Contractile Responses of Isolated Bovine Retinal Microarteries to Acetylcholine

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The contractile responses of isolated and activated bovine retinal microarteries (BRA) (diameter, 204 ± 4 μm; n = 48) to acetylcholine (ACh) were studied. These responses depended on the nature of the activating agent. The ACh relaxed BRA activated by prostaglandin F2α (PGF2α) and circumferential stretching in a dose-dependent manner but had no significant effect on K+-activated BRA. The effects of ACh also depended on the degree of BRA activation: the stronger the PGF2α-induced contractions, the weaker the relaxation produced by ACh. In equivalent PGF2α-induced contractions, the ACh effects were reproducible. The muscarinic antagonist, atropine, reversed the relaxation caused by ACh of PGF2α-activated BRA. Physostigmine, an inhibitor of acetylcholinesterase, did not potentiate or prolong the relaxant action of ACh. Selective removal of the BRA endothelium (by gassing the BRA lumen, checked by scanning electron microscopy) blocked the relaxation caused by ACh of PGF2α-induced contractions and unmasked a constricting action of ACh. This suggests that, in BRA with functional endothelium, the direct constricting effects of ACh on smooth muscle are masked by the more potent dilating activity, mediated by endothelial muscarinic receptors. Acetylcholinesterase was not found in BRA. Invest Ophthalmol Vis Sci 32:1996-2005, 1991

In the eye, cholinergics act on muscarinic receptors in the ciliary muscle and iris sphincter. Topical cholinergics are, therefore, commonly used in ophthalmology. However, little is known about the responsiveness of the retinal circulation to these agents. Recent biochemical evidence suggests that retinal arteries have muscarinic binding sites.1 We wished to establish whether these binding sites are functional muscarinic receptors and, if so, determine (1) the effects of receptor stimulation and (2) the exact localization of these receptors in the arterial wall. Therefore we studied the contractile effects of acetylcholine (ACh) in isolated segments of bovine retinal arteries under various conditions.

Materials and Methods

Biologic Material and Organ Bath

Forty-eight bovine retinal microarteries (BRA) were used in this study. The bovine eyes were obtained at the local abattoir, at the latest 30 min after death. The eyes were transported to the laboratory in physiologic salt solution (PSS) at 4°C, containing: NaCl, 118 mM; KCl, 4.7 mM; MgSO4, 1.2 mM; KH2PO4, 1.2 mM; NaHCO3, 24 mM; CaCl2, 2.5 mM; and glucose, 4.5 mM. One BRA segment (located between the optic disc and the first intraretinal arterial branching; length, 1.95 ± 0.01 mm) was isolated, mounted in an organ bath, and connected to an electromagnetic force-length transducer with two stainless-steel wires threaded through the lumen. This was described extensively in a previous publication.2 The PSS in the organ bath (5 ml) was maintained at 37°C and at pH 7.35–7.45 by gassing it with a mixture of 95% O2 and 5% CO2. The solution could be rapidly replaced by prewarmed and oxygenated PSS from a reservoir.2

Transducer and Recording System

Isometric contractions were studied. Contracting BRA were not allowed to shorten circumferentially but developed force instead (units, 1 mN = 0.1 g). Constant circumference and force measurements were accomplished by the force–length transducer.2 Force and length signals were captured with a microcomputer data acquisition system (Schneider CPC 6128, Amstrad, Zaventem, Belgium, and data acquisition system module made at the departmental workshop). All parameters were displayed on the screen as a function of time and stored on floppy disks. The figures were plotted on a Hitachi 672 Graph-Plotter (Hitachi, Tokyo, Japan) from the data stored on the floppy disks.
Activation of BRA

The BRA showed no spontaneous contractile activity in PSS. We used three activating agents: (1) a prewarmed and oxygenated solution obtained from a second reservoir and containing 125 mM K⁺ (K⁺-PSS), an equimolar replacement of Na⁺; K⁺-induced contractions were characterized previously;² (2) prostaglandin F₂α (PGF₂α) dissolved in distilled water and added to the organ bath to produce sustained contractions; and (3) rapid circumferential stretching (accomplished by the specific design of the force-length transducer) to induce transient contractions. To obtain reproducible contractile responses to stretching, each stretch-induced contraction was preceded by two K⁺-induced contractions at 5-min intervals.²

