Evidence for the involvement of myoendothelial gap junctions in EDHF-mediated relaxation in the rat middle cerebral artery

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ABSTRACT

The mechanisms underlying endothelium-dependent hyperpolarizing factor (EDHF) in the middle cerebral artery (MCA) remain largely unresolved. In particular, very little is known regarding the way in which the signal is transmitted from the endothelium to the smooth muscle. The present study tested the hypothesis that direct communication via myoendothelial gap junctions contributes to the EDHF response in the male rat MCA.

EDHF-mediated dilations were elicited in rat MCAs by the luminal application of ATP or UTP in the presence of L-NAME and indomethacin. Maximum dilation to luminal ATP ($10^{-4}$M) was reduced significantly following incubation with a gap peptide cocktail (9±4%; n=6) compared with a scrambled gap peptide cocktail (99±1%; n=6; p<0.05). A gap peptide cocktail was without effect on the amplitude of endothelial cell hyperpolarization in response to UTP ($3\times10^{-5}$M; 22±3mV versus 22±1mV; n=4) while smooth muscle cell hyperpolarization was significantly attenuated (17±1mV versus 6±1mV; n=4; p=0.004). Connexin 37 (Cx37) was localized to the smooth muscle and Cx43 was localized to the endothelium, while Cx40 was found in both endothelium and smooth muscle. Electron microscopy revealed the existence of frequent myoendothelial junctions (MEJs). The total number of MEJs/µm MCA sectioned was 2.5±0.5. Our results suggest that myoendothelial communication contributes to smooth muscle cell hyperpolarization and EDHF dilation in male rat MCA.

Keywords: connexins; endothelium-derived hyperpolarizing factor; gap junctions; vascular smooth muscle
INTRODUCTION

Endothelium-derived hyperpolarizing factor (EDHF) remains an incompletely described phenomenon whose identity appears to vary in different vascular beds (2). In the rat middle cerebral artery (MCA), we know that it originates in the endothelium and is not nitric oxide (NO), prostacyclin or a cyclooxygenase metabolite (41). Stimulation of the endothelial cells evokes an increase in endothelial cell (EC) intracellular calcium (24), triggering the opening of EC intermediate calcium-sensitive K⁺ channels (IK₁Ca), and leading to EC hyperpolarization (25). These events in the EC are followed by hyperpolarization of the underlying smooth muscle cells (SMC) (41). There are two possibilities by which hyperpolarization of the EC may be communicated to the SMC—either a paracrine factor is released from the EC that diffuses to the SMC or there exists a functional communicating junction between the EC and SMC.

In some vessels, a paracrine factor has been shown to mediate SMC hyperpolarization. One that has been considered extensively is the family of arachidonic acid metabolites known as the epoxyeicosatrienoic acids (EETs). Elevation in intracellular EC Ca²⁺ can activate phospholipase A₂, releasing arachidonic acid that is a precursor of EETs. The EETs are thought to diffuse to the SMC, activate the large-conductance, Ca²⁺-activated K channels (BK₁Ca), leading to hyperpolarization of the smooth muscle (3, 14). However, the EDHF response in rat MCA is resistant to the BK₁Ca inhibitor, iberiotoxin (25) and inhibitors of EET formation (39).

In other vessels, K⁺ has been shown to act as a paracrine factor. The efflux of K⁺ through EC IK₁Ca channels results in an accumulation of K⁺ in the extracellular space that in turn activates inwardly rectifying K channels (Kᵢr) and Na⁺-K⁺ ATPase on the SMC.
thereby producing hyperpolarization (8). However, events in MCA are controversial, with evidence for (31) and against (41) an involvement of $K_{ir}$ channels and $Na^+-K^+$ATPase. Such discrepancies may reflect differences between agonists or other methodologies.

Hydrogen peroxide has also been considered to be a paracrine factor by hyperpolarizing the SMC by activation of $K_{Ca}$ channels. There is evidence supporting a role for hydrogen peroxide in some vessels (29, 30, 32) but not others (10, 18) including the rat MCA (39).

