Electrical Stimulation of the Endothelial Surface of Pressurized Cat Middle Cerebral Artery Results in TTX-Sensitive Vasoconstriction

David R. Harder and Jane A. Madden

Previous studies suggest that, in most arteries, nerve axons and their terminals are found within the adventitial layer, where the synaptic cleft can range between 800 Å and many microns. This finding would lead to the assumption that adventitial muscle cells receive greater innervation and a higher concentration of transmitter compared with muscle layers in the center or intimal areas of the artery that may, depending on diffusional barriers, receive less transmitter or none at all.

This study was designed to determine the electrical and mechanical responses of cat middle cerebral arteries to electrical stimulation of the adventitial vs. intimal surface of the vessels and to measure the resultant responses as a function of transmural pressure. Middle cerebral arteries were cannulated at both ends. Within each cannula was a stimulating electrode. Electrical stimulation (0.5-msec square pulses at 0.5 Hz yielding 160 µA of current between electrodes) resulted in significant reduction in diameter that was greater at both 40 and 80 mm Hg vs. 100 or 140 mm Hg. Conversely, adventitial stimulation of perivascular nerves with transmural platinum stimulating electrodes resulted in significant vasodilation. The constrictor response to intimal stimulation, as well as the dilatory response to adventitial stimulation, was blocked by tetrodotoxin. The constrictor response to luminal stimulation was enhanced by scorpion toxin demonstrating a functional role for tissues containing fast Na+ channels. Perfusion with collagenase to disrupt the endothelium also abolished the constrictor response to luminal stimulation. The divergence of responses between adventitial and luminal surface stimulation may suggest that different cell layers within a blood vessel serve different functions, one to increase resistance and another to decrease resistance. For example, in cat middle cerebral arteries, the adventitial nerves (i.e., via reflexes) may increase flow, while blood-borne substances may mediate release of agents that reduce flow. (Circulation Research 1987;60:831-836)

Materials and Methods

Adult mongrel cats (2.5-4.0 kg) of either sex were anesthetized with sodium pentobarbital (30 mg/kg), decapitated, and the brain removed. The middle cerebral arteries (MCAs) were dissected out and immediately placed in cold (4°C) physiologic saline solution (PSS) containing (in mM): Na+ 141, Cl- 125, Ca2+ 2.5, K+ 4.7, Mg2+ 0.76, H2PO4- 1.7, HCO3- 25, glucose 11, and N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) buffer 5.

For diameter measurements and electrical studies, one end of an 8-mm segment of MCA was threaded onto a 50-µm diameter plastic cannula and tied in place with 22-µm silk suture as described previously. In brief, the opposite end was likewise threaded onto a cannula that could either be left open for a flow-through system or clamped off to maintain a fixed pressure in the system. All side branches of the artery were tied off, and the vessel was adjusted to its approximate length in vivo. The exterior of the vessel was suffused with PSS at 37°C and aerated with 94% O2-6% CO2 for a pH of 7.4. The PSS was also perfused through the inflow cannula into the vessel lumen. The vessel was pressurized with PSS to 100 mm Hg for equilibration. This was accomplished by pressurizing a PSS-filled reservoir. Parallel between the pressure reservoir and the arterial preparation was a pressure transducer to monitor applied transmural pressure. Pressure measurements were made when the outflow cannula was clamped (blind system) so the flow resistance of the vessel did not need to be accounted for. During the 60-minute equilibration period, the pressure in the flow-through system declined gradually to about 80 mm Hg. Internal and external
diameter and wall thickness were observed with a video system containing a camera (RCA), TV monitor (Sony), and VCR (Panasonic) and were measured throughout the experiment with a Colorado Video, Inc., measuring system. Internal diameter and membrane potential ($E_m$) were measured with the outflow cannula clamped off so that the transmural pressure remained constant. All drugs were administered through the pressure transducer into the inflow cannula. Drugs and their concentrations perfused through the vessel were: collagenase (1 $\mu$g/ml), elastase (0.05 $\mu$g/ml), tetrodotoxin (TTX) (10$^{-6}$ M), and scorpion venom (10$^{-6}$ M). Potassium chloride (30 mM) and serotonin (3 x 10$^{-7}$ M) were applied via suffusion solutions. Arterial segments were perfused with the above enzymes for 2 minutes to disrupt or remove the endothelium. After this 2-minute period, cold (20° C) PSS was perfused through the vessel at 100 mm Hg to inactivate and wash out the enzymes. To test the integrity of the muscle within the vessel wall after endothelial disruption with enzymes, the response to 30 mM KC1 and 3 x 10$^{-7}$ M serotonin was examined before and after this procedure. Transmural pressure during this procedure did not exceed 100 mm Hg. When phentolamine (10$^{-6}$ M) is perfused through the vessel at 100 mm Hg 20 minutes prior to endothelial stimulation, all contractile responses are abolished (see Figure 6). To further explore the types of receptors responsible for this action of phentolamine, we blocked $\alpha_1$-receptors with prazosin (10$^{-6}$-10$^{-3}$ M), and stimulated $\alpha_2$-receptors with clonidine (10$^{-6}$-10$^{-3}$ M) and serotoninergic receptors with methysergide (10$^{-6}$ M). All vessels were perfused with the above agonists for 20 minutes at a transmural pressure of 100 mm Hg prior to electrical luminal stimulation with intact endothelium.