Experimental Protocols

After mounting in the organ bath, the BRA were allowed to stabilize for at least 1 hr at a small circumference. Subsequently, lₘₐₓ, ie, the circumference at which the BRA developed maximal active force, was determined as described previously.² All experiments were done at lₘₐₓ. The BRA internal circumference at lₘₐₓ was 640 ± 11 μm (internal diameter, 204 ± 4 μm; range, 132–255 μm). Passive force at lₘₐₓ, ie, the force needed to stretch the BRA to lₘₐₓ, was 0.23 ± 0.01 mN. The ratio of passive to total force (active developed force plus passive force) during K⁺-activation was determined, and only BRA with ratios smaller than 0.15 were used, ensuring that experiments were done only on BRA not affected extensively by the isolation procedure or in vitro conditions.

After stabilization, pharmacologic interventions were used. The drugs were dissolved in distilled water and added as 5-μl or 15-μl volumes to the organ bath. With a first procedure, ACh or atropine was added after the K⁺- or PGF₂α-induced contraction stabilized, ie, when sustained K⁺- or PGF₂α-induced force was constant. The responses were expressed with reference to the force before drug application. With a second procedure, resting BRA were equilibrated with ACh, after which active responses to stretching were obtained in the presence of ACh. The effects of the drug were compared with values obtained from contractions before ACh application and after washing.

Physostigmine was added to resting BRA, after which PGF₂α-contractions were induced in the presence of the drug. The ACh was added after at least 8 min of equilibration with physostigmine.

Scanning Electron Microscopy

At the end of an experiment, the BRA were fixed by immersion in 1% glutaraldehyde in 0.1 M Millonig buffer for at least 1 hr. After postfixation for 1 hr with 0.2% OsO₄ in 0.1 M cacodylate buffer, BRA were dehydrated and critical-point dried in a Balzer’s apparatus with CO₂ as the transition fluid. Dried BRA were glued on aluminum stubs with Tempfix (Neubauer, Munster, Germany), and the BRA lumen was opened with a small scalpel. The luminal surface was coated with a 10-nm layer of gold in a Balzer’s sputter coater and observed in a Leitz AMR-1200B (Wild-Leitz, Wetzlar, Germany) or a Jeol JSM-U3 (Jeol, Tokyo, Japan) scanning electron microscope operated at 15 or 20 kV.

Removal of the Endothelium

Gas (95% O₂ and 5% CO₂) was blown on the endothelium for 50 sec through a micropipette placed at one end of the mounted BRA segment. The gas bubbles escaped at the other end or at both ends. Before this procedure, the two intraluminal wires were approximated so that these wires no longer touched the BRA internal surface during gassing. This allowed gassing of all parts of the endothelium without over-stretching the BRA circumferentially. Immediately after the procedure, the wires were reset to the initial position. Therefore we were still able to compare passive force before and after gassing. These controlled changes in position of the wires were accomplished by the specific design of the force-length transducer.² After gassing, the arterial lumen retained a large gas bubble. This bubble was removed by irrigation of the lumen with PSS through another micropipette. If not removed, this bubble might have formed a diffusion barrier to the fixation fluid for scanning electron microscopy that could have resulted in a falsely endothelium-denuded preparation. After the procedure, the BRA were allowed to reequilibrate for at least 30 min.

Statistics and Calculations

Force was normalized by the vessel wall surface as described.² All data were expressed as means ± standard error and were statistically analyzed using paired student t-tests and taking P < 0.05 as the limit of significance. For each experimental protocol, the number of BRA reported was also the number of animals used.

Drugs Used

The drugs were obtained from the following sources: PGF₂α from Upjohn (Puurs, Belgium) and ACh, atropine, and physostigmine from Sigma (St. Louis, MO).

Results

Characterization of the Response to PGF₂α

Cumulative doses of PGF₂α (10⁻⁷–10⁻⁴ M) were added to resting BRA (n = 6). The PGF₂α induced
sustained contractions from $10^{-6}$ M onward in a dose-dependent manner (Fig. 1). In five BRA, PGF$_{2\alpha}$-induced contractions showed an immediate large force transient (phasic part) before stabilization at a plateau (tonic part). The phasic part of the contraction became less prominent at higher doses of PGF$_{2\alpha}$. During the PGF$_{2\alpha}$-induced contractions, five BRA showed rhythmic activity, ie, small and rapid oscillatory contractions of variable amplitude.

