Endothelial coordination of cerebral vasomotion via myoendothelial gap junctions containing connexins 37 and 40

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There is clear heterogeneity in the mechanisms that underlie vasomotion, and specifically whether the endothelium plays a mandatory or modulatory role. In the rat iris arteriole, rabbit ear, and superior mesenteric artery, vasomotion is reported to be endothelium independent (3, 18, 39), whereas in the rat mesenteric, rabbit femoral artery, and hamster aorta, vasomotion is suggested to be endothelium dependent, as shown by its abolition with endothelium denudation (12, 21, 34, 38, 40).

Endothelium-dependent effects on vasomotion have been attributed to the vasodilatory factors nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). In some studies, endothelial cells play an essential role in synchronizing the activity of smooth muscle cells through the release of NO, with endothelium denudation desynchronizing adjacent smooth muscle cell [Ca2+]i oscillations, NO inhibition abolishing vasomotion, and exogenous cGMP activating vasomotion (11, 30, 41). In contrast, in other studies, NO has an apparent inhibitory effect on vasomotion (17, 18, 20, 34, 38, 48, 51, 52). Loss of vasomotion and desynchronization of smooth muscle cell [Ca2+]i oscillations, which mimic endothelium removal, have also been reported in rat mesenteric artery after incubation in the classical EDHF and calcium-activated potassium channel (KCa) blockers apamin and charybdotoxin, thus implying a role for EDHF in vasomotion (8, 34, 38).

Cerebral artery vasomotion occurs in vivo and in vitro (9, 13) and may be integral for regulation of cerebral blood flow. The physiological control of the cerebral circulation differs in many respects from that of other systemic vessels, and mechanisms reliant on changes in membrane voltage play a critical role (7). Thus the hypothesis that the endothelium modulates spontaneous vasomotion of cerebral vessels by direct electrical coupling, rather than by the release of diffusible vasodilators, was investigated in the rat basilar artery.

MATERIALS AND METHODS

The investigation was conducted under protocols approved by the Animal Experimentation and Ethics Committees of the Australian National University and the University of New South Wales.

Electrophysiology. Male Wistar rats (14–17 days postnatal) were anesthetized with ether and killed by decapitation before removal of the brain into cold dissection buffer containing (in mM) 3 MOPS, 1.2 NaH2PO4, 4.6 glucose, 2 pyruvate, 0.02 NaEDTA, 0.15 albumin, 145

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NaCl, 4.7 KCl, 2 CaCl₂, and 1.2 MgSO₄. The basilar artery and its side branches, in a segment of pial membrane, were pinned via the pia to Sylgard in the base of a recording chamber to secure vessels for electrode impalement and superfused with Krebs solution (3 ml/min, 33–34°C), as described previously (13). Electrophysiological studies were conducted on the primary and secondary branches of the basilar artery. In some vessels the endothelium was removed by rubbing the intimal surface with a tungsten wire before electrical recordings. Endothelial cell damage was confirmed by the absence of vasodilation to acetylcholine (10 μM), while the integrity of the smooth muscle cells was determined by the presence of relaxation to the smooth muscle cell-dependent vasodilator and NO donor S-nitroso-N-acetylpenicillamine (SNAP; 10 μM).

Preparations were equilibrated for 20 min or until spontaneous activity was constant in frequency and amplitude. This spontaneous activity indicated that the in vitro preparations used in the present study mimicked those in vivo, where similar myogenic activity has been previously described in this vascular bed (9). Vessel diameter was continuously monitored using video microscopy (Hamamatsu Performance Vidicon camera) and Diamatrak edge detection program (36); vascular cells were impaled with sharp microelectrodes (120–220 MΩ; Flaming Brown micropipette puller, Sutter Instrument) filled with propidum iodide (0.2% in 0.5 M KCl) to confirm cellular identity. Membrane potentials were defined as the most negative values recorded. Records were amplified with an Axoclamp 2B (Axon Instruments). Simultaneous changes in membrane potential and vessel diameter in the region where the cell was impaled were stored for analysis using pclamp software (Axon Instruments).