The endothelium was stimulated electrically with 30-$\mu$m diameter silver wires placed 1.5 cm apart inside of both the inflow and outflow cannulae. Most of the current was assumed to flow longitudinally through the vessel (Figure 1). The wires were connected to a Grass Stimulus Isolation Unit and S44 stimulator set (Quincy, Mass.) at 90 V, 0.5 Hz, and 0.5 msec. The square wave stimulus was applied for 30 seconds and resulted in 160 $\mu$A of current flow between the wires. Transmural nerve stimulation was accomplished via platinum plate electrodes placed 0.5 mm from the vessel on either side. Stimulation parameters were 240 V, with a square wave pulse 0.5 msec in width at 8 Hz for 20 seconds.

Intracellular $E_m$ was measured according to techniques previously described. Glass microelectrodes, filled with 3 M KCl, had tip resistances of 50–80 M$\Omega$ and tip potentials less than 4 mV. The primary criteria for a successful impalement were a sharp drop in voltage from baseline on entry of the microelectrode into the cell and a sharp return to zero on exit. Cell impalements were made with the aid of a sliding micromanipulator (Aus Jena).

The efficacy of endothelium disruption and/or removal with collagenase and elastase was documented by electron microscopy and discussed in a separate communication.

Statistical analysis of all data used paired and nonpaired Student's $t$ tests.

**Results**

**Mechanical Response of Isolated Cat MCA to Intimal Surface Electrical Stimulation**

Isolated cat MCA were prepared and stimulated as described in "Materials and Methods." Electrical stimulation via the intimal surface resulted in significant reduction in diameter in cannulated, pressurized arteries (Figure 2). This constrictor response was greater at lower transmural pressures and leveled off at pressures beyond 100 mm Hg. The percent reduction in diameter ranged between 7 and 3%. Again, as can be seen, perfusion of the vessel lumen with TTX (10$^{-6}$ M) to block activation of fast Na$^+$ channels eliminated the response to luminal surface electrical stimulation at all transmural pressures studied (Figure 2).

To examine the role of the endothelium in this regard, the cannulated MCAs were perfused with collagenase and elastase to disrupt the endothelial surface. Efficacy of endothelial disruption was assessed by transmission electron microscopy as described previously.

Figure 3 represents a separate series of experiments using vessels before and after endothelial disruption with collagenase. Disruption of endothelium abolished the constrictor response to electrical stimulation of the intimal surface. In all cases, the constrictor response with intact endothelium was significantly greater than zero, again with this response being greater at lower transmural pressures. After perfusion with collagen-
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• STIMULATION ONLY

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FIGURE 2. Absolute decrease in internal diameter of cat middle cerebral arteries cannulated at both ends and electrically stimulated by silver wires inside each cannula. Stimulation parameters were 0.5-msec square wave pulses at 0.5 Hz and supramaximal voltage yielding total current flow between electrodes measured at 160 μA. Solid bars, control conditions at each transmural pressure, i.e., suffused and perfused with control physiologic salt solutions; shaded bars, response to electrical stimulation when isolated vessel segments were perfused and pressurized with solutions containing tetrodotoxin (TTX, $10^{-6}$ M) to block excitation of neuronal tissue or cells activated through changes in conductance through fast Na$^+$ channels. Lines on top of each bar represent the SEM of 6 separate arteries. *p < 0.05. Note that after TTX treatment, vessel diameter did not decrease in response to electrical stimulation.