To study the short-term reproducibility of PGF$_{2\alpha}$-induced contractions, three sequential responses (time intervals ± 17 min) to a single dose ($10^{-5}$ M) of PGF$_{2\alpha}$ were induced in five BRA. No significant change in the PGF$_{2\alpha}$ response was noted. Active phasic force was $2.75 \pm 0.23$, $2.66 \pm 0.25$, and $2.88 \pm 0.32$ mN/mm$^2$ in the first, second, and third trials, respectively; active tonic force was $1.08 \pm 0.25$, $0.97 \pm 0.34$, and $1.09 \pm 0.29$ mN/mm$^2$, respectively.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933388/)

**Figure 1.** (A) Representative example of the response to cumulative doses of PGF$_{2\alpha}$ (M): (a): $10^{-6}$; (b): $3.10^{-6}$; (c): $10^{-5}$; (d): $3.10^{-5}$; (e): $10^{-4}$. (1) Phasic and (2) tonic parts of the contractions. (B) Mean active phasic (○) and tonic (○) force during cumulative doses of PGF$_{2\alpha}$.

### Table 1. Subdivision of the PGF$_{2\alpha}$-induced contractions

<table>
<thead>
<tr>
<th>Degree of contraction</th>
<th>$AF$ (mN/mm$^2$)</th>
<th>$AF$ (%)</th>
<th>$PGF_{2\alpha}$ (M)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak</td>
<td>1.12 ± 0.08</td>
<td>108 ± 8</td>
<td>$10^{-8}$ to $3.10^{-5}$</td>
<td>9</td>
</tr>
<tr>
<td>Moderate</td>
<td>1.71 ± 0.29</td>
<td>166 ± 13</td>
<td>$10^{-5}$ to $10^{-4}$</td>
<td>5</td>
</tr>
<tr>
<td>Strong</td>
<td>3.93 ± 0.42</td>
<td>370 ± 52</td>
<td>$10^{-4}$ to $10^{-4}$</td>
<td>4</td>
</tr>
</tbody>
</table>

$AF$ (mN/mm$^2$): mean PGF$_{2\alpha}$-induced active tonic force; $AF$ (%): mean PGF$_{2\alpha}$-induced active tonic force as % of $K^+$-induced active tonic force; $PGF_{2\alpha}$ (M): range of the dose of PGF$_{2\alpha}$ used; N: number of BRA studied.

The long-term reproducibility of PGF$_{2\alpha}$-induced contractions was assessed in five BRA by adding PGF$_{2\alpha}$ $10^{-5}$ M and retesting the response after an interval of ± 105 min. In the second trial, active tonic force had increased by 60 ± 21% (significantly different) while active phasic force remained constant (ie, 111 ± 10% of the force in the first trial).

### Characterization of the Response to ACh

**Response during PGF$_{2\alpha}$ activation:** Increasing doses ($10^{-9}$–$10^{-6}$ M) of ACh were added to weak, moderate, and strong PGF$_{2\alpha}$-induced contractions (Table 1). In weak PGF$_{2\alpha}$-induced contractions, ACh induced a marked, dose-dependent relaxation from $10^{-9}$ M onward (Fig. 2). In moderate PGF$_{2\alpha}$-induced contractions, significant responses to ACh only appeared at $10^{-7}$ M, and a diminution of the response was observed. Strong PGF$_{2\alpha}$-induced contractions were resistant to ACh.

To test the reproducibility of ACh-induced relaxation, two sequential weak and equivalent PGF$_{2\alpha}$-induced contractions were subjected to a single dose ($10^{-6}$ M) of ACh (n = 5). In the first trial, PGF$_{2\alpha}$-induced active tonic force was 1.22 ± 0.26 mN/mm$^2$ during control conditions and 0.42 ± 0.21 mN/mm$^2$ after adding ACh (ie, 26 ± 11% of control). In the second trial, PGF$_{2\alpha}$-induced active tonic force was 1.18 ± 0.21 mN/mm$^2$ during control conditions and 0.42 ± 0.21 mN/mm$^2$ during ACh (ie, 29 ± 10% of control).