In some preparations with either intact or damaged endothelium, short segments were cut to 1.3 ± 0.1 mm (n = 28) in length using a microsurgical knife (Sharpoint) to determine input resistance by injection of hyperpolarizing current pulses (0.1–1.0 nA, duration 30–45 s). In these studies, microelectrodes were dipped in silicone, and the fluid level of the organ bath was reduced to a minimum to reduce electrode tip capacitance. Recordings were made in discontinuous-current-clamp mode with switching frequencies of 200–500 Hz, and the electrode capacitance was compensated by monitoring the head-stage voltage to ensure that the settling of tip capacitance did not distort the voltage recording.

Changes in [Ca²⁺]. Measurement of [Ca²⁺] in smooth muscle cells was made after incubation in the ratiometric fluorescent dye fura-2 AM, as described previously (13). To facilitate loading, a section of the pia was removed from over the vessel, resulting in an apparently smaller diameter than that of vessels used for electrophysiology. Changes in [Ca²⁺], in the arterial wall were measured with a photometry system (TILL Photonics; pclamp 8 software), while changes in individual smooth muscle cell [Ca²⁺], were measured with an intensified cooled CCD (Princeton Instruments; Axon Imaging system). Simultaneous measurements of vessel diameter and [Ca²⁺], were made by illuminating the preparation with infrared light (775 nm; Hamamatsu Performance Vidicon camera). Measurements of [Ca²⁺], were made before and after incubation for 20 min in drugs. Electrophysiological and calcium data were obtained from different preparations.

Quantitative PCR and mRNA expression. The basilar artery and its branches were removed from the meninges and immersed in RNA-later (Ambion). Three samples, each containing vessels from 12 animals, were snap frozen in liquid nitrogen, homogenized, and processed using the RNeasy Mini Kit (Qiagen), including 20 min incubation with DNase, according to the manufacturer’s instructions. mRNA was reverse transcribed to cDNA (42°C 1 h, 50°C 1 h, 90°C, 10 min) by using oligo(dT) primers (500 ng/μl Invitrogen) and Superscript II (200 U/μl Invitrogen). Reactions from which reverse transcriptase or RNA was omitted were run in parallel to control for contaminating DNA.

Quantitative PCR was used to determine absolute copy numbers of connexins (Cx37, 40, and 43) at high magnification, as described previously (43). For high-resolution antigen localization studies, segments of basilar artery from four rats were frozen at high pressure (BAL TEC, HPM 010 or Leica EM HPF; −2,100 bar), freeze-substituted (Leica, AFS; −90°C) in 0.1% uranyl acetate in acetone over 4 days, infiltrated, and embedded in LR gold at −25°C polymerized under UV light. Individual serial thin transverse sections were mounted on Formvar- and carbon-coated slot grids and incubated in 1% normal donkey serum/
BSA, 0.2% Tween-20 in PBS (30 min), followed by rabbit anti-rat Cx37 (AbD), rabbit anti-mouse Cx40 (Chemicon), rabbit anti-rat Cx43 (Zymed) or mouse anti-smooth muscle α-actin (Sigma) in blocking buffer (18 h at 37°C or 4°C), and followed by 5 nm colloidal gold-conjugated secondary antibody (1:40; British Biocell; 2 h) in 0.01% Tween-20. Specificity of Cx antibodies has been previously demonstrated by using Western blotting and immunohistochemistry on tissues and transfected cells (42, 44). Sections were treated with 1% glutaraldehyde in PBS and stained conventionally.

Drugs and solutions. The following drugs were obtained commercially: apamin, charybdotoxin, ACh, and SNAP (Sigma Chemical), N\(^{\text{G}}\)-nitro-L-arginine methyl ester (l-NAME; Saphire Bioscience), fura-2 AM and pluronic acid (Molecular Probes), cGMP sodium salt (DBcGMP, Alexis Biochemicals). Charybdotoxin was dissolved in PBS containing 0.1% BSA. Cx-mimetic peptides were synthesized by the Australian Cancer Research Foundation Biomolecular Resource Facility, John Curtin School of Medical Research; purity >97%. All other drugs were made up as ×1,000 stock solutions in distilled water and diluted into Krebs solution.

Analysis of results. Frequency of depolarizations or contractions in both control and drug solutions was determined over 5 min, while the amplitude was the average of 15 consecutive responses. Changes in vascular tone were measured in different vessels. Analysis of results. Frequency of depolarizations or contractions in both control and drug solutions was determined over 5 min, while the amplitude was the average of 15 consecutive responses. Changes in [Ca\(^{2+}\)]\(_{\text{cyt}}\) were expressed as the ratio of the fluorescence emission recorded in response to 340 nm and 380 nm (F\(_{340/380}\)) excitation, whereby the F\(_{340/380}\) in control is defined as 100%. Results are means ± SE of N preparations where each preparation was from a different rat. Data analysis and graphs were prepared with GraphPad Prism (GraphPad Software). Results were analyzed for statistical significance (P < 0.05) by using paired or unpaired t-tests as appropriate.

