Neurogenic Muscarinic Vasodilation in the Cat
An Example of Endothelial Cell-Independent Cholinergic Relaxation

Joseph E. Brayden and John A. Bevan
From the Department of Pharmacology, University of Vermont, College of Medicine, Burlington, Vermont

SUMMARY. Nerve-mediated and acetylcholine-induced dilator behavior of feline posterior auricular arteries was studied in vitro. We evaluated the muscarinic nature and endothelial cell-dependence of the vasodilations and attempted to determine if there are inhibitory muscarinic receptors located directly on the smooth muscle cells in this artery. Transmural nerve stimulation of arteries which were pretreated with guanethidine (5 X 10^-6 M) and constricted with prostaglandin F2α (3 X 10^-4 M) caused a frequency-dependent, tetrodotoxin-sensitive relaxation of up to 50% of induced tone. Atropine (10^-7 M) blocked more than 95% of this response at all frequencies. Removal of the endothelium by rubbing the intimal surface did not affect the magnitude of the response, but prolonged it slightly. Neurogenic relaxations in rubbed preparations were atropine-sensitive, although less so than control at higher stimulation frequencies. Relaxation of this artery to the calcium ionophore A23187 was completely endothelial cell-dependent. However, exogenous acetylcholine caused dose-dependent relaxations both in control and rubbed preparations. We conclude that the posterior auricular artery is an example of a blood vessel which has muscarinic receptors located directly on its smooth muscle cells which, when activated by acetylcholine released from perivascular nerves, mediate a smooth muscle cell relaxation. This finding contrasts with models of the vascular smooth muscle cell which indicates an excitatory role for muscarinic receptors. (Circ Res 56: 205-211, 1985)

ALTHOUGH adrenergically mediated neural control of blood vessels is established and widespread within the cardiovascular system of most animals (Bevan et al., 1980; Vanhoutte et al., 1981), evidence for a functional cholinergic neural supply to arteries and veins is less common and less convincing. Tissues such as the lung (Fisher, 1965), heart (Brown, 1968), uterus (Bell, 1968), skeletal muscle (Bulbring and Burn, 1935) and kidneys (Stinson et al., 1968) are thought to receive a neurogenic, cholinergic vasodilator input. However, many of the conclusions are based on histological evidence of acetylcholinesterase activity, a measure which is no longer considered to be conclusive evidence for cholinergic nerves (Koelle, 1955; Barajas and Wang, 1975). Other studies have examined the effects of exogenously applied acetylcholine as an index of cholinergic innervation. As muscarinic receptors are known to occur in many blood vessels where there is no evidence of cholinergic innervation, the latter type of evidence is without value. Perhaps some of the best evidence for cholinergic vasodilator nerves comes from studies of the cerebral and extracerebral cephalic circulation (Pinard et al., 1979; Bevan et al., 1982a) where at least part of nerve-mediated vasodilation seems to be under muscarinic control. In addition to pharmacological evidence for cholinergic innervation in cephalic vascular beds, biochemical indices of a cholinergic nerve supply, including high choline acetyltransferase levels (Bevan et al., 1982b) and a high affinity choline uptake system (Florence and Bevan, 1979), have been found in these arteries.

As is now clear, largely from the work of Furchgott et al. (1983), a wide variety of blood vessels dilate upon exposure to exogenous acetylcholine through a mechanism which is endothelial cell-dependent. Acetylcholine acts upon muscarinic receptors located on the endothelial cells. These cells in turn release an as yet unidentified endothelium-derived relaxing factor which leads ultimately to relaxation of the vascular smooth muscle cells. In those arteries so far examined, removal of the endothelium abolishes the vascular relaxation caused by acetylcholine (Furchgott and Zawadzki, 1980; De Mey et al., 1982). It is not known whether cholinergic neurogenic vasodilation is dependent upon the endothelium or whether it is mediated through muscarinic receptors located at postjunctional sites on the vascular smooth muscle cells themselves. In the present study, we have characterized the neurodilator behavior of one vessel from the cephalic circulation, the feline posterior auricular artery. The neural relaxation in this artery is almost completely cholinergic. Our intent was to evaluate the endothelial cell dependence of a muscarinic vasodilator response which clearly is mediated by nerves and, as a corollary, to determine whether or not there are inhibitory muscarinic receptors located directly on the vascular smooth muscle cells in this artery.
Methods

Cats weighing 3-4 kg and of either sex were anesthetized with sodium pentobarbital (50 mg/kg) and exsanguinated. The posterior auricular artery, a branch of the proximal external carotid artery, was then rapidly removed and placed in oxygenated Krebs bicarbonate solution of the following composition (mM): Na+, 144.2; K+, 4.9; Ca**, 1.6; Mg**, 1.2; Cl-, 126.7; HCO3-, 25.0; SO4-, 1.99; glucose, 11.1; and disodium ethylenediamine tetraacetate, 0.023.