**Response during $K^+$-induced activation:** Increasing doses ($10^{-8}$–$10^{-6}$ M) of ACh were added to stabilized $K^+$-activated BRA (n = 9). Although ACh relaxed some of the BRA partially, it did not have a significant effect. Mean $K^+$-induced active tonic force was 1.40 ± 0.20 mN/mm$^2$ during control conditions and 1.28 ± 0.28 mN/mm$^2$ with ACh $10^{-6}$ M.

**Response during stretch-induced activation:** Rapid circumferential stretching was applied to BRA (n = 6) from 0.5 $l_{max}$ to $l_{max}$ in the absence and presence of
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Fig. 2. (A) Representative example of the response to ACh during a weak PGF$\textsubscript{2\alpha}$-induced contraction. Cumulative doses of ACh were added (M): (a): 10$^{-8}$; (b): 3.10$^{-8}$; (c): 10$^{-7}$; (d): 3.10$^{-7}$; (e): 10$^{-6}$. At the lower doses, the effects of ACh partially reversed spontaneously. At higher doses, the effects of ACh were sustained. Adding atropine 10$^{-6}$ M (f) reversed the effect of ACh 10$^{-6}$ M. (B) mean PGF$\textsubscript{2\alpha}$-induced active tonic force during cumulative doses of ACh, as percent of PGF$\textsubscript{2\alpha}$-induced active tonic force prior to ACh application. (O): weak, (•): moderate and (▲) strong PGF$\textsubscript{2\alpha}$-induced contractions (*P < 0.05; **P < 0.01; ***P < 0.001).

ACh 10$^{-8}$ M, 10$^{-7}$ M, and 10$^{-6}$ M. The ACh relaxed stretch-induced contractions in a dose-dependent manner (Fig. 3).

Effects of Physostigmine

The dose–response relationship for ACh (10$^{-9}$–10$^{-6}$ M) in weak PGF$\textsubscript{2\alpha}$-induced contractions was studied in the absence and presence of physostigmine 10$^{-6}$ M (n = 4). Incubation of BRA with physostigmine increased PGF$\textsubscript{2\alpha}$-induced force. Therefore, during physostigmine administration, a lower dose of PGF$\textsubscript{2\alpha}$ was chosen to match the control PGF$\textsubscript{2\alpha}$-induced contraction. Physostigmine had no potentiating effects; it did not shift the dose–response relationship for ACh to the left (Fig. 4A). On the contrary, in the presence of physostigmine, a diminution of the response to ACh was noted.

The time course of the effects of a single dose of ACh (5.10$^{-8}$ M) on weak and matched PGF$\textsubscript{2\alpha}$-induced contractions was studied before and after adding physostigmine 10$^{-6}$ M (n = 8, Fig. 4B). During both conditions, the relaxation by ACh partially reversed spontaneously. Physostigmine did not prolong the relaxation.

Role of the BRA Endothelium

The endothelium in control preparations: Six BRA were examined with scanning electron microscopy at the end of one experiment (Figs. 5A–B). The endothelium was well preserved, except near both BRA endings where damaged cells and denuded basal lamina were present. Endothelial cells were elongated with their long axis parallel to the direction of flow. Inter cellular ridges and some small microvilli were the only surface structures on the endothelial surface besides occasional crater-like structures that were found along intercellular borders. Even at sites where the intraluminal wires had touched the BRA internal surface, the endothelium was not damaged, although the endothelial cells in this area were less flattened and had more microvilli or bleb-like structures than the cells not touched by the wires.

The endothelium after gassing: Seven BRA were examined with scanning electron microscopy after gassing (Figs. 5C–D). In three BRA, all endothelial cells were removed, leaving a fine fibrillar meshwork representing the basal lamina. In the other BRA, endothelial cells were retained in small areas. However, most of these cells were severely damaged, showing permeabilized cell membranes and widened inter cellular openings. Many cells were almost completely detached from the subendothelial surface.