RESULTS

Rhythmic activity in dye-identified cells. Microelectrode recordings from a propidium iodide-identified endothelial cell (Fig. 1A) and smooth muscle cell (Fig. 1B) showed that during spontaneous vasomotion, rhythmical depolarizations were present in both cell types, and these preceded rhythmical contractions (Fig. 1, C and D, respectively). No difference was observed in the resting membrane potential between the two cell types when impalements were made in the same preparation (endothelial cell −45 mV; smooth muscle cell −45 mV, n = 1). A single recording was made from an identified endothelial cell in a total of 32 stable impalments in intact vessels.

Endothelium removal. Endothelium removal tended to depolarize smooth muscle cell membrane potential (+endothelium: −42.2 ± 0.9 mV, n = 20, −endothelium: −39.9 ± 0.7 mV, n = 20, unpaired t-test; P > 0.05), and rhythmical depolarizations were decreased in amplitude (+endothelium: 6.3 ± 0.7 mV, n = 20; −endothelium: 3.6 ± 0.2 mV, n = 20; P < 0.05, unpaired t-test) and increased in frequency (Fig. 2A; +endothelium: 16.2 ± 1.0 min\(^{-1}\), n = 20; −endothelium: 31.2 ± 1.7 min\(^{-1}\); n = 20; P < 0.05, unpaired t-test). Endothelium denudation led to loss of coordinated rhythmical contractions (Fig. 2, A and B) without significant effect on vascular tone (+endothelium: 45.9 ± 2.2 μm, n = 53; −endothelium: 50.7 ± 2.8 μm, n = 40, unpaired t-test), although measurements were made in different vessels.

Calcium oscillations were smaller and irregular with endothelium removal, although no significant change in basal calcium levels occurred (Fig. 2B; +endothelium: 0.53 ± 0.026 F\(_{340/380}\), n = 33; −endothelium: 0.53 ± 0.017 F\(_{340/380}\), n = 20, unpaired t-test). Smooth muscle cell imaging showed that oscillations in [Ca\(^{2+}\)]\(_{\text{cyt}}\) were still present in individual cells, but these were asynchronous with neighboring cells (Fig. 2C).

Application of hyperpolarizing current pulses to short arterial segments showed that smooth muscle cell input resistance
A + endothelium - endothelium

Membrane potential

Diameter

B Wall calcium

Diameter

C SMC calcium

Fig. 2. Influence of the endothelium on rhythmic activity. Rhythmic depolarizations and contractions (A) and calcium oscillations and contractions in the arterial wall (B) and in individual SMCs (C; n = 6 cells), recorded from either endothelium-intact (+ endothelium; left) or different endothelium-denuded (− endothelium; right) preparations. Upward deflection indicates increased intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). Basal SMC [Ca\(^{2+}\)]\(_i\) levels in endothelium-intact preparations and endothelium-denuded preparations were 0.64 \(F_{340/380}\) and 0.52 \(F_{340/380}\), respectively, where \(F_{340/380}\) is the ratio of the fluorescence emission recorded in response to 340 nm and 380 nm excitation.

was significantly greater in endothelium-denuded vessels (+ endothelium: 5.2 ± 0.9 mV; n = 12; − endothelium: 11.7 ± 0.9 mV; n = 16; P < 0.05, unpaired t-test).