An alternative mechanism by which events in the EC may be communicated to the SMC is by direct contact. Ultrastructural evidence suggests that ECs can extend cellular protrusions through perforations in the internal elastic lamina to come into close contact with SMCs (myoendothelial gap junctions, MEGJs) in rabbit carotid artery (36) and rat mesenteric artery (34, 35). Furthermore, electrical coupling between ECs and SMCs has been demonstrated in hamster retractor muscle feed arteries (11), rat mesenteric arteries (35) and guinea-pig submucosal arterioles (7). Further support for a role of gap junctions in mediating EDHF has been provided by the use of connexin inhibitor proteins (1, 4, 9). However, not all blood vessels possess MEGJs (9, 20, 35).

Evidence to date suggests that the existence of a universal EDHF is highly unlikely (2). Vessel size, location and age may all be critical factors determining the underlying mechanism of EDHF. In particular, mechanisms of dilation in cerebral arteries can be different compared with peripheral arteries. For example, activation of $IK_{Ca}$ channels alone are sufficient to elicit EC hyperpolarization and EDHF-mediated dilation in rat cerebral arteries (25), while in peripheral arteries both $IK_{Ca}$ and a small-conductance, $Ca^{2+}$-activated K channel ($SK_{Ca}$) is additionally involved (8). Since we have
no evidence that a paracrine factor mediates EDHF in rat MCA, we turned to the possibility that there is direct communication between ECs and SMCs. The present study tested the hypothesis that MEGJs exist in MCA, that they provide a pathway for current spread between EC and SMC, and that this contributes to EDHF in this vascular bed.
MATERIALS AND METHODS

Experiments were carried out in accordance with the NIH guidelines for the care and use of laboratory animals and were approved by the Animal Protocol Review Committee at Baylor College of Medicine and in accordance with the National Health and Medical Research Council of Australia and approved by the Monash University Animal Ethics Committee. Male Long-Evans rats (275-325g) were housed under a 12 hr light/12 hr dark cycle with unrestricted access to food and water.

Harvesting and Mounting Cerebral Vessels

Male Long-Evans rat (n=12) were placed in an anesthetic chamber, allowed to spontaneously breathe isoflurane and then decapitated. The brain was removed from the cranium and placed in cold physiological salt solution (PSS). The MCA was excised, cleaned of surrounding connective tissue and cannulated with micropipettes in a custom-made teflon-coated vessel chamber (ChuelTech, Houston, TX). The temperature was maintained at 37°C via a microprocessor controlled heating block. PSS was circulated abluminally (6ml) and perfused luminally. Continuous monitoring of intraluminal pressure was achieved via in-line transducers, which were connected to two strain gauge panel meters (Omega, Stamford, CT). Once mounted, vessels were tested for leaks by clamping the proximal and distal tubing and monitoring intraluminal pressure. Vessels that did not maintain a steady pressure were discarded. The vessel chamber was mounted on the stage of an inverted microscope. Transmural pressure was set at 85 mmHg with a flow of 150 µl/min through the lumen, and the vessels allowed to equilibrate for 1 hr. During this time they developed spontaneous tone by constricting from their fully dilated
diameter at initial pressurization. After the development of tone, the experiment was initiated (see *EDHF-Mediated Dilations*).

**EDHF-Mediated Dilations**

Following the development of spontaneous tone, the luminal and abluminal compartments were exposed to either a cocktail of connexin mimetic peptides (gap peptides) or their scrambled sequences and incubated for 1 hr (300µM of each peptide). The gap peptide cocktail (courtesy of Dr Dale Pelligrino, University of Chicago at Illinois) consisted of a combination of the synthetic peptide homologous to a region of the second extracellular loop of connexin 37 (Cx37) and Cx43 (SRPTEKTIFII; \(^{37,43}\text{Gap 27}\)), the peptide homologous to a region of the first extracellular loop of Cx43 (VCYDKSFPIHV\(^{33}\text{Gap 26}\)) and the peptide homologous to a region of the second extracellular loop of Cx40 (SRPTEKNSFIV; \(^{40}\text{Gap 27}\) (5). A cocktail of scrambled peptides, negative controls, consisted of the scrambled peptide of \(^{37,43}\text{Gap 27}\) (FKTIRTISIEP), the scrambled peptide of \(^{43}\text{Gap 26}\) (PSDVFRSCVHKYI) and the scrambled peptide of \(^{40}\text{Gap 27}\) (VTNIEVPSFR). All peptides were made to a purity >95%. S-Nitroso-N-acetylpenicillamine (SNAP; \(5\times10^{-5}\text{M}\)) was used to verify that SMC vasodilator function was intact in the presence of gap peptide inhibition.