Contractile characteristics of gassed BRA: Passive force (at $l_{\text{max}}$) and active responses to high K$^+$ or PGF$\textsubscript{2\alpha}$ 10$^{-5}$ M were studied before and after gassing (n = 6). There were no significant effects of gassing on passive force (Fig. 6A). Passive force was 0.20 ± 0.02 mN/mm$^2$ before and 0.22 ± 0.03 mN/mm$^2$ after gassing. The first response to high K$^+$ after gassing was depressed. However, during the following exposures to K$^+$, K$^+$-induced force gradually increased. After stabilization, K$^+$-induced active tonic force reached significantly higher levels than before gassing (Fig. 6B). Similarly, PGF$\textsubscript{2\alpha}$-induced active tonic force increased significantly. Both K$^+$- and PGF$\textsubscript{2\alpha}$-induced active phasic force stabilized close to the pregassing values. The time interval between PGF$\textsubscript{2\alpha}$ trials, before and after gassing, varied considerably depending on the duration for stabilization of the high K$^+$ response. The mean time interval was 107 ± 22 min.
**Effects of ACh in gassed BRA:** The effects of ACh $10^{-6}$ M in weak PGF$_{2\alpha}$-induced contractions were studied before and after gassing ($n = 8$, Fig. 7). After gassing PGF$_{2\alpha}$-induced active tonic force increased, and a lower dose of PGF$_{2\alpha}$ was chosen to match the PGF$_{2\alpha}$-induced contraction before gassing. The ACh decreased PGF$_{2\alpha}$-induced force in all BRA (to 42 ± 9% of control) before gassing. By contrast, after gassing, ACh increased PGF$_{2\alpha}$-induced force in seven BRA to 191 ± 37% of control (significantly different, $P < 0.05$). One BRA showed a residual relaxation to ACh of 37% of the initial response despite the fact that scanning electron microscopy showed extensive destruction of the endothelium.

**Importance of the Position of the Intraluminal Wires During Gassing**

To assess the importance of approximating the intraluminal wires during gassing, six BRA were gassed without changing the position of these wires, ie, with the BRA internal circumference at $l_{\text{max}}$ (Fig. 8). Gassed in this way, BRA showed no increases of $K^+$- and PGF$_{2\alpha}$-induced active tonic force (mean time interval between PGF$_{2\alpha}$ trials, 109 ± 4 min). The PGF$_{2\alpha}$-induced active phasic force was depressed significantly. Passive force was 0.25 ± 0.02 mN/mm$^2$ before and 0.27 ± 0.01 mN/mm$^2$ after gassing.

Before gassing, ACh $10^{-6}$ M decreased PGF$_{2\alpha}$-induced force in all BRA to 30 ± 8% of control. After gassing the drug did not increase PGF$_{2\alpha}$-induced force significantly. The PGF$_{2\alpha}$-induced tonic force during ACh was 103 ± 18% of control. In one BRA, the relaxation to ACh was blocked, and two BRA contracted during ACh. Scanning electron microscopy of these BRA showed totally destroyed endothelium. However, the other three gassed BRA had a residual relaxation response to ACh. Scanning electron microscopy of these BRA confirmed the persistence of endothelium in those parts covered by the wires.

**Discussion**

**Characterization of the Relaxant Effects of ACh**

In most experiments on vascular preparations, norepinephrine was used to produce a sustained contraction on which ACh has then been tested for its relaxing activity.$^{3,4}$ However, alpha$\_1$-adrenergic agonists do not produce sustained contractions in BRA.$^2$ Therefore, we used PGF$_{2\alpha}$ to induce contractions because in other vascular preparations essentially the same sensitivity to the relaxing effect of ACh as produced by norepinephrine has been shown.$^3$ The ACh relaxed PGF$_{2\alpha}$-activated BRA in a dose-dependent manner. Atropine reversed this relaxation, indicating the muscarinic nature of the receptor on which ACh acts.
The relaxing activity of ACh depended on the degree of BRA activation. Weak PGF$_{2\alpha}$-induced contractions were highly sensitive to ACh. In stronger PGF$_{2\alpha}$-induced contractions, the relaxing effects of ACh appeared only at higher doses and were less marked. The drug was not able to relax near-maximal PGF$_{2\alpha}$-induced contractions. In equivalent PGF$_{2\alpha}$-induced contractions, ACh had reproducible effects. Consequently, a self-pairing experimental design can be used to study the effects of ACh during different conditions if the BRA is precontracted with PGF$_{2\alpha}$ to approximately similar levels of contraction.