Confirmation of endothelium removal and integrity of smooth muscle cells. In endothelium-intact preparations, ACh (10 μM) hyperpolarized the smooth muscle (control: −42.2 ± 0.9 mV; ACh: −51.7 ± 1.7 mV; n = 20; P < 0.05, paired t-test), decreased basal calcium levels in the arterial wall (ACh: 89.9 ± 0.1% of control \(F_{340/380}\); n = 33, P < 0.05, paired t-test), abolished vasomotion, and caused the vessel to relax (control: 45.9 ± 2.2 μm; ACh: 50.0 ± 2.2 μm; n = 53; P < 0.05, paired t-test). In contrast, in endothelium-denuded preparations, ACh had no significant effect on smooth muscle cell membrane potential (control: −39.9 ± 0.8 mV; ACh: −39.0 ± 1.0 mV; n = 20, P > 0.05, paired t-test), basal calcium levels in the arterial wall were increased (ACh: 109.5 ± 0.5% of control \(F_{340/380}\); n = 20, P < 0.05, paired t-test), and a small but significant constriction was observed (control: 50.7 ± 2.8 μm; ACh: 49.9 ± 2.8 μm; n = 40; P < 0.05, paired t-test). These results confirmed successful removal of the endothelium because vasodilation following the addition of ACh was abolished (P < 0.05, unpaired t-test). ACh-induced vasconstriction in endothelium-denuded vessels confirmed the integrity of the smooth muscle because similar responses have been described in the cerebral, coronary, mesenteric, and renal circulation under conditions in which the endothelium has either been removed or has been shown to be dysfunctional (10, 28).

In endothelium-intact preparations, the effects of SNAP on membrane potential (SNAP: −52.0 ± 1.3 mV; n = 20, paired t-test), basal calcium levels (SNAP: 91.1 ± 0.3% of control \(F_{340/380}\); n = 33, paired t-test), or vessel diameter (SNAP: 50.2 ± 2.2 μm; n = 53, paired t-test) were indistinguishable from ACh. In endothelium-denuded preparations, SNAP hyperpolarized the smooth muscle cells (SNAP: −51.1 ± 1.2 mV; n = 20; P < 0.05, paired t-test), decreased basal calcium levels in the arterial wall (SNAP: 91.0 ± 1.7% of control \(F_{340/380}\); n = 20; P < 0.05, paired t-test), and caused a significant relaxation (SNAP: 53.8 ± 2.8 μm; n = 40; P < 0.05, paired t-test). These effects were indistinguishable from those of SNAP in intact preparations, providing further confirmation of the integrity of the smooth muscle cells.

Role of NO. Endothelium denudation did not mimic inhibition of NO synthase with L-NAME (10 μM; Fig. 3). L-NAME increased the frequency (control: 15.4 ± 0.7 min\(^{-1}\); L-NAME: 19.6 ± 0.9 min\(^{-1}\); n = 8; P < 0.05, paired t-test; Fig. 3A) and amplitude of rhythmic depolarizations (control: 5.3 ± 1.1 mV; L-NAME: 9.4 ± 1.0 mV; n = 4; P < 0.05, paired t-test; Fig. 3A), contractions (control: 3.3 ± 0.4% resting vessel diameter; L-NAME: 5.8 ± 0.5% resting vessel diameter; n = 8; P < 0.05, paired t-test, Fig. 3, A and B), arterial wall [Ca\(^{2+}\)]\(_i\) oscillations (L-NAME: 153.0 ± 3.3% of amplitude of control oscillations in the arterial wall (B). Oscillations in individual SMC [Ca\(^{2+}\)]\(_i\) were more synchronized (C). Upward deflection indicates increased [Ca\(^{2+}\)]\(_i\). The representative control basal calcium level in the arterial wall was 0.73 \(F_{340/380}\). Dashed horizontal lines are a visual aid at the same comparative point for comparing left and right column data.

Fig. 3. Effect of nitric oxide synthase (NOS) inhibition on spontaneous activity. The NOS inhibitor N\(^o\)-nitro-L-arginine methyl ester (L-NAME; 10 μM) increased the frequency and amplitude of rhythmic depolarizations (A) and contractions (A and B) and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) oscillations in the cerebral, coronary, mesenteric, and renal circulatory systems. The NOS inhibition with SNAP hyperpolarized the smooth muscle because similar responses have been described in the cerebral, coronary, mesenteric, and renal circulation under conditions in which the endothelium has either been removed or has been shown to be dysfunctional (10, 28).

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F$_{340/380}$ oscillation; $n = 4$; $P < 0.05$, paired $t$-test; Fig. 3B) and individual smooth muscle cell [Ca$^{2+}$]$_i$ (Fig. 3C). L-NAME decreased vessel diameter (control: 46.9 ± 3.9 μm; L-NAME: 44.9 ± 3.5 μm; $n = 8$; $P < 0.05$, paired $t$-test; Fig. 3, A and B), without significant effect on membrane potential (control: −40.5 ± 0.5 mV; L-NAME: −39.5 ± 2.5 mV; $n = 4$, paired $t$-test; compare in Fig. 3A) or arterial wall basal calcium levels (L-NAME: 104 ± 2.9% of control F$_{340/380}$; $n = 4$, paired $t$-test; Fig. 3B). Moreover, in endothelium-denuded preparations in which vasomotion was disrupted and oscillations in [Ca$^{2+}$]$_i$ in adjacent smooth muscle cells were asynchronous, addition of DBcGMP (300 μM) did not restore rhythmicity (Fig. 4).