To determine if disruption of the endothelium with enzymes resulted in any significant damage to the muscle within the vessel wall, a separate series of experiments was done ($n = 5$ different vessels) comparing the response to suffusion of 30 mM KCl and $3 \times 10^{-7}$ M serotonin before and after enzyme perfusion (Figure 4). The contractile response, observed as a reduction in internal diameter, was not significantly different in control vs. enzyme-perfused vessels (Figure 4). The responses to 30 mM KCl and serotonin are compared to the maximum potassium chloride response (i.e., 90 mM) in untreated arteries. The initial diameter in all vessels used to generate the data in Figure 3 was between 320 and 405 μm. Perfusion of collagenase and elastase tended to increase the diameter at 100 mm Hg compared with the control but not significantly.

Beyond the obvious dilation vs. contraction action, these data are markedly different from those obtained in vessels that were electrically stimulated intraluminally. First, endothelial disruption had no significant effect on the response to electrical stimulation, and second, the electrically mediated response increased as adventitial nerve stimulation before and after endothelial removal (Figure 5).

Cat MCAs were prepared identically to those studied above. However, rather than stimulating the vessels via electrodes within the pressurization and outflow pipettes, they were stimulated via platinum electrodes across the adventitial surface (i.e., transmural nerve stimulation). Vessels were studied at transmural pressures of 80, 120, and 160 mm Hg before and after disruption of the endothelium with collagenase and elastase. Transmural nerve stimulation resulted in dilation, which became greater at higher transmural pressures (Figure 5). There was no significant difference in the increase in internal diameter on adventitial nerve stimulation before and after endothelial removal (Figure 5).

FIGURE 3. Effect of endothelial cell disruption on constrictor response to intimal surface electrical stimulation in cat middle cerebral arteries. Solid bars, absolute change in internal diameter in double cannulated isolated vessels in response to electrical stimulation via silver electrodes inside each cannula (0.5-msec square wave pulses at 5 Hz for 30 seconds and supramaximal voltage); shaded bar, response to same electrical stimulus in same arterial segments after perfusion of vessel interior with collagenase (at 80 mm Hg) to disrupt endothelium. The lines through each bar give the SEM of 6 arteries, before and after collagenase, in response to intimal electrical stimulation. *p < 0.05. No response to intimal surface stimulation after collagenase perfusion was significantly different from 0.

FIGURE 4. Decrease in diameter by middle cerebral arteries in response to 30 mM KCl and $3 \times 10^{-7}$ M serotonin in 6 arteries. Solid bars, vessel response before perfusion with collagenase; shaded bars, response by same vessel after collagenase perfusion. Responses are compared to that elicited in a control vessel by maximal stimulation with 90 mM KCl. Lines on top of each bar represent the SEM of measurements from 5 arteries. Collagenase treatment did not significantly change response to 30 mM KCl or serotonin.
Figure 5. Increase in diameter of middle cerebral arteries in response to adventitial stimulation. Solid bars, vessel response before perfusion with collagenase; shaded bars, response by same vessel after collagenase perfusion. Lines on top of each bar represent the SEM of measurements from 6 arteries. Vessel diameter increased more at higher transmural pressures. These findings are observed when the tone is myogenic as a result of pressure and not induced by an agonist.

A function of transmural pressure. Luminal stimulation-mediated contraction was abolished on endothelial disruption, and the response was less at higher transmural pressures.

Action of Phentolamine on Intimal Surface Stimulation

Pressurized cat MCAs were perfused with phentolamine to block α-adrenergic receptors in an initial attempt to define the nature of the excitatory substance presumably released from cerebral vascular endothelium (Figure 6). As can be seen, phentolamine blocked the response to electrical stimulation of the endothelial surface in its entirety.