\(\text{N}^{0}\text{-nitro-L-arginine methyl ester (L-NAME; 3x10^{-5}\text{M}) and indomethacin (10^{-5}\text{M}) were added to the luminal and abluminal solutions for 30 min in order to remove the NO synthase and cyclooxygenase contributions, respectively. EDHF-mediated dilations were assessed by concentration response curves to luminal application of ATP (10^{-7} to 10^{-4}\text{M}).} \)
Experiments were terminated by replacing PSS with calcium-free PSS containing 1mM EGTA in order to determine the maximum diameter of the vessel.

**Electrophysiological Experiments**

Long-Evans male rats (n=30) were anesthetized with chloroform and decapitated. The MCA was isolated as described above. One end was cut open longitudinally and pinned to the base of a vessel chamber with the ECs facing uppermost, allowing for EC impalement. The other end remained intact with the outer SMCs facing uppermost, allowing for SMC impalement. Preparations were superfused with PSS and allowed to equilibrate for 30min. The vessels were exposed to L-NAME and indomethacin in all experiments.

EC and SMC membrane potentials were recorded independently using intracellular glass micropipettes, tip filled with 2% Lucifer Yellow, back filled with 1M KCl and had resistances of approximately 100 MΩ (35). The Lucifer Yellow diffused into the cell during impalement, permitting identification of each cell impaled. Lucifer Yellow was the dye of choice to unequivocally identify the cell impaled since it does not spread to the other cell type (23). Criteria for successful impalement of a cell included an abrupt change in voltage upon cell entry, a return to baseline voltage upon exiting the cell and a maintenance of pre-entry electrode resistance.

The EC was stimulated using UTP (3x10⁻⁵M), applied for 1 min. We have previously shown that both ATP and UTP produce comparable EDHF-mediated dilations (15, 41) and SMC hyperpolarization in rat MCA (unpublished data). MCAs were incubated for 1 hr with the gap peptide cocktail or vehicle (PSS). In some experiments,
membrane potential responses to UTP were assessed in the presence of charybdo
toxin (5x10^{-8}M) and apamin (5x10^{-7}M; both from Auspep, Australia), inhibitors of IK_{Ca} and
SK_{Ca} channels, respectively.

Connexin Immunofluorescence

Preparation of Whole Mounts: Rats were anesthetized with sodium pentobarbital
(60mg/kg, ip) and the vascular system perfused by a transcardial approach with
heparinized saline solution (20 units/ml). Once cleared of blood, animals were perfusion
fixed with 2% paraformaldehyde in 0.1M phosphate buffer (PBS). After decapitation,
MCAs were removed and immersion fixed in the same solution. The MCA was cut open
longitudinally, with the intimal side facing upwards, and pinned flat on silicone
elastomer. These preparations were stored in PBS at 4ºC until ready to use in the
immunofluorescence protocol.

Preparation of Cross-Sections: Rats were prepared as described above. MCAs
were dissected from the brain, placed in optimum cutting temperature compound and then
snap frozen in dry-ice chilled 2-methylbutane. Tissues were cut at 10µm-thick cross-
sections using a cryostat (-20°C), mounted on glass slides and allowed to air dry. The
sections were stored at −20°C until ready to use in the immunofluorescence protocol.

Immunofluorescence Light Microscopy: MCA whole-mounts or cross-sections
were washed in cold PBS, permeabilized (0.1% Triton-X 100) and blocked (0.1% Tween-
20, 10% goat or donkey serum in PBS). Primary antibody was added for either 2 hr at
room temperature (Cx43), overnight at room temperature (Cx40), or overnight at 4ºC
(Cx37). Cx37 and Cx43 were localized using rabbit polyclonal anti-peptide antibodies
(6µg/ml; Alpha Diagnostics and Sigma, respectively). Cx40 was localized using a guinea pig polyclonal antibody (courtesy of Dr Robert Gourdie, University of South Carolina).