After removal of surrounding connective tissue, 4-mm-long cylindrical segments of artery having unstretched lumen diameters of approximately 0.8 mm were mounted between two wires, one of which was connected to an isometric force transducer and the other to a micrometer displacement device. Tissues were then immersed in organ baths containing 50 ml of oxygenated Krebs solution (pH 7.4), and changes in tone were measured as previously described (Bevan et al., 1982a). In brief, after temperature equilibration for 15 minutes at 37°C, resting tension was adjusted to 1.0 g, which in pilot studies was found to be an optimal pre-load for force development, and platinum-stimulating electrodes were positioned, one on either side of the artery. After a 60-minute equilibration period, vessels were exposed to an EC70 dose of norepinephrine (3 X 10^-8 M) and then washed. Guanethidine (5 x 10^-6 M) was added to the tissue bath and replaced after every wash to block adrenergic constriction. Blockade occurred in all experiments and was verified by a lack of constriction in response to transmural nerve stimulation after 30 minutes of exposure to guanethidine. Dilator responses to nerve stimulation were examined after induction of tone by prostaglandin F2alpha (PGF2alpha, 3 X 10^-6 M). Transmural nerve stimulation was applied as 20-second trains of 0.3-msec pulses at a current strength of 70 mA. Stimulation frequencies ranging from 1 to 16 Hz were used. In every experiment, the dilator responses to such stimulation were shown to be tetrodotoxin-sensitive (3 X 10^-7 M) at all frequencies of stimulation. Frequency-response characteristics and effects of various drugs were determined at least 30 minutes after washout of the tetrodotoxin. Peak dilation was expressed as percentage decrease in the level of active tone.

In experiments designed to test the effect of disruption of the endothelium, arteries were rubbed briefly over the entire intimal surface, using a short length of stainless steel tubing, 0.2 mm in diameter, and then were mounted as usual in the organ bath. Destruction of endothelial cells was verified by means of a pharmacological criterion—absence of relaxation to the calcium ionophore A23187—and, at the end of experiments, by direct observation of the intimal surface after en face silver staining (Poole et al., 1958).

Dose-response relationships to acetylcholine and calcium ionophore A23187 were obtained by cumulative addition of the agents to the organ bath following induction of tone with PGF2alpha.

Data are presented as mean ± SEM, and statistical comparisons were made using Student's t-test.

Results

Evidence for Removal of Endothelial Cells

Removal of endothelial cells was confirmed pharmacologically by loss of dilator action of the calcium ionophore A23187—

![Graph showing the calcium ionophore A23187 dose-response relationships of control (n = 5) and rubbed posterior auricular arteries (n = 5). Data are expressed as percent change in PGF2alpha-induced tone. Arteries with endothelium dilated due to this agent at a threshold dose of 10^-8 M. Arteries without endothelial cells showed only a constrictor response to A23187. *P < 0.001 vs. control. Insets: examples of en face silver-stained preparations of rubbed (inset A) and control (inset B) posterior auricular arteries following in vitro experiments.](http://circres.ahajournals.org/)

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ionophore A23187 in rubbed preparations (Fig. 1). Although intact preparations underwent a dose-dependent relaxation to concentrations of this agent ranging from $3 \times 10^{-9}$ to $3 \times 10^{-8}$ M, rubbed vessels did not respond to doses less than about $10^{-7}$ M, and contracted at higher doses. In addition, the state of the endothelium was routinely examined histologically at the conclusion of experiments. After 3–4 hours in the organ bath, vessels which were not subjected to the rubbing procedure maintained an intact endothelium (inset, Fig. 1) over nearly all of the intimal surface, with the exception of those areas which had been in continual contact with the mounting wires. Arterial segments that had been intentionally rubbed were, in all cases, completely devoid of endothelial cells (inset, Fig. 1).