Since phentolamine can block both α₁ and α₂ and also occupy serotonin receptors, we examined the action of agents specific for those receptors both on resting tone and the response to electrical stimulation of the arterial lumen. Clonidine, a pure α₂-agonist, had no effect on resting tone nor did it significantly alter the response to electrical stimulation (Figure 7A). Prazosin, an α₁-antagonist, was also without significant effect on resting tone or response to electrical stimulation (Figure 7A). Clonidine was chosen in this regard because if α₂-receptors were involved in the activation process observed here, we should see an excitatory effect, and because of its inhibitory effect on norepinephrine release, we might have seen a reduced response to electrical endothelial stimulation if conventional synaptic mechanisms were involved.9

In sharp contrast, however, perfusion of the serotonin antagonist methysergide both markedly dilated all vessels at a pressure of 100 mm Hg (by around 70%) and blocked all responses to electrical stimulation (Figure 7), suggesting that an intact serotonergic system may be necessary for this excitatory response. The response to methysergide indicated the use of clonidine rather than the α₁-blocker yohimbine, in that yohimbine blocks peripheral serotonin receptors as well.10

Figure 6. Effect of luminal stimulation on internal diameter of cat middle cerebral artery following 20 minutes' phentolamine perfusion. Following this 20-minute exposure in 6 vessels there was no significant reduction (or change) in response to electrical endothelial stimulation at any transmural pressure studied. Above each bar is the mean and SEM related to vertical lines through each bar.

Figure 7. To further explore the specific effect of phentolamine in blocking the contractile response to luminal stimulation we examined Panel A: Same experimental paradigm as in Figure 6 following 20 minutes' suffusion of the α₂-agonist clonidine (10⁻⁵ M) and the α₁-antagonist prazosin (10⁻⁵ M). Panel B: Comparison of response to electrical luminal stimulation following a 20-minute suffusion of serotonin antagonist methysergide (10⁻⁴ M) with control response. There were no significant differences in any of the data in Panel A (means and SEM given). In Panel B, response to stimulation is blocked significantly (p < 0.001) by methysergide.
Action of Scorpion Toxin

From the data presented thus far it appears that there is a constrictor substance released from a cell type or types within the endothelial layer of cat MCA. The data obtained following TTX suffusion suggested that the cell type may be neuronal in origin or may at least possess fast TTX-sensitive Na+ channels found in nerve but absent in smooth muscle.11

To test this hypothesis further, cannulated MCAs were perfused at a transmural pressure of 100 mm Hg with 10−7 g/ml scorpion toxin (Androctonus australis, Sigma Chemical Co., St. Louis, Mo.). The action of scorpion toxin is to "hold open" fast Na+ channels in neuronal tissue, thus maintaining a depolarized state and resulting in tonic release of neurotransmitter.12 If there are TTX-sensitive cells that release an agonist within the endothelial environment, scorpion toxin should release an agonist and mimic the response to electrical stimulation. This was, indeed, the case. Perfusion of scorpion toxin reduced the internal diameter of all 5 isolated MCAs studied by 11.8 ± 2.0% from a mean internal diameter of 258.0 ± 38 μm. The internal diameter after scorpion toxin infusion was significantly different from control (p < 0.002). The arteries were maintained at a transmural pressure of 100 mm Hg.

Effects of Intimal Surface Electrical Stimulation on Intracellular Electrical Activity

Glass microelectrodes filled with 3 M KCl were used to measure membrane potential in pressurized cerebral arteries in response to intimal surface stimulation. The resting E_m in 12 cells from 6 arteries was −50 ± 1.3 mV. Electrical stimulation of the intimal surface resulted in a 10.2 ± 1.8 mV change in resting E_m. At a transmural pressure of 100 mm Hg, intimal electrical stimulation resulted in excitatory electrical events associated with regenerative electrical activity similar to excitatory junction potentials (Figure 8). These responses were blocked by perfusion with TTX.

Obviously, it was not possible to know which cell layers we were in at the time of the impalement; however, attempts were made to pass through adventitial surface muscle cell layers to measure E_m in muscle cells close to the endothelial surface. (Transient impalements as the microelectrode was passed through the vessel were used as criteria for going through muscle cell layers.) Attempts at injecting markers into the impaled cells were unsuccessful due to the high resistance of the microelectrodes (70 MΩ). Conversely, stimulation of the adventitial surface results in membrane hyperpolarization.13

Discussion

This study provides evidence for the existence of specific cell types within the intact endothelium of cat cerebral arteries possessing fast Na+ channels and mediating vasoconstriction. Recent reports have demonstrated the existence of vasoconstrictors released from arterial endothelial cells.14-16 However, there are no reports in the literature documenting the existence of fast (TTX-sensitive and scorpion-toxin-sensitive) Na+ channels in endothelial cells.