Following two wash steps, labeling was assessed by secondary detection with either Alexa Fluor 488 fluorochrome conjugated to goat anti-rabbit IgG (Cx43), donkey anti-rabbit FITC (Cx37), or goat anti-guinea pig biotin (Sigma, 1:250 dilution) followed by streptomyacin-fluorescein (Cx40; Amersham, 1:250 dilution).

The specimens were then treated with DAPI (1µM, Molecular Probes) for nuclear detection and mounted in Airvol. Aortic sections were used as positive control for Cx43 to demonstrate the specificity of the antibody. For negative controls, sections were treated with non-immune rabbit or guinea-pig IgG and the primary antibody was omitted. Deconvolution microscopy (Delta Vision, Applied Precision, Issaquah, WA) was used to evaluate connexin immunolabeling.

**Electron Microscopy**

Anesthetized rats (n=3) were perfusion fixed as described above. Fixation was achieved using Sorenson’s PBS containing 3% glutaraldehyde and the brain was removed and immersed in this fixative overnight at 4°C. The following day, the MCA was dissected from the brain and placed in PBS at 4°C. Tissue samples were post-fixed in 1% tannic acid (5 min) followed by 1% osmium tetroxide (1 hr) and then aqueous uranyl acetate (1 hr). Samples were subsequently dehydrated in a graded ethanol series, embedded in Araldite resin and ultrathin sections (100nm) were obtained using an ultramicrotome (RMC 7000, RMC, AZ) equipped with a diamond knife. Sections were stained with uranyl acetate and lead citrate before viewing with a JEOL 200CX electron microscope.
microscope. Gap junctions are defined by the pentalaminar appearance of the membranes at points of cell-to-cell contact where the distance between opposing membranes is \( \leq 3.5\text{nm} \) (33). In the present study we used this criterion to define a myoendothelial gap junction (MEGJ). In this case, both the EC and SMC basal laminae are breached and the two cell types are in close contact (\( \leq 3.5\text{nm} \)). However, there were instances where the distance between the opposing membranes exceeded 3.5nm, and this type of cell-cell association was defined as a myoendothelial junction (MEJ).

**Reagents and Buffers**

All chemicals used to assess EDHF-mediated dilations were purchased from Sigma (St Louis, MO, USA). The composition of PSS contained the following (mM): NaCl 119, NaHCO\(_3\) 26, KCl 4.7, KH\(_2\)PO\(_4\) 1.18, MgSO\(_4\) 1.17, CaCl\(_2\) 1.6, glucose 5.5 and EDTA 0.026. Stock solutions of ATP (10\(^{-2}\)M), UTP (10\(^{-2}\)M), and L-NAME (3x10\(^{-2}\)M or 10\(^{-1}\)M) were prepared in distilled water, aliquotted and frozen. Indomethacin stock solution (10\(^{-2}\)M) was in Na\(_2\)CO\(_3\): distilled water (1:1 by weight). Gap peptides and scrambled peptides were dissolved directly in PSS.

**Data Analysis and Calculations**

Data were expressed as mean±sem and n indicates the number of animals tested. Diameter measurements were averaged over 2 mins immediately following luminal exposure to ATP. Changes in vascular diameter are presented as a percentage of the maximum diameter of the MCAs, as described previously (15).
Statistical comparisons of the concentration-response curves to ATP were made using a two-way analysis of variance with repeated measures and multiple comparisons were made using a Student-Newman-Keuls test. For the electrophysiological data, statistical significance was tested using a one-way analysis of variance with repeated measures. Post-hoc comparisons were made using a Student-Newman-Keuls test. Differences were considered significant at error probabilities less than 0.05 (p<0.05).
RESULTS

Effect of Gap Peptides on EDHF-Mediated Dilations

After equilibration, resting MCA diameter was similar between groups: 220±15µm (scrambled peptides) and 229±21µm (gap peptides). To assess the participation of gap junctions in the EDHF-mediated response, MCAs were tested randomly following incubation with a gap peptide cocktail (n=6) or a scrambled peptide cocktail as the control (n=6). Figure 1 illustrates the diameter changes to increasing concentrations of luminal ATP. The maximum dilation to ATP was significantly reduced in the presence of the gap peptide cocktail (9±4%; n=6) compared with scrambled gap peptides (99±1%; n=6; p<0.05). Gap peptides selectively attenuated EDHF-mediated dilations, without affecting dilation to luminal delivery of the NO donor, SNAP (5x10⁻⁵M; 95±2%; n=6; data not shown).