**Dilator Responses of Intact Arteries**

Traces of the typical pattern of dilator responses of an intact posterior auricular artery are shown in Figure 2A. Responses to transmural nerve stimulation were frequency dependent and reached a maximum during the 20-second stimulation or within a few seconds after the stimulus was removed. Redevelopment of tone to prestimulation levels also was rapid and generally occurred within the 60 seconds following cessation of stimulation. Ten minutes after treatment with atropine ($10^{-7}$ M), the effect of transmural nerve stimulation was nearly abolished at all stimulation frequencies (Fig. 2A). Dilator responses to papaverine ($10^{-5}$ M, Fig. 2A) were not affected by the presence of atropine. After washout of drugs,

**FIGURE 2.** Typical pattern of dilator responses of posterior auricular arteries with (panel A) and without (panel B) endothelium. Active tone was induced with PGF$_2\alpha$ ($3 \times 10^{-8}$ M) in the presence of guanethidine ($5 \times 10^{-7}$ M). Time of stimulation is indicated by bar above frequency labels. Intervals between stimulations are 10 minutes, except where indicated. Ten-minute treatment with atropine ($10^{-7}$ M) blocked more than 95% of the response at all frequencies when the endothelium was present, and more than 60% of the response when the endothelium was absent.

**FIGURE 3.** Summary of effects of atropine ($10^{-7}$ M) on neurogenic relaxations of posterior auricular arteries with (n = 8) and without (n = 8) endothelium. Data are expressed as percent decrease in PGF$_2\alpha$-induced tone following transmural nerve stimulation at 2, 4, 8, and 16 Hz. Error bars are standard errors.

***P < 0.001 vs. responses prior to addition of atropine.
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Relaxations of up to 45% of induced tone were common at higher frequencies of stimulation, and in all cases responses were inhibited nearly completely by atropine (Fig. 3). As would be predicted for a classical muscarinic response, physostigmine \((10^{-6} \text{M})\) increased both the magnitude and duration of the dilator response (Fig. 4A), and the subsequent addition of atropine reversed this effect.

**Effects of Endothelial Cell Removal**

Removal of the endothelium had no significant effect on the ability of the posterior auricular artery to contract to PGF\(_2\alpha\) (Table 1, legend). The magnitudes of the neurogenic dilator responses of rubbed arterial segments were not different from those of intact preparations (Figs. 2B, 3, and 4B; Table 1). The potentiating effect of physostigmine also was not different in rubbed arteries compared with control (Fig. 4B; Table 1).

Nevertheless, some differences in dilator behavior of the two preparations were apparent (Table 1). The \(t_v\) for recovery of relaxations to prestimulation levels of tone in vessels lacking endothelial cells was significantly greater than that for control vessels at all frequencies of stimulation. In addition, an atropine-resistant component ranging from 20% to 40% of the total relaxation was observed in rubbed vessels at stimulation frequencies of 4, 8 and 16 Hz. Increased concentrations of atropine up to \(5 \times 10^{-6} \text{M}\) did not effect this response.

In three experiments, 10 minutes of exposure to indomethacin \((10^{-5} \text{M})\) or 5,8,11,14-eicosatetraynoic acid \((3 \times 10^{-5} \text{M})\), a lipoxygenase and cyclooxygenase inhibitor, did not alter the atropine-resistant component (data not shown). Atropine-resistant relaxations were tetrodotoxin-sensitive, again indicating their neurogenic origin (Fig. 2B). Relaxations of rubbed arteries to papaverine occurred in the presence of atropine and tetrodotoxin. The effects of all drugs were reversible, and the dilator capability of rubbed preparations did not decline during the

**Table 1**

Summary of Transmural Nerve Stimulation-Induced Relaxations in Presence (+) and Absence (−) of Endothelium

