Vasoactivity of Intraluminal and Extraluminal Agonists in Perfused Retinal Arteries

Dao-Yi Yu, Valerie A. Alder, Stephen J. Cringle, Er-Ning Su, and Paula K. Yu

Purpose. To evaluate the vasoactive response of isolated perfused arteries of the pig to K+ and adrenergic agonists and to compare the effects of intraluminal (IL) and extraluminal (EL) drug delivery.

Methods. A new microperfusion system was developed, in which short lengths of porcine retinal arteries (outer diameter 90.4 ± 2.7 μm) were cannulated at both ends and perfused at a controlled rate (5 μl/min) with outflow through a single side branch. The diameter of the vessel and the intraluminal pressure were monitored, and the effect of intraluminally and extraluminally applied agonists was determined. Endothelial cell function and the integrity of the blood retinal barrier was verified.

Results. Consistent vasoactive responses were obtained from most vessels. The resting diameter of the vessel was not greatly influenced by changes in flow rate or intraluminal pressure over the physiological range. Adrenaline and noradrenaline caused dose-dependent contractions, which were larger when applied intraluminally than they were when applied extraluminally. The largest contraction for adrenaline was 19.0% ± 2.1% (n = 13) IL and 8.4% ± 1.5% (n = 13) EL, and for noradrenaline, 17.8% ± 1.9% (n = 13) IL and 6.8% ± 1.1% (n = 13) EL. The IL contraction to 124-mM K+, 19.0% ± 1.6% (n = 21), was also greater than that for EL application, 5.0% ± 1.0% (n = 13). We found that the existence of myogenic contractions was restricted to the special case in which vessels with no branches were pressurized under zero flow conditions.

Conclusions. Pig retinal arteries exhibited asymmetry in their responses to adrenergic agonists and K+, with contractions significantly larger when the drug was applied to the intraluminal surface rather than the extraluminal surface. This asymmetry may reflect an important property of retinal vessels. Microperfusion systems of this type may prove valuable in developing a better understanding of control mechanisms in retinal circulations. Invest Ophthalmol Vis Sci. 1994; 35:4087-4099.

The retinal circulation is a highly specialized vascular bed where the requirement of guaranteeing a good optical image appears to have taken precedence over the provision of a plentiful blood supply. It is relatively sparse, and even under normal conditions it operates with a large arteriovenous oxygen difference. The main arteries divide to form two to three capillary beds in most holangiotic mammalian retinas. Adrenergic autonomic innervation ceases at the optic nerve head, and there is no evidence of parasympathetic or peptidergic innervation in retinal arteries, so the dynamic process of matching local tissue blood flow to local metabolic needs must be controlled by other factors. Likely candidates for such control mechanisms are: smooth muscle myogenic responses to blood pressure and intraocular pressure changes, release of metabolic signalers from neural and glial tissue, blood-borne factors, autocoids such as nitric oxide and endothelin, which may be released from endothelial cells, and feedback communication between the venous and arterial sides of the circulation. It is possible that considerable heterogeneity exists in the vasoactive response of different segments of the retinal circulation from first order arteriole to capillary and retinal veins. Loca-
tional heterogeneity has already been reported in in vitro investigations of the ophthalmic artery, and the observation of a differential shunting of the red cell moiety of blood between the superficial and deep capillary beds in the retina also implies localized control mechanisms. If such heterogeneity is a consistent feature of the retinal circulation, then in vitro preparations could provide the means to help unravel this differential control. The local control of retinal arteries has already been explored in in vitro preparations of the larger (>200 μm diameter) bovine retinal arteries, using a ring segment technique. The ring segment technique that we and others have used has the limitation that candidate vasoactive agents act on both sides of the vessel wall simultaneously, and that small diameter vessels such as those found in the human cannot be used. The recent proliferation of papers demonstrating differential responses to intraluminal (IL) and extraluminal (EL) application of vasoactive candidates in vessels from other organs such as the brain, car, tail, mesentery, and cheek pouch, as well as the carotid artery, has driven us to question whether retinal vessels display a preference for IL or EL application of candidate vasoactive agents. To differentiate between IL- and EL-induced responses, it is necessary to perfuse the vessel segment with a physiological solution so that the agents can be added either to the perfusate or to the bathing solution separately. This constant perfusion technique is arguably closer to the true physiological situation than can be achieved with the ring segment preparation or with vessels that are pressurized under no flow conditions. Indeed, perfusion may be functionally important to vasoactivity, because changes in flow through vessels have been shown to mediate vessel responses both in vivo and in vitro.

We have therefore performed pilot studies that have allowed us to develop a perfusion-based technique for retinal vessels based on the work of Chonko et al in the kidney, and Duling and Rivers for mesenteric vessels. It also owes some of its features to the microperfusion system used by Murta et al for blood retinal barrier studies in rabbit retinal vessels. Although this study relates exclusively to retinal vessels from pigs, the same system has proved suitable for retinal vessels from human, cat, and dog. We initially concentrated on optimizing and validating the perfusion technique itself and then investigated the differential sensitivity of in vitro pig retinal arteries to IL and EL application of catecholamines and K+. Histology of these vessels has been performed in parallel so that the structure of the vessel wall may be related to vascular responses. We regard this as a necessary first step in the process of determining controlling factors for the retinal circulation.

MATERIALS AND METHODS

Animals
A total of 60 pig eyes was used in this study. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The eyes were obtained either from a local abattoir immediately after slaughter or from animals under Halothane anesthesia in our own surgical facility. In both instances the eyes were enucleated and placed in a sealed bottle of oxygenated Krebs solution containing 0.6% dialyzed albumin (BSA) and kept on ice for transfer to the laboratory (10 to 45 minutes).

Histology of the Retinal Artery
In a preliminary histologic study, two freshly enucleated pig eyes were used for structural studies of the retinal artery. The ophthalmic artery was cannulated, and the whole eye was perfused initially with Krebs solution for 10 minutes at an input pressure of 50 to 70 mm Hg, followed by 2.5% gluteraldehyde for 30 minutes. After making an entry hole into the vitreous at pars plana ciliare to allow easy diffusion into the ocular tissues, the whole eyeball was further fixed in 2.5% gluteraldehyde for 24 hours. Pieces of major arteries and adjacent retina were excised, post fixed in osmium tetroxide, dehydrated in graded ethanol’s, and embedded in epoxy resin for light and electron microscopy.

Preparation of Vessel for In Vitro Study
Using a dissecting microscope (Zeiss, Oberkochen, Germany), the eyes were sectioned at pars plana ciliare, separating the anterior segment and adherent vitreous body from the posterior pole. The retina, choroid, and sclera were divided into quadrants using a razor blade, taking care not to section any major retinal arteries. The retina was carefully separated from the underlying