Effect of Gap Peptides on EDHF-Mediated Hyperpolarizations

Resting membrane potentials of dye-identified ECs and SMCs (Figure 2A) were -34±0.4mV (n=21) and -37±1mV (n=22), respectively. UTP (3x10⁻⁵M) produced hyperpolarizations of similar amplitudes in EC (22±2mV; n=12) and SMC (16±4mV; n=12). Exposure to gap peptides had no effect on the resting membrane potential in either EC (-35±1mV versus -35±4mV, n=4) or in SMC (-39±2mV versus -37±2mV, n=4).

In the presence of gap peptides, UTP-mediated hyperpolarizations in SMC were significantly attenuated (34±8% of the initial response; n=4; Figure 2B). In contrast, UTP-mediated hyperpolarizations in ECs were maintained (103±16% of the initial response; n=4; Figure 2B). The hyperpolarization to UTP in EC and SMC was
abolished by charybdotoxin and apamin. EC hyperpolarization to UTP was $21\pm5\text{mV}$ (n=4) and $0\pm0\text{mV}$ (n=4) prior to and following charybdotoxin and apamin, and in SMC the values were $17\pm5\text{mV}$ (n=3) and $0\pm0\text{mV}$ (n=3).

**Localization of Connexin Immunofluorescence**

Connexin immunofluorescence was detected using both whole mount sections and frozen cross-sections of rat MCA. In whole mount sections, EC nuclei appeared in focus first (arranged along the longitudinal axis of the vessel wall) followed by SMC nuclei (arranged circumferentially). This allowed easy distinction between the two cell types. In pilot studies, the endothelial layer was confirmed with anti-von Willebrand factor (data not shown). In frozen cross-sections, the SMC were positively identified with anti-smooth muscle $\alpha$-actin (data not shown). The internal elastic lamina, separating the endothelial layer from the SMC, could be visualized by autofluorescence through the FITC filter-set (green autofluorescence). The immunocytochemistry in cross sections revealed good evidence of punctate staining which suggests connexin labeling at gap junctions.

When viewed *en face*, Cx40 and Cx43 fluorescence decorated the borders of each EC with punctate staining (Figures 3A and B). Electron microscopy subsequently confirmed the presence of gap junction plaques at EC-EC contacts (Figure 6). Cx40 was also found in SMC (Figure 4B) while Cx43 was also found in the adventitia and parenchyma (Figure 4C). Note the intense Cx43 staining in the parenchyma, which has been reported previously in tissue directly beneath the pial surface (38). Positive staining for connexin proteins was confirmed when compared with sections incubated without the
primary antibody, sections incubated with the antigen plus the primary antibody, or with the appropriate non-immune IgG (data not shown). Neither Cx37 (data not shown) nor the IgG control showed EC staining (Figure 3C). However Cx37 was apparent in SMC (Figure 4A).

**Identification of Myoendothelial Gap Junctions**

Using electron microscopy, we examined MCA serial cross-sections (100nm thick) and performed a systematic evaluation of perforations (holes) in the internal elastic lamina (IEL). Sets of serial sections were analyzed for each perforation in the IEL. Of the total number assessed, 15% (15 of 101) of the perforations were not filled with any cellular projections. 83% (84 of 101) of the perforations contained an EC projection. Of these 84 perforations, 35 had serial sections encompassing the entire perforation. The average dimension of EC projections (measured in the long axis of the vessel) as they passed through the IEL was 434±41nm. In the remaining 49 perforations the serial sections were incomplete. The average dimension of these projections was 492±32nm. Analysis of these 84 perforations showed that 24% (20 of 84) of the time, the EC contacted, but did not breach, the basal lamina of the SMC.

