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Evidence for Involvement of Both IK\textsubscript{Ca} and SK\textsubscript{Ca} Channels in Hyperpolarizing Responses of the Rat Middle Cerebral Artery

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Background and Purpose—Endothelium-derived hyperpolarizing factor responses in the rat middle cerebral artery are blocked by inhibiting IK\textsubscript{Ca} channels alone, contrasting with peripheral vessels where block of both IK\textsubscript{Ca} and SK\textsubscript{Ca} is required. As the contribution of IK\textsubscript{Ca} and SK\textsubscript{Ca} to endothelium-dependent hyperpolarization differs in peripheral arteries, depending on the level of arterial constriction, we investigated the possibility that SK\textsubscript{Ca} 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 K\textsubscript{Ca} 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 K\textsubscript{Ca} 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 IK\textsubscript{Ca} (TRAM-34, 1 μmol/L) or SK\textsubscript{Ca} (apamin, 50 nmol/L) alone or in combination. In contrast, the associated smooth-muscle hyperpolarization 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 SK\textsubscript{Ca} and IK\textsubscript{Ca} within the endothelium.

Conclusions—In the absence of NO, IK\textsubscript{Ca} underpins endothelium-dependent hyperpolarization and relaxation in cerebral arteries. However, when NOS is active SK\textsubscript{Ca} 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 SK\textsubscript{Ca} 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 IK\textsubscript{Ca} channels alone is sufficient to block endothelium-derived hyperpolarizing factor (EDHF)—mediated relaxation and hyperpolarization in rat middle cerebral arteries.\(^1\) This is a dramatic contrast to other peripheral vessels, where in general EDHF–mediated responses are abolished by combined inhibition of both SK\textsubscript{Ca} and IK\textsubscript{Ca} in the endothelium.\(^3\)

In arteries where blockade of EDHF–mediated responses require the simultaneous block of SK\textsubscript{Ca} and IK\textsubscript{Ca}, 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 IK\textsubscript{Ca} channels in cerebral vessels may reflect a differential expression of K\textsubscript{Ca} subunits. In middle cerebral arteries, for example, SK\textsubscript{Ca} subunits may be absent, have a low expression level, or perhaps be masked by high IK\textsubscript{Ca} expression levels. Alternatively, the cellular location of the K\textsubscript{Ca} subtypes may differ between the endothelium and the smooth muscle. However, there is currently little evidence on the relative expression and cellular distribution of SK\textsubscript{Ca} and IK\textsubscript{Ca} in rat middle cerebral arteries, although functional data does suggest that IK\textsubscript{Ca} are localized to endothelial cells.\(^3\)

The apparent dominant role of IK\textsubscript{Ca} in EDHF responses in the middle cerebral artery may reflect experimental parameters. IK\textsubscript{Ca} and SK\textsubscript{Ca} mediate individual components of endothelium-dependent hyperpolarization in rat mesenteric arteries. SK\textsubscript{Ca} underpin smooth-muscle hyperpolarization, whereas IK\textsubscript{Ca} reverse agonist-induced depolarization–repolarization.\(^7\) Thus, the extent of vasoconstrictor stimuli influences the relative contribution of IK\textsubscript{Ca} and SK\textsubscript{Ca} for subsequent changes in membrane potential, so that when the smooth-muscle cells...
are depolarized and constricted by $\alpha$ stimulation (with phenyl-
ephine), block of endothelium-dependent hyperpolarization and
relaxation requires inhibition of endothelial SK$_{Ca}$ and IK$_{Ca}$. 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. For example, in rat posterior
cerebral artery mounted at 10 mm Hg, smooth-muscle cell
resting membrane potential is $\approx -67$ mV, whereas after develop-
opining myogenic tone at 60 mm Hg the membrane potential is
$\approx -38$ mV. Inhibition of nitric oxide synthase (NOS) in
cerebral arteries will often constuct and depolarize the smooth
muscle further. The observation that EDHF in rat middle
cerebral artery is solely dependent on IK$_{Ca}$ might reflect some
physiological masking of SK$_{Ca}$ 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 SK$_{Ca}$ and define any modulation by endothelium-derived NO in the rat middle cerebral artery. Furthermore, using immunohistochemistry we investigated the expression and distribution of K$_{Ca}$ 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 $\mu$m) were mounted in a Multivy-Halpern myograph (model 400A, Danish Myotechnology) in Krebs solution containing (mmol/L): NaCl, 118.0; NaCO$_3$, 24; KCl, 3.6; MgSO$_4$, 1.12; glucose, 11.0; CaCl$_2$, 2.5; gassed with 95% O$_2$ and 5% CO$_2$ 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 140 mm Hg) in order to increase spontaneous myogenic constric-
tion. Smooth-muscle tension was recorded with an isometric
pressure transducer and Powerlab software (ADI, Australia). Vessel
response was equilibrated for at least 20 minutes before vasodilator responses were
assessed by relaxation to papaverine (150 $\mu$mol/L) 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.