**Role of EDHF.** Apamin (0.5 μM) and charybdotoxin (60 nM) caused smooth muscle cell depolarization (control: −44.6 ± 3.5 mV; apamin + charybdotoxin: −27.8 ± 2.2 mV; $n = 4$; $P < 0.05$, paired $t$-test; Fig. 5A), vessel constriction (control: 65.8 ± 5.6 μm; apamin + charybdotoxin: 52.3 ± 5.2 μm; $n = 4$; $P < 0.05$, paired $t$-test), and the development of irregular uncoordinated contractions (Fig. 5, A and B). Rhythmic depolarizations were increased in frequency (control: 21.5 ± 3.5 min$^{-1}$; apamin + charybdotoxin: 43.5 ± 8.4 min$^{-1}$; $n = 4$; $P < 0.05$, paired $t$-test) and amplitude (control: 2.7 ± 0.5 mV; apamin + charybdotoxin: 8.8 ± 2.2 mV; $n = 4$; $P < 0.05$, paired $t$-test; Fig. 5A). Oscillations in [Ca$^{2+}$]$_i$ in the arterial wall became irregular (Fig. 5B), and in adjacent smooth muscle cells, oscillations in [Ca$^{2+}$]$_i$ were reduced in amplitude ($P < 0.05$) and unsynchronized (Fig. 5C). Similar results on vessel tone (endothelium: 64.2 ± 3.1 μm; apamin + charybdotoxin: 52.8 ± 4.8 μm; $n = 4$; $P < 0.05$, paired $t$-test), resting membrane potential (endothelium: −37.2 ± 1.1 mV; apamin + charybdotoxin: −27.5 ± 0.6 mV; $P < 0.05$, paired $t$-test), and arterial wall basal calcium levels in control is 0.44 F$_{340/380}$. Dashed horizontal lines are a visual aid at the same comparative point for comparing left and right column data.

Fig. 4. Effect of DBcGMP (300 μM) on rhythmical activity in the absence of the endothelium. In endothelium-denuded preparations, incubation in DB-cGMP (300 μM) had no significant effect on rhythmical depolarization and SMC membrane potential (A, top) or small contractions and vessel diameter (A, bottom). Basal calcium levels and the small irregular calcium oscillations observed in the arterial wall were also not different from control (B). Calcium oscillations in adjacent SMCs remained unsynchronized (C; $n = 6$ cells). Upward deflection indicates increased [Ca$^{2+}$]$_i$. The basal arterial wall [Ca$^{2+}$]$_i$ level in control was 0.44 F$_{340/380}$. Dashed horizontal lines are a visual aid at the same comparative point for comparing left and right column data.

Fig. 5. Effect of combined apamin (0.5 μM) and charybdotoxin (60 nM) (Apa/ChTx) on basilar artery vasomotion and conventional immunohistochemistry of IK1 (SK4) expression. Small-conductance (SKCa) and intermediate/large-conductance Ca$^{2+}$-activated potassium channel (I/BKCa) inhibition with apamin and charybdotoxin (ChTx) increased rhythmical depolarization frequency and amplitude and depolarized membrane potential (A). Arterial wall [Ca$^{2+}$]$_i$, oscillations became irregular (B); rhythmic contractions were reduced, and vessel movement became irregular (A and B). [Ca$^{2+}$]$_i$ oscillations recorded in individual adjacent SMCs became asynchronous (C; $n = 6$ cells). Upward deflection indicates increased [Ca$^{2+}$]$_i$. The representative basal calcium level in control is 0.44 F$_{340/380}$. Dashed horizontal lines are a visual aid at the same comparative point for comparing left and right column data. Whole mount IK1 (SK4) localization, indicative of IKCa, was found in smooth muscle (D, left) and endothelial cells (EC; D, right). Smooth muscle and endothelial images are of the same field of view but from different focal planes. No staining was found when the primary antibody was omitted from the incubation solution (insets). Longitudinal vessel axis runs left to right. Bar, 25 μm.
Because apamin and charybdotoxin block small (SKCa)-, intermediate (IKCa), and large-conductance KCa (BKCa), and voltage-gated potassium (Kv) channels, and our previous electrophysiological studies showed similar effects with IKCa inhibition, but no effect of individual inhibition of either SKCa, BKCa, or Kv, (13), the distribution of IKCa protein was investigated using immunohistochemistry. Localization of IK1 (SK4), indicative of IKCa, was found in both smooth muscle and endothelial cells (Fig. 5, inset), while staining was absent when the primary antibody was omitted (Fig. 5, inset).