The effects of ACh also depended on the nature of the activating agent. The K$^+$-induced contractions (K$^+$-induced tonic force equivalent to weak PGF$_{2\alpha}$-induced tonic force) were considerably less sensitive to ACh. In large arteries, K$^+$-induced contractions also have shown considerably less sensitivity to the action of ACh than have equivalent contractions produced by norepinephrine. The mechanisms underlying this apparent difference are unknown but may be at the level of the response to cyclic guanosine monophosphate (cGMP) (Fig. 9). Since the role of PGF$_{2\alpha}$ in modulation of BRA vascular tone is not yet established, we also studied the effects of ACh on stretch-induced contractions. As we discussed previously, stretch-induced activation is thought to be a major determinant of vascular tone. The drug relaxed stretch-induced contractions in BRA in a dose-dependent manner. This indicates that cholinergic mechanisms are important to the regulation of retinal vascular tone, if there is a local source of ACh. Recently, it was shown that retinal vessels are able to synthesize ACh.

**Presence of Acetylcholinesterase**

Acetylcholinesterase (AChE) is the enzyme responsible for the breakdown of ACh. We investigated the presence of AChE in BRA by using physostigmine, which inhibits AChE. Physostigmine did not increase the sensitivity to ACh in BRA. This contrasts with its effects in ciliary muscle where it can potentiate the action of ACh approximately 100 times. However, the potentiating effects of physostigmine in this smooth muscle preparation are due to the dense cholinergic innervation. Since retinal arteries lack cholinergic nerve endings, physostigmine could have more subtle effects in BRA. Therefore we also studied the effects of physostigmine on the duration of the relaxation by a single dose of ACh. Again, physostigmine did not prolong this relaxation. Consequently, we were unable to demonstrate the presence of AChE in BRA.

**Mechanism of the Relaxation by ACh**

Muscarinic receptors located on prejunctional adrenergic nerve endings have been implicated in the vasodilating effect of ACh. It acts on the prejunctional receptors and can inhibit the release of norepinephrine evoked by nerve stimulation and the stimulation-evoked vasoconstriction. However, the absence of adrenergic nerve endings excludes such a possibility in BRA.

Since 1980, the principal mechanism for ACh-induced vasodilatation in vivo has been attributed to an endothelium-dependent mechanism (Fig. 9). For maximal endothelium-dependent relaxation by ACh, isolated vascular preparations have to retain 60–75% of the endothelial cells by the end of an experiment. To examine the condition of the endothelium in our preparations, we used scanning electron microscopy.
Fig. 5. Scanning electron microscopy of BRA. (A) Control preparation. The endothelial cells had an elongated cell shape, intercellular borders (arrowheads), some microvilli, and an occasional crater-like structure (arrow) (original magnification ×5000). (B) Control preparation. Endothelial cells that were located behind the intraluminal wires during the experiment, had a cylindrical shape; intercellular borders were not found; the cell surface was smooth or covered with blebs (arrowheads) and small microvilli (original magnification ×5000). (C) Internal surface after gassing, showing one partly loosened endothelial cell (arrow) on a denuded basal lamina (BL) (original magnification ×3500). (D) Internal surface after gassing: basal lamina (BL); one remnant of an endothelial cell (*), showing folded edges and holes in the cell membrane; two intact cells (which were rare) with free borders (arrows) and a nearly detached cell covered by bleb-like protrusions (arrowheads) (original magnification ×5000). Bars = 5 μm.

because this technique enables us to examine the entire BRA internal surface. The BRA retained sufficient endothelial cells to allow complete endothelium-dependent relaxation by ACh. After selective removal of the BRA endothelium, the relaxation to ACh was blocked. This demonstrates that, in agreement with most vascular preparations, the endothelial cells are required for relaxation of BRA to ACh. Moreover, removing the endothelium unmasked a direct constricting action of ACh on the smooth muscle (Fig. 7). Thus, in retinal arteries with functional endothelium, the constricting effect of ACh is masked by the more potent dilating component. It is conceivable however that, during pathologic conditions that impair endothelial function, the constricting component may predominate.