Statistical Analysis

Results are expressed as the mean±SE mean of n animals. Tension values are in mN (always per 2 mm segment) and E_m as mV. Vasodilatation is expressed as percentage reduction of the total
vascular tone (myogenic tone plus vasoconstrictor response) as-
essed by relaxation to papaverine (150 $\mu$mol/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 TRAM-34 alone (20.3±3.6%, n=6), and apamin had no further 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).
Morphology and K\textsubscript{Ca} 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- derothelial 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 (K\textsubscript{Ca}2.2), SK3 (K\textsubscript{Ca}2.3) and IK1 (K\textsubscript{Ca}3.1) 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 SK\textsubscript{Ca} 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 SK\textsubscript{Ca} was not dependent on the level of prior arterial constriction. In contrast, when either endothelial NOS or soluble guanylyl cyclase were inhibited, IK\textsubscript{Ca} alone underpinned endothelium-dependent hyperpolarization and the associated relaxation, as previously reported.\textsuperscript{1,2} We also show, using immunohistochemistry, that both SK\textsubscript{Ca} and IK\textsubscript{Ca} 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.\textsuperscript{2}

It is unclear why SK\textsubscript{Ca} 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.\textsuperscript{7} In quiescent mesenteric artery, EDHF...
NO may indirectly activate SK

Ca.

SKCa was inhibited by blockade of BK

Ca. Hyperpolarization produced by addition of an NO donor, 20-HETE, a mediator involved in development of myogenic constriction, cytochrome P450, via the hydroxylase pathway, produces

of cytochrome P450–dependent enzymes.

Arterial stimulation can alter the contribution of IK

and IK

on EC derived projections with a thin (B and inset), whereas those between adjacent smooth-muscle cells (C and inset) and adjacent endothelial and smooth-muscle cells were typically small (D and insets). These latter myoendothelial gap junctions were present on EC derived projections with a thin (∼100 nm wide) stalk passing through the internal elastic lamina (IEL) with a bulbous enlargement at the EC-SMC interface (asterisk; ∼1.2 μm at the widest point; D and D, lower inset). The lower inset in D shows a section with a pentalaminar structure (upper inset) characteristic of a gap junction 4 serial sections (∼400 nm) past that shown in D (main panel). Bar, A, 5 μm; B through D, 0.5 μm; B, C, insets, 50 nm; D, upper inset, 25 nm; D, lower inset, 1 μm.

Hyperpolarization is almost totally attributable to SK

activity whereas during vasoconstriction with phenylephrine this changes, so both SK

and IK

contribute to the EDHF response. Thus, at least in the mesenteric artery, the extent of arterial stimulation can alter the contribution of IK

and SK

to endothelium-dependent hyperpolarization. This is clearly not the case in the middle cerebral artery, as IK

contributed to endothelium-dependent hyperpolarization over a range of tensions equal to or greater than observed in the presence of l-NAME, when SK

activity was not detected.

An alternative explanation could be that NO, or an associated reactive oxygen species, facilitates SK

activation. NO donors have been shown to stimulate SK

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 SK

channels, but was inhibited by blockade of BK

channels. Thus, a direct effect of NO on SK

seems unlikely, although an additive effect perhaps requiring rises in intracellular Ca

at sites near the SK

channels cannot be excluded. Another possibility is that NO may indirectly activate SK

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 BK

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 SK

channels.

Another possibility relates to the ultrastructural and morphological characterization of the arterial cells and K

channels. Throughout this study, we used the same sized vessels (diameter ∼175 μmol/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 IK

and SK

were expressed in the endothelium, and SLIGRL stimulates an endothelium-dependent response through a receptor coupled by G

to rises in intracellular Ca

, 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 IKCa 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 SKCa and IKCa.4,15,24

IKCa expression was observed in both the smooth muscle and smooth-muscle cells. The endothelial cell expression was associated with the plasma membrane, consistent with the role of IKCa in endothelium-dependent hyperpolarization. This is similar to functional data for this channel obtained in porcine coronary,5 rat mesenteric6 and rat middle cerebral arteries.7 Evidence for expression of IKCa 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 IKCa was apparent both at the plasmalemma and within the cytoplasm. The veracity of our data regarding IKCa expression in the middle cerebral arterial support is by the use of 3 different IKCa (KCa3.1,16,17) antibodies. Although further investigation of a possible functional role for muscle IKCa 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 Ca2+ levels were elevated in association with myogenic tone.

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

Acknowledgments

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Figure 1. 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.