Effect of gap junction uncouplers. 37,43Gap27 (100 μM; n = 12) abolished rhythmical depolarizations, contractions (Fig. 6A), and oscillations in [Ca2+]i in the arterial wall and in individual smooth muscle cells (Fig. 6B, C, respectively). Smooth muscle cells hyperpolarized (control: −42.8 ± 2.3 mV; 37,43Gap27: −54.3 ± 2.8 mV; n = 4; P < 0.05, paired t-test), and the vessels relaxed (control: 37.6 ± 4.6 μm; 37,43Gap27: 41.8 ± 4.8 μm; n = 12; P < 0.05, paired t-test). 40Gap27 (100 μM) also abolished vasomotion but did not hyperpolarize or significantly relax the arteries (Fig. 7, A and B; control: −38.7 ± 1.9 mV; 40Gap27: −39.4 ± 1.3 mV; n = 4; control: 31.9 ± 3.5 μm; 40Gap27: 32.7 ± 4.3 μm; n = 8).

Rhythmical depolarizations and individual smooth muscle cell [Ca2+]i oscillations were significantly reduced in amplitude (P < 0.05, paired t-test) and were no longer synchronized (Fig. 7, A and C).

Connexins and endothelial and myoendothelial gap junctions. Uniform punctate Cx37 was detected by immunohistochemistry in the media (Fig. 8A). In contrast, Cx40 was absent, and Cx43 was only detected in the cytoplasm of occasional smooth muscle cells (Fig. 8B and C, respectively). Cx37, Cx40, and Cx43 were detected at adjacent endothelial cell borders (Fig. 8D–F and K–M). Pentalaminar membrane, characteristic of gap junctions, was present between adjacent endothelial cells (Fig. 8, J and N).

Confocal imaging of Cx37- and Cx40-labeled tissue at the internal elastic lamina-smooth muscle cell interface suggested the presence of Cx37 and Cx40 but not Cx43 at these sites (Fig. 9, A–D). High-resolution ultrastructural labeling confirmed this distribution of myoendothelial Cxs (Fig. 10).

The media of the main side branch of the basilar artery consisted of two to three smooth muscle cell layers (2.4 ± 0.2, n = 6, Fig. 10, A and B). Serial sections through 5-μm-long vessel segments contained myoendothelial gap junctions (Fig. 10, B–F) and homocellular endothelial cell gap junctions (Fig. 8, J and N). All myoendothelial gap junctions were characterized by the presence of pentalaminar membrane structures between endothelial cells and smooth muscle cells (Fig. 8, C–F, inset). Myoendothelial gap junction density was 3.2 ± 0.9 per endothelial cell (n = 6; mean endothelial cell size 388 ± 18.6 μm², n = 6).

The ultrastructural integrity of the vessel wall was well maintained in tissue prepared for high-resolution antigen studies (Fig. 10, A and D–F), with pentalaminar membrane, char-
characteristic of gap junctions, found between adjacent endothelial cells. No subendothelial or sub-smooth muscle space adjacent to the internal elastic lamina was present, in contrast to conventionally prepared tissue (Fig. 10A; compare with Fig. 10, B and C, respectively). Controls conducted on serial sections that were exposed to either primary antibody preincubated in antigenic peptide or to secondary antibody alone showed no specific gold label (Fig. 10E, inset).