The endothelium of cerebral arteries is very tight, comprising a functional blood brain barrier. Na+ permeability across this endothelial cell layer is blocked by amiloride, and its transport is furosamide sensitive.17 However, the Na+ permeability of the endothelium is largely unknown. If cerebral arterial endothelial cells do not contain fast Na+ channels, then the action of TTX, which blocks fast Na+ channels in nerve and nonsmooth muscle and scorpion toxin, which retards inactivation of fast Na+ channels in nerve,18 documents the existence of a cell type possessing some of the characteristics of neural tissue. However, until complete electrophysiologic analysis of ion conductance systems in cerebral arterial endothelium documents the presence or absence of fast Na+ channels, the possibility that this is not a normal function of these endothelial cells cannot be ruled out. Our data demonstrates that enzymatic disruption of cerebral artery endothelium does not significantly alter the medial layer muscle cell response to agonists.

One explanation of the different responses from ad-
ventitial and luminal surface electrical stimulation may be that within the cell layers of these cerebral arteries, some may function to decrease resistance to flow (ad- ventitial cell layers) while those muscle cells close to the endothelium may function to increase resistance. This would, of course, depend on the nature of the stimulus. For example, the substances released from vascular endothelium appear to be regulated by specific receptors mediating the responses to a specific chemical stimulant, such as acetylcholine, are usually dilatory, and are seen primarily in peripheral vessels.

It is interesting that the dilatory response to adventitial nerve stimulation becomes greater at higher transmural pressures in contrast to the constriction mediated by luminal stimulation, which is more prominent at lower transmural pressures. It is possible that, during adventitial stimulation, an equivalent amount of transmitter may exert a larger effect on a more depolarized membrane (these muscle cells depolarize as a function of increasing transmural pressures) or that more transmitter is released at higher pressures. Regardless of the mechanisms, such divergence further supports the idea that different systems are involved in these two diametric responses.

A divergence in membrane electrical responses of the muscle cells to adventitial vs. luminal stimulation brings up another interesting possibility. The muscle cell electrical response to luminal stimulation is excitatory, displaying depolarization and spike activity, whereas the muscle cell response to adventitial stimulation is inhibitory, displaying hyperpolarization and inhibition of spontaneous spike activity. Given that the muscle cells impaled with microelectrodes come from various cell layers because of the difficulty in impaling only adventitial or luminal surface cells, one could imagine several different responses depending on which stimulus (adventitial nerves or endothelial) was predominating: 1) the entire vessel would respond to only adventitial or endothelial factors, depending on the extent of cell-to-cell communication; 2) simultaneous release of adventitial and endothelial factors may tend to cancel in that the change in membrane potential would be somewhere between the maximum depolarized and hyperpolarized state; or 3) the different systems may act in concert to achieve a “mid-range” level of arterial tone and resistance to flow.

In summary, we have shown that electrical stimulation of luminal and adventitial surfaces results in release of substances with divergent mechanisms and diametric responses. There is strong evidence suggesting that VIP is the substance released from adventitial nerves. We have no good data regarding the identity of the substance released from the endothelial surface except for data showing that phenolamine can block the response to electrical stimulation. However, phenolamine can block responses to a number of agonists, including norepinephrine and serotonin, and thus, extensive pharmacologic analysis needs to be done before we can speculate on the nature of this constrictor substance. Our preliminary data suggest that an intact serotonergic pathway may be required, and further study, including histochemical and pharmacologic, is required. Finally, since neural tissue has not been demonstrated near endothelial cells, we need to do specific electrophysiologic experiments on cerebral arterial endothelium to determine the existence of TTX-sensitive and scorpion-toxin–sensitive Na+ channels.

References


Key Words • Middle cerebral artery • luminal electrical stimulation • adventitial electrical stimulation • tetrodotoxin • endothelium
Electrical stimulation of the endothelial surface of pressurized cat middle cerebral artery results in TTX-sensitive vasoconstriction.

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Circ Res. 1987;60:831-836
doi: 10.1161/01.RES.60.6.831

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1987 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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