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 Hz</th>
<th>4 Hz</th>
<th>8 Hz</th>
<th>16 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak dilator response (g)</td>
<td>(+)</td>
<td>−</td>
<td>(+)</td>
<td>−</td>
</tr>
<tr>
<td>(t_v) for recovery of initial tone (sec)</td>
<td>23 ± 3</td>
<td>50 ± 5†</td>
<td>22 ± 1</td>
<td>48 ± 3†</td>
</tr>
<tr>
<td>Atropine-resistant dilation (% of total dilation)</td>
<td>0 ± 0</td>
<td>9.9 ± 8.8</td>
<td>4.2 ± 2.3</td>
<td>22.1 ± 8.1*</td>
</tr>
<tr>
<td>Peak dilator response in presence of physostigmine (g)</td>
<td>0.8 ± 0.5</td>
<td>0.9 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

\* \(P < 0.05\) vs. control; † \(P < 0.01\) vs. control; ‡ \(P < 0.001\) vs. control. \(n = 4\) for experiments testing effects of physostigmine. For other parameters, \(n = 10\) for control experiments and \(n = 8\) for experiments testing effects of endothelial cell removal. Peak constrictor responses to PGF\(_2\alpha\) \((3 \times 10^{-6} \text{M})\) were 2.9 ± 0.2 g and 3.2 ± 0.2 g in control \((n = 10)\) and rubbed vessels \((n = 8)\), respectively, and were not significantly different from each other. ND: not determined.
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FIGURE 5. Acetylcholine dose-response relationships with (n = 6) and without (n = 6) endothelium. Data are expressed as percent of the maximum response to acetylcholine. Maximum relaxations (% of active tone) were 83.9 ± 3.9% with endothelium and 58.6 ± 4.1% (P < 0.001 vs. unrubbed) without endothelium. Negative log EC50 values (mean with 95% confidence intervals) were 7.99 (7.80-8.33) with endothelium and 7.56 (7.36-7.95) (P < 0.05 vs. unrubbed preparations) without endothelium. Inset: tracings of typical responses to exogenous acetylcholine of arteries with and without endothelium. Numbers above arrows are negative log acetylcholine values.

course of the experiments, as indicated by the responses to nerve stimulation at the end of experiments (Fig. 2B).

Responses to Exogenous Acetylcholine

Addition of exogenous acetylcholine to both rubbed and intact arteries caused a dose-dependent relaxation (Fig. 5). The peak dilator response occurred at an acetylcholine concentration of 3 × 10^-6 M in both control and rubbed preparations. The EC50 for this effect of acetylcholine in rubbed vessels was significantly different from that for control vessels (Fig. 5, legend). In addition, the maximum acetylcholine-induced relaxation of PGF2α-induced tone in rubbed vessels was significantly less than relaxation of control arteries (Fig. 5, legend).

Discussion

The results from this study support the notion that cephalic vessels are subject to inhibitory neural inputs which could have significant effects on vascular resistance and distribution of blood flow. Many examples of neurogenic dilator responses of cerebral blood vessels have been described (D'Alecy, 1977; Lee, 1982). In the extracerebral cephalic circulation, the tongue (Eccles and Wallis, 1974), nasal mucosa (Anggard, 1974), eye (Stjernschantz and Bill, 1980), and salivary glands (Lundberg, 1981) all receive well-documented vasodilator innervation. Previous reports from this laboratory (Bevan et al., 1982a) have demonstrated that neurodilator responses of many feline cephalic arteries consist of two components, one of which is cholinergic, mediated through muscarinic receptors, and that the contribution of this component can vary considerably from vessel to vessel. The posterior auricular artery apparently falls at one end of this spectrum, since its neural dilator mechanism is mediated predominantly by one transmitter substance, i.e., acetylcholine.

The ability of the posterior auricular artery to relax upon activation of its perivascular nerves does not seem to be dependent on the presence of endothelial cells. Peak dilator responses were nearly identical in magnitude in arteries with and without endothelial cells. This finding is in agreement with similar studies of feline cerebral arteries (Lee, 1982) in which transmural nerve stimulation caused relaxations of nearly 50% of developed tone in arteries with and without endothelium, although, in this case, the
dilation, since it was resistant to atropine, was not cholinergic.

In the present study, nerve-mediated relaxations of posterior auricular arteries were atropine-sensitive, both in control and in rubbed preparations. Thus, these experiments suggest that acetylcholine released from perivascular nerves can act directly on the smooth muscle cells to cause relaxation via a muscarinic receptor. In preliminary experiments, dilator responses also were examined after induction of tone with 5-hydroxytryptamine (10^{-6} M). Identical neurogenic dilator behavior was observed, regardless of the agonist used to induce tone. This finding argues against the involvement of dilator mechanisms which may be specific to PGF_2alpha or its receptors. Responses to PGF_2alpha were maintained for longer periods of time than those to 5-hydroxytryptamine, and, hence, the prostaglandin was used throughout these studies.