As in conventionally prepared tissue (Fig. 10, B and C), endothelial cell-derived projections were found to abut smooth muscle cells (Fig. 10, D–F). At these points of close association, small regions of pentalaminar membrane were present, and Cx37- and Cx40-conjugated gold label, but not Cx43-conjugated gold, was localized (Fig. 10, D and F). These sites were typical of myoendothelial gap junctions found in arteries using conventional ultrastructural methods (compare with Fig. 10, B and C).

mRNA for Cx37, Cx40, and Cx43 was detected in the basilar artery, with Cx37 expression being 20- and 60-fold higher than Cx40 and Cx43, respectively (Cx mRNA expression: Cx37, 38,381 ± 5,534; Cx40, 2,218 ± 605; Cx43, 607 ± 115, as normalized mRNA copy number/10⁶ copies of 18S rRNA; n = 3 RNA samples).

DISCUSSION

In the primary branch of the rat basilar artery, the present data show that the endothelium plays an essential role in initiating vasomotion by synchronizing [Ca²⁺]ᵢ oscillations among adjacent smooth muscle cells. This effect is not mediated by either NO or EDHF but rather by coupling of the endothelium and smooth muscle by myoendothelial gap junctions consisting of Cx37 and Cx40. These junctions enable electrical coupling between the two cell layers and facilitate the synchronization of vasomotor activity along the vessel length.

A similar loss of vasomotion and desynchronization of adjacent smooth muscle cell [Ca²⁺]ᵢ oscillations has been...
Fig. 10. High-resolution immunohistochemistry of myoendothelial connexin expression. High-pressure frozen, freeze-substituted vessels showed good preservation with no shrinkage around the IEL (A) compared with tissue prepared with conventional ultrastructural methods (B and C). Schwann cells (sc) surrounded perivascular varicosities (v) containing synaptic vesicles (A, inset). Myoendothelial gap junctions (MEGJs) showed pentalaminar membrane in conventional (B and C; asterisk and inset between arrows in C) and high-pressure frozen tissue (D–F, between arrows). Pentalaminar EC-EC gap junctions (C, between arrowheads; F, left inset) were found close to MEGJs. Gold-conjugated antibodies against Cx37 and Cx40 were localized to MEGJs (D and inset; F and right inset). Incubation of the adjacent serial section to that labeled with Cx40-gold in the absence of antibody showed no gold labeling (E and inset). Cx43 was not observed at MEGJs. Bar: A–F and A, inset, 1 μm; C–F, inset, 50 nm.

reported in rat mesenteric artery after endothelial denudation. This was attributed to loss of endothelium-derived NO and failure to activate a cGMP-dependent depolarizing current essential for the initiation of agonist-induced vasomotion in this vessel (11, 41). In contrast, in the present study, incubation with the NO synthase inhibitor l-NAME augmented vasomotion of the basilar artery and improved the synchronization of [Ca²⁺]i oscillations in individual smooth muscle cells, consistent with previous studies of cerebral artery vasomotion (5, 17, 24). Furthermore, a cGMP-activated depolarizing current is unlikely to be important for initiating vasomotion in the basilar artery because the smooth muscle membrane potential was not altered by endothelium removal or inhibition of NO. In addition, application of DBcGMP to endothelium-denuded preparations failed to resynchronize adjacent smooth muscle cell [Ca²⁺]i oscillations or restore vasomotion.

EDHF was also not responsible for the initiation of vasomotion in the basilar artery. While incubation with apamin and charybdotoxin did result in loss of regular contractions and appearance of asynchrononous smooth muscle cell [Ca²⁺]i oscillations, these effects were accompanied by significant depolarization and constriction, an effect similar to that found previously after individual inhibition of IKCa but not BKCa, SKCa, or Kᵦ (13). Because apamin and charybdotoxin produced the same effect in the absence of the endothelium, an action at IKCa in the smooth muscle is likely, and, indeed, IKCa is present in the media of the basilar artery, as shown in the present study, and in other cerebral vessels (35). Thus the loss of basilar artery vasomotion after Kᵦ inhibition can be attributed simply to an increase in smooth muscle cell [Ca²⁺]i, after membrane depolarization, leading to augmented vasocostriction and a reduction in the amplitude and synchronization of oscillatory behavior of smooth muscle cells. The modeling of Koenigsberger et al. (23) suggests that when [Ca²⁺]i is low or high, smooth muscle cells may be in a steady state, while between these extremes they may exhibit calcium oscillations.

Thus any effect that results in an increase in mean calcium levels may reduce or abolish oscillatory behavior by movement toward a more stable state. In the present study, this effect followed closure of smooth muscle IKCa rather than release of EDHF from the endothelium.