MEJs require cellular penetration of the basal lamina of both the EC and SMC, thereby allowing the two cell types to come into close proximity. There was a 17% (14 of 84) incidence of this occurring. Our data suggest that of these, 6 appeared to be touching and, in some cases, a pentalaminar membrane structure characteristic of gap junctions was discernible (Figure 5). Based on these observations, the total number of MEJs/5μm MCA sectioned was 2.5±0.5 (n=3 rats). In 5 out of 6 cases, the MEJ consisted of an EC
projecting through the IEL and perforating the SMC basal lamina. In one case, the MEJ appeared at a site where both the EC and SMC perforated their respective basal laminae and extended towards one another (Figure 5). In an additional 8 cases a myoendothelial association was observed where the distance between the EC and SMC membranes was between 20 and 250nm.

Very few (2%) perforations showed a SMC projecting through the IEL towards the EC and none of these perforated the EC basal lamina. Note that during inspection of the MCA cross-sections, gap junctions between ECs were routinely observed (Figure 6).
DISCUSSION

The results of the present study suggest that in rat MCA (1) there is strong electrical communication between the EC and SMC (2) myoendothelial junctions are present and (3) gap junctions mediate the EDHF response, suggesting an important role for myoendothelial communication in mediating EDHF dilations in this vessel.

Effect of Gap Peptides on EDHF-Mediated Dilations

Historically, inhibition of gap junctions has been achieved using a variety of compounds, many of these possessing indirect effects. Aliphatic alcohols (octanol and heptanol) are believed to limit gap junction communication by dissolving in the lipid membrane while lipophilic compounds (18α-glycyrrhetinic acid and oleamide) may produce their effect through activation of protein kinases or G-proteins (12). In contrast, gap peptides are short synthetic peptides that possess sequence homology with conserved domains of the extracellular connexin loops. While these peptides do not discriminate between homocellular or heterocellular gap junctions, Gap27 has been shown to inhibit myoendothelial dye transfer in an endothelial-smooth muscle cell co-culture system (27). Although modulation of dye transfer does not necessarily reflect modulation of electrical transfer, the data suggest that the gap peptides may inhibit communication between EC and SMC. We elected to use a gap peptide cocktail directed towards Cx37, Cx40 and Cx43 since we observed positive immunofluorescence staining in the MCA for these proteins.

EDHF-mediated dilations were assessed in perfused and pressurized MCAs by applying ATP specifically to the lumen of the vessel in the presence of L-NAME and
indomethacin. We have shown previously that during this process, (1) a viable endothelium is required, (2) dilation persists when NO and prostanoid components are removed, (3) the smooth muscle is hyperpolarized and (4) $K_{Ca}$ channels are involved (40, 41). Therefore this response can be attributed to EDHF. Incubation with scrambled gap peptides was without effect on concentration-dependent dilation in the MCA (Figure 1). Following incubation with the gap peptide cocktail, maximum dilation to ATP was reduced significantly. The effect of gap peptides was not a general inhibition of dilation, since the NO donor, SNAP, produced near maximal dilations.

Gap junctions have been implicated previously in the EDHF response of rabbit carotid artery (4), guinea-pig carotid artery (9), rabbit mesenteric artery (21), rat mesenteric artery (28, 35) and the rabbit MCA (37). To our knowledge, this is the first study implicating a role for gap junctions in the rat MCA and demonstrating this in perfused and pressurized vessels. This is an important consideration since this preparation closely resembles the physiological situation of luminal pressure and shear stress.