It is not clear whether or not the nerve-released acetylcholine, in addition to having a direct effect on smooth muscle cells, caused arterial relaxation by inducing secondary release of a relaxing factor from the endothelial cells. The actual magnitude of the atropine-sensitive relaxation, i.e., that remaining after the atropine-insensitive component is subtracted from the total response, is decreased in rubbed arteries. This would indicate a possible endothelial cell contribution to the cholinergic relaxation in intact preparations. In a recent study, Angus and co-workers (1983) showed that acetylcholine applied to the adventitial side of canine femoral arteries in vivo was able to traverse the arterial wall and cause an endothelium-dependent relaxation. Thus, it is conceivable that acetylcholine release from nerves could diffuse through the vessel wall and reach the endothelial cell layer, although evidence to the contrary also has been presented (Cohen et al., 1984).

The mechanism by which endothelial cell removal unmasked the atropine-insensitive dilator response in this artery is not known. It is possible that transmural nerve stimulation caused a tetrodotoxin-sensitive release of a constrictor substance from the endothelial cells (De Mey and Vanhoutte, 1983) which offset the effects of a noncholinergic dilator transmitter substance. Removal of endothelial cells might then have revealed the atropine-insensitive relaxation. The lack of effect of indomethacin and 5,8,11,14-eicosatetraynoic acid argues against the involvement of arachidonic acid metabolites in the atropine-resistant vasodilator response.

Furchgott and Zawadzki (1980) first reported that removal of endothelial cells from rabbit aortic strips completely abolished the relaxation responses of this preparation to acetylcholine and, in later work (Furchgott et al., 1983), to ATP, bradykinin and the calcium ionophore A23187. These authors also found that both control and rubbed preparations constricted when high concentrations of acetylcholine (>10^{-6} M) were applied. Subsequent work by others has confirmed these findings in a variety of blood vessels (Altura and Chand, 1981; De Mey et al., 1982). As a result of these studies, current models of the vascular smooth muscle cell imply that activation of muscarinic receptors located on this cell leads to constriction (Furchgott et al., 1981, Vanhoutte, 1981). The present study represents an example of a blood vessel where this clearly is not the case. Arteries lacking endothelial cells still undergo significant relaxation in response to low doses of exogenous acetylcholine. This finding indicates that muscarinic receptors which mediate smooth muscle cell relaxation to exogenous acetylcholine indeed are present on the vascular smooth muscle cells. The dilator response of the posterior auricular artery to the calcium ionophore A23187 showed the same endothelial dependence as has been seen in other blood vessels (Furchgott et al., 1983). This suggests that, although the dilator response of the posterior auricular artery to acetylcholine is unique when compared to that seen in all other vessels so far examined, it is unlikely that this artery is unusual in other respects. Indeed, the greater relaxation response of control posterior auricular arteries to exogenous acetylcholine compared with the response in arteries which have been rubbed probably is due to the combined effects of an endothelial-derived relaxation factor, as well as the direct relaxant effect of acetylcholine on the smooth muscle cells.

The functional implications of this cholinergic outflow are not clear. The posterior auricular artery supplies middle ear structures, as well as skeletal muscle near the ears (Crouch, 1969). It has been suggested that dilator innervation in arteries supplying other feline cephalic structures may be activated during times of thermal stress (Bevan et al., 1981, Gibbins et al., in press). However, based on the nature of the tissues supplied by the posterior auricular artery, a thermoregulatory role for these nerves seems unlikely. It is possible that this system is analogous to the sympathetic, muscarinic dilator innervation which has been demonstrated in arteries supplying other skeletal muscles (Bulbring and Burn, 1935), but, pending further work, a specific role for this particular dilator outflow must remain speculative. Regardless of the physiological conditions which might lead to activation of these dilator nerves, the direct muscarinic inhibitory response which occurs in this artery upon activation of its neural input is pronounced, and is sufficient to play an important role in determining blood flow in the tissues supplied by this artery.
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