The electrophysiological demonstration of increased smooth muscle cell input resistance in endothelium-denuded preparations and similar voltage oscillations in dye-identified endothelial and smooth muscle cells provide evidence that the two cell layers of the basilar artery are electrically coupled. The anatomic data confirm the presence of extensive myoendothelial gap junctions composed of Cx37 and Cx40. At the mRNA level, Cx37 was the predominant Cx expressed in the basilar artery, with these latter data being consistent with the extensive Cx37 protein expression among endothelial cells and weak Cx37 expression in smooth muscle cells in this and previous studies (19). The absence of Cx40 and Cx43 in smooth muscle, but presence of both Cxs in the endothelium (19), suggests that there may be greater potential for intimal than medial coupling in the basilar artery. Interestingly, at the ultrastructural level in the media of muscular arteries, gap junctions are sparse and small (45, 46), implying that the potential for control of the synchronization of smooth muscle cells within the media alone is limited.

The data of the present study suggest that cell coupling through the endothelium might play a critical role in the generation of basilar artery vasomotion by synchronizing smooth muscle cell activity. While previous studies have pointed to the involvement of gap junctions in vasomotion in a number of vascular beds through the use of putative gap junction blockers, such as heptanol, octanol, and 18β-glycyrrhetinic acid (2, 22, 31, 48), these inhibitors have been shown to exert additional nonspecific effects (3, 4, 16, 31, 50, 53). Peptides mimicking segments of the amino acid sequence of the extracellular loops of Cx proteins have also been used to examine the gap junctional dependence of vasomotion in rabbit
superior mesenteric artery (3). The involvement of gap junctions in basilar artery vasomotion was examined in a similar manner using the Cx-mimetic peptides 37,43Gap27 and 40Gap27, with both peptides abolishing vasomotion and 40Gap27 desynchronizing adjacent smooth muscle cell \([Ca^{2+}]_i\) oscillations, in a similar manner to endothelium removal. However, the peptides also reduced \([Ca^{2+}]_i\) oscillations in smooth muscle cells, and 37,42Gap27 hyperpolarized the membrane, effects not readily explained by an action on gap junctions alone.

The apparent nonjunctural effects of 37,43Gap27 and 40Gap27 in the basilar branch are similar to nonjunctural effects of heptanol and glycyrretinic acid in rat mesenteric artery (31). Interestingly, hyperpolarization, relaxation, and decreased wall \([Ca^{2+}]_i\), in pressurized mesenteric and cerebral arteries incubated in 37,43Gap27 or glycyrretinic acid were attributed to heterogeneous responses of individual smooth muscle cells to exogenous stimuli, cell coupling thus facilitating a uniform response throughout the muscle (6, 25). Non-specific effects were discounted due to the failure of gap junction antagonists to affect agonist- or depolarization-induced vasoconstriction (6, 25), although an effect on hemichannels cannot be ruled out (29). Unfortunately, other than the technically difficult method of pinocytically loading endothelial cells with Cx antibodies (32), no other selective antagonists for gap junctions are currently available.

It should be noted that the arteries used in the present study were stretched and pinned to the bottom of the recording chamber. These conditions enabled us to continuously monitor activity in the same cell over extended periods of time despite the oscillating vessel diameter and changes in tension after drug additions. However, caution should be exercised when extrapolating our results to the in vivo state because the arterial segments were not intraluminally perfused. Nevertheless, even under these conditions, the vessels exhibited spontaneous vasomotion as described in vivo (9), and the smooth muscle cell membrane potentials in our vessels were depolarized to a similar extent as pressurized vessels in vitro (6).

The present data support the concept that there is considerable heterogeneity in the mechanisms that underlie vasomotion in different segments of the vasculature. While the endothelium is essential for vasomotion in both the mesenteric and basilar artery, in the former the role of the endothelium is to produce vasodilatory factors to synchronize smooth muscle cell activity (34, 38, 41). In contrast, in the basilar artery these factors do not initiate vasomotion, but rather the endothelium facilitates current spread along the vascular wall by indirectly coupling smooth muscle cells through myoendothelial gap junctions composed of Cx37 and Cx40. Thus the endothelium acts to coordinate voltage changes in individual smooth muscle cells and synchronize calcium fluxes through voltage-dependent calcium channels, which are responsible for the coordination of smooth muscle cell \([Ca^{2+}]_i\), oscillations (13).

**REFERENCES**


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