**Effect of Gap Peptides on EDHF-Mediated Hyperpolarizations**

In the current study, the hyperpolarization was sustained in the endothelium in the presence of gap peptides while being significantly reduced in the smooth muscle, consistent with a role for myoendothelial communication in mediating the EDHF response. The gap peptides may impair EC-EC, SMC-SMC as well as EC-SMC gap junction communication. In rat mesenteric artery, where MEGJs are involved in the EDHF response, the hyperpolarization was also selectively impaired in the smooth muscle by gap peptides (35). In the same artery, selective loading of a connexin antibody
into EC depressed EDHF-mediated relaxation (28). However, in the study of Mather and colleagues (28), extracellular application of gap peptides was without effect on EDHF-mediated responses. In a wider range of arteries, gap peptides markedly reduce EDHF-mediated hyperpolarization (9, 17). Our data are consistent with those in other vessels (9, 17) and together infer a role for MEGJ in the EDHF response in a number of arteries including the MCA.

Our finding that EC and SMC resting membrane potentials were similar is consistent with functional electrical connectivity between the two cell types (see below). Sandow and colleagues (35) reported that in the rat femoral artery, the resting membrane potentials in EC and SMC were significantly different and no MEJs were detected. Therefore, the existence of MEJs may be commensurate with comparable resting membrane potentials in endothelium and smooth muscle. In the current study there were also some preparations that exhibited spontaneous action potential activity in ECs (data not shown). This is further support for electrical coupling between EC and SMCs as ECs cannot generate action potentials. Spread of action potentials from SMCs to ECs has been reported in highly coupled vessels such as guinea-pig arterioles (6).

To our knowledge, this study is the first to implicate a role for gap junctions in mediating EDHF dilations in a rat cerebral artery. Gap junctions have been implicated in the EDHF response in a variety of vessels (see above). It is always possible that a diffusible factor may also contribute to EDHF-mediated responses in MCA, which could account for the residual 7 mV hyperpolarization that persists in the presence of gap peptides. This response was consistently, but variably, delayed in onset and appeared to consist of a mixture of opposing depolarization and hyperpolarization (Figure 2A).
we have previously found no effect of ouabain or Ba$^{2+}$ on EDHF-mediated responses evoked by ATP in MCA of Long-Evans rats (41), the PAR-2 agonist SLIGRL evokes release of a ouabain and Ba$^{2+}$ sensitive paracrine dilator in MCA of Wistar rats (31).

**Localization of Connexin Immunofluorescence**

Immunocytochemistry of the rat MCA, revealed the presence of Cx40 in both the endothelium and smooth muscle. In agreement with our studies, Little and colleagues (22) observed Cx40 in ECs and SMCs of rat brain pial arterioles. Interestingly, Hong and Hill (19) reported that Cx43 staining was virtually absent from both ECs and SMCs of the rat MCA. It is possible that the disparity in findings may be attributed to the difference in rat strain (Wistar-Kyoto rats versus our Long-Evans rats) or age (5-7 wk old versus our 8-10 wk old animals).

While our data suggest that at least three connexin proteins are expressed in the rat MCA, it is not known which combination are located at MEGJs. Positive immunogold labeling would be a prerequisite in order to confirm the presence of connexin proteins at these junctions. However, this is a challenging prospect in the rat MCA. Since a MEGJ can be contained within a single 100nm section and appeared to involve a single point-point contact between ECs and SMCs, the number of connexin proteins available for gold labeling is most likely to be very small; perhaps even below the limit of detection.
Identification of Myoendothelial Gap Junctions

We have provided a detailed ultrastructural evaluation of the perforations in the IEL of the rat middle cerebral artery. It is interesting that while we observed many instances of endothelial cell projections passing through the IEL, the prevalence of MEGJs was significantly less. We employed rigorous morphologic criteria to permit accurate identification of gap junctions. The membranes of potential MEGJs could not be resolved on 5 occasions, even when goniometer tilting was utilized. Hence, we may be underestimating the presence of MEGJs by 5. Possible reasons that MEGJs are more technically challenging to resolve than gap junctions between ECs are related to the nature of the cell-cell contact in MEGJs. EC gap junctions (defined by pentalaminar membrane apposition) are typically larger and remain visible in multiple serial sections, making extensive lateral contacts at their cell-cell margins (Figure 6). In contrast, ECs and SMCs make small point-point contacts that are contained within a single 100nm serial section (Figure 5) that appear to be smaller than those reported previously in the rat mesenteric artery (34).