Contractile Characteristics of Endothelium-Denuded BRA

Under normal conditions, K⁺-induced tonic force remains constant during the time course of an experiment.² By contrast, after removal of the endothelium, K⁺-induced tonic force progressively and significantly increased. Similarly, PGF₂α-induced tonic force, which increased under normal conditions, increased more after endothelium removal. We have no ready explanation for these findings at this time. In various studies, different mechanisms for the increased constricting responses of endothelium-denuded vascular preparations have been proposed. In one study it was suggested that the endothelium forms a diffusion barrier to contractile agents.¹⁵ Removing such a
agents, is by the release of endothelium-derived relaxing factor (EDRF, Fig. 9). The contractile agents nor-
epinephrine and serotonin stimulate the endothelium to release EDRF in coronary arteries.16 Alternatively, the endothelium might release EDRF continuously and spontaneously.17-19 In rat aorta, the latter mechanism accounts for the depression of the force induced by a range of contractile agents, including PGF\(_{2\alpha}\).17 Therefore, this is the most likely explanation for our PGF\(_{2\alpha}\) data. If the BRA endothelium releases EDRF spontaneously, this release does not affect the basal tone of the resting BRA preparation. In our study, passive force did not increase after endothelium removal. This means that in the absence of any contractile agent, the muscle cells are totally relaxed, ie, they do not have a low level of basal activation. In addition spontaneous release of EDRF may not account for the depression of the K\(^+\)-induced contractions because these contractions were little affected by the ACh-stimulated release of EDRF. Although further investigation is needed, the observation of the increased responses of endothelium-denuded BRA to contractile agents is important. Such an increase of vascular reactivity to contractile substances may occur in retinal arteries when the endothelium is damaged by pathologic events.

**Comments on the Removal of BRA Endothelium**

Several agents, in addition of ACh, have now been found to depend on the endothelial cells for producing all or part of their effects on vascular preparations. Because future studies on the effects of these agents on BRA will also require removal of the endothelium, we wish to comment on the technique we used. To demonstrate the obligatory role of the endothelium in the action of an agent, the endothelium has to be removed entirely. If only a small amount of the endothelial cells remains, ACh (for instance) can produce an important residual endothelium-dependent relax-
ation.12 Furthermore, the endothelium should be removed with minimal trauma to the underlying smooth muscle cells. Therefore, the technique commonly used in isolated large arteries (ie, mechanical removal of the endothelium by rubbing the internal arterial surface) is not applicable to isolated microarteries such as BRA. Seeking an alternative technique, we first tried intraluminal injection of the detergent Triton-X 100 (Sigma, St. Louis, MO, unpublished ob-
servations). Detergents were used successfully in isolated large vessels20 and perfused vascular beds.21 This method failed in BRA; rapid diffusion of the detergent caused extensive smooth muscle damage (as shown by the important depression of K\(^+\) - and PGF\(_{2\alpha}\)-induced contractions after the procedure).
Next, we tried a modification of the gassing technique, which has been successfully applied to perfused rat arteries. Gassing the BRA was an easily applicable and adequate technique. Little if any damage was done to the smooth muscle cells. After the procedure the contractile responses to high $K^+$ and PGF$_{2\alpha}$ even increased. Furthermore, scanning electron microscopy showed that the endothelial cells were removed efficiently or destroyed. In gassed BRA, scanning electron microscopic evaluation of the endothelium correlated well with the altered responses to ACh. During the gassing procedure, the intraluminal wires should be approximated. This is done first to avoid overstretching of the BRA (beyond $I_{\text{max}}$), which inevitably results in smooth muscle damage. Indeed, BRA gassed without approximating the wires (ie, at $I_{\text{max}}$) did poorly; the responses to high $K^+$ and PGF$_{2\alpha}$ were attenuated. Second, we follow this procedure to permit gassing of those parts of the endothelium which are, during the experiment, covered by the intraluminal wires. Scanning electron microscopy showed that these parts of the endothelium under normal conditions were intact. Furthermore, three of six BRA gassed without approximating the wires showed a residual relaxation to ACh, due to the persistence of parts of this endothelium.

**Fig. 8.** Contractile characteristics of BRA gassed without changing the position of the wires. See legend, Figure 6B.

**Fig. 9.** The endothelium-dependent relaxation by ACh is initiated by an action of this agent on a muscarinic receptor (M) of the endothelial cells. The action of ACh on this receptor stimulates the endothelial cells to release some factor(s), called EDRF or endothelium-derived relaxing factor(s). Recent evidence suggests that in most circumstances, nitric oxide (NO) is the major EDRF. EDRF then acts on the smooth muscle cells of the artery to make them produce cyclic GMP. In response to the rise in intracellular cyclic GMP, the smooth muscle cells relax.
Key words: resistance arteries, endothelium, muscarinic receptors, gassing, physostigmine

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References