribution from SKCa was not dependent on the level of prior arterial constriction. In contrast, when either endothelial NOS or soluble guanylyl cyclase were inhibited, IKCa alone underpinned endothelium-dependent hyperpolarization and the associated relaxation, as previously reported.\(^1\)\(^2\) We also show, using immunohistochemistry, that both SKCa and IKCa are present on endothelial cells in the middle cerebral artery. In order to record membrane potential in isobaric preparations, pharmacological interventions, such as the addition of nifedipine, are required and prevent simultaneous measurement of the dilator response. Therefore, all experiments were conducted in a wire myograph to enable simultaneous recording of changes in smooth-muscle tension and smooth-muscle membrane potential. We have previously compared the EDHF response under isometric and isobaric conditions and found no major differences with SLIRL–induced dilator responses.\(^2\)

It is unclear why SKCa channels do not contribute to hyperpolarization when NOS is inhibited in the rat middle cerebral artery. However, this is in contrast to the rat mesenteric artery.\(^7\) In quiescent mesenteric artery, EDHF

![Figure 3](image1)

**Figure 3.** Summary of the effect of KCa channel inhibitors on responses to 20 μmol/L SLIGRL in rat middle cerebral arteries at different background tensions. Average paired hyperpolarization (top) and relaxation (bottom) responses stimulated by SLIGRL in arteries stretched to normal tension (1 mN; a), high tension (4 mN; b) or normal tension in the presence of L-NAME–induced vasoconstriction (c) under control conditions or in the presence of either apamin (50 nmol/L), TRAM-34 (1 μmol/L) or their combination. *P<0.05 indicates a significant difference from control, N=3.

![Figure 4](image2)

**Figure 4.** Summary of the effect of inhibitors on responses to 300 nM DEA-NNOate in rat middle cerebral arteries at normal tension in the presence of L-NAME–induced vasoconstriction. Average paired hyperpolarization (A) and relaxation (B) responses stimulated by DEA-NNOate under control conditions and in the presence of either TRAM-34 (1 μmol/L), apamin (50 nmol/L), iberiotoxin (bTx, 100 nmol/L), ODQ (10 μmol/L), or glibenclamide (10 μmol/L). *P<0.05 indicates a significant difference from control, n=4.
hyperpolarization is almost totally attributable to SKCa activity whereas during vasocostriction with phenylephrine this changes, so both SKCa and IKCa contribute to the EDHF response. Thus, at least in the mesenteric artery, the extent of arterial stimulation can alter the contribution of IKCa and SKCa to endothelium-dependent hyperpolarization. This is clearly not the case in the middle cerebral artery, as IKCa contributed to endothelium-dependent hyperpolarization over a range of tensions equal to or greater than observed in the presence of L-NAME, when SKCa activity was not detected.

An alternative explanation could be that NO, or an associated reactive oxygen species, facilitates SKCa activation. NO donors have been shown to stimulate SKCa in rat fundus by a cGMP-dependent mechanism. However, in the rat middle cerebral artery, either in the absence or presence of L-NAME, hyperpolarization produced by addition of an NO donor (DEA-NONOate) was unaffected by inhibition of SKCa, but was inhibited by blockade of BKCa. Thus, a direct effect of NO on SKCa seems unlikely, although an additive effect perhaps requiring rises in intracellular Ca2+ at sites near the SKCa channels cannot be excluded. Another possibility is that NO may indirectly activate SKCa possibly through inhibition of cytochrome P450–dependent enzymes. In cerebral arteries, cytochrome P450, via the hydroxylase pathway, produces 20-HETE, a mediator involved in development of myogenic constriction, at least in part, attributable to inhibition of BKCa channels. Thus, removing an inhibitory influence of NOS may increase 20-HETE, with consequent vasoconstriction. An effect of this kind would be consistent with the L-NAME–induced vasoconstriction and depolarization we observed. However, there is currently no evidence that 20-HETE or other cytochrome P450 metabolites might inhibit SKCa.

Another possibility relates to the ultrastructural and morphological characterization of the arterial cells and KCa channels. Throughout this study, we used the same sized vessels (diameter \( \approx 175 \mu \text{mol/L} \)), taken from the second branch of the middle cerebral artery, because we found there to be no significant difference in relaxation responses to SLIGRL compared with either a larger or smaller branch (data not shown). In this branch of the middle cerebral artery, interendothelial cell and intersmooth-muscle cell gap junctions could be observed, and in addition myoendothelial gap junctions were present suggesting that both homo- and heterocellular coupling exists between the different cell types; these findings are similar to those described in human cerebral arteries. Because both IKCa and SKCa were expressed in the endothelium, and SLIGRL stimulates an endothelium-dependent response through a receptor coupled by G protein \( \alpha_\text{11} \) to rises in intracellular Ca2+, the likely explanation is that endothelial cell hyperpolarization may be transmitted to the muscle layers through myoendothelial gap junctions. However, it appears that when considering nonnuclear staining, both the SK2 and SK3 channels are very diffuse within the endothelium, suggesting low levels of expression. Therefore, it is possible that
their contribution to functional responses is less marked than through IK_{Ca} channels, which had a more uniform distribution. This is in marked contrast to the high expression levels of SK3 at adjacent endothelial cell borders in porcine coronary artery,^{4} and juvenile rat saphenous artery (Figure 6), arteries which exhibit an EDHF response that is clearly dependent on the activation of both SK_{Ca} and IK_{Ca}.^{2,4,15,24}

IK_{Ca} expression was observed in both the endothelial and smooth-muscle cells. The endothelial cell expression was associated with the plasma membrane, consistent with the role of IK_{Ca} in endothelium-dependent hyperpolarization. This is similar to functional data for this channel obtained in porcine coronary,^{5} rat mesenteric^{6} and rat middle cerebral arteries.^{1} Evidence for expression of IK_{Ca} in the smooth muscle was unexpected and contrasted to its absence from smooth muscle in the rat mesenteric artery using the same protocols (S.L.S. and C.J.G., unpublished results, 2005). Smooth muscle staining for IK_{Ca} was apparent both at the plasmalemma and within the cytoplasm. The veracity of our data regarding IK_{Ca} expression in the middle cerebral artery is supported by the use of 3 different IK_{Ca} (K_{Ca}-3.1,16,17) antibodies. Although further investigation of a possible functional role for muscle IK_{Ca} is called for, a tonic role for these channels in hyperpolarizing and relaxing the smooth muscle seems unlikely. The addition of TRAM-34 did not stimulate any increase in arterial tone,^{2} which would be predicted when smooth-muscle Ca^{2+} levels were elevated in association with myogenic tone.

In summary, when endothelial NOS is inhibited, IK_{Ca} activity alone can explain EDHF-mediated hyperpolarization and relaxation in middle cerebral arteries. When the NO/cGMP pathway is active, both SK_{Ca} and IK_{Ca} then contribute to SLIGRL–induced hyperpolarization. Thus, functional SK_{Ca} activity can be uncovered in the rat middle cerebral artery, although the input it makes to hyperpolarization and relaxation does not appear marked under normal conditions. However, sex hormones such as estrogen can modulate the relative contribution made to vasodilation by EDHF and NO in the cerebral circulation,^{25} and estrogen can upregulate SK_{Ca} expression.^{26,27} Therefore, it is likely that the importance of SK_{Ca} differs significantly in disease states where estrogen levels are altered or NO release is in some way compromised.

Acknowledgments

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References


Figure I. Validation of rabbit anti-hiK1 antibody M20. 10 μg total cell extract from untransfected and stable CHO cell lines expressing hSK1, hSK2, hSK3 and hiK1 was loaded in each lane. The arrow indicates the predicted size of tetrameric channel complex. The lower molecular mass bands are likely a combination of degradation products or trimers, dimers and monomers of the hiK1 complex.