As discussed in a recent review (13), many published electron micrographs of putative MEGJs show membrane separation greater than that defined for a gap junction. In our preparation, we observed projections between EC and SMC, through holes in the IEL, where the distance between the two membranes was 20-250nm. This could ensure rapid paracrine communication, or could contain a small number of hemichannels or MEGJs, so small in area as not to be distinguishable as a pentalaminar MEGJ, or represent a dynamic structure capable of forming closer communications within a limited time frame.
In conclusion, the results of the present study demonstrate that MEGJ communication is involved in EDHF-mediated SMC hyperpolarization and dilation of the male rat MCA. Our findings expand the potential mechanisms associated with disrupted EDHF responses following pathological conditions such as stroke (26) and traumatic brain injury (16) and underscores the fact that cell-to-cell coupling plays a critical role in mediating cerebrovascular responses.

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LEGENDS TO FIGURES

Figure 1: Bar graph showing the concentration response curve for EDHF mediated vasodilation evoked by luminal ATP in the presence of L-NAME and indomethacin. Dilation to luminal ATP was significantly attenuated following incubation with the gap peptide cocktail (37,43Gap 27, 43Gap 26 and 40Gap 27; 300µM each; n=6; open bars) compared with the scrambled peptide cocktail (300µM each; n=6; solid bars). (* indicates p<0.05 compared with the scrambled peptide control; 2-way RM ANOVA).

Figure 2: (A) Membrane potential responses obtained in the endothelium and smooth muscle with and without gap peptides. UTP (3x10^{-5}M) was applied to the vessel for 1 min. Lucifer Yellow-filled endothelial cells and smooth muscle cells are shown. Dashed lines indicate orientation of the blood vessel. The solid arrow indicates the cell that was directly impaled with the microelectrode. The scale bar represents 50µm. (B) In the presence of gap peptide cocktail, endothelial cell hyperpolarization was sustained (open bars; n=4) while smooth muscle cell hyperpolarization was significantly attenuated (solid bars; n=4) in the same arteries.

Figure 3: *En face* immunostaining in the rat MCA of Cx40 (A), Cx43 (B), and IgG (C). Both Cx40 and Cx43 decorated the borders of endothelial cells while IgG control showed an absence of staining.

Figure 4: Immunofluorescence staining (green) of rat MCA cryosections with rabbit anti-rat Cx37 (A), guinea pig anti-rat Cx40 (B), and rabbit anti-rat Cx43 (C). Nuclei are
counterstained with DAPI (blue). Images are a maximum projection composite taken from 60 optical sections spaced at 0.15µm intervals. Punctate staining of Cx37 and Cx40 was apparent in the smooth muscle (arrowheads) while Cx43 staining was found in the adventitia and parenchyma (arrowheads).

Figure 5: Transmission electron micrographs of a rat MCA taken from three 100nm serial cross-sections showing a myoendothelial gap junction. (A) The low magnification shows a small endothelial cell (EC) protrusion extending towards a larger smooth muscle cell protrusion through a gap in the internal elastic lamina (indicated with *) where they meet. The area denoted by the box is magnified four times (C) showing the point-to-point contact between the EC and SMC (arrow). The width of the gap is ~ 3.5nm. Note that the serial sections preceding (B) and succeeding (D) the section containing the myoendothelial gap junction show the distance between the two cell membranes to be greater (20nm), demonstrating the point-point contact nature of the myoendothelial gap junction. The scale bar represents 500nm.

Figure 6: Transmission electron micrographs of a rat MCA taken from three 100nm cross-sections showing an endothelial cell-endothelial cell contact. (A) The low magnification shows a gap junction between endothelial cell 1 (EC1) and endothelial cell 2 (EC2). * indicates the internal elastic lamina. The area denoted by the box is magnified 7 times in (B) and (C) where one can identify the multi-laminar nature of the gap junction and observe the width of the gap to be ~3.5nm. In contrast to myoendothelial gap junctions appearing in a single cross-section, a single gap junction plaque between ECs
was visible in multiple serial sections spanning 600nm. The arrows indicate the depth of the gap junction within the endothelial cleft. The scale bar represents 200nm.
A. Cx40

B. Cx43

C. IgG Control
A. Cx37

B. Cx40

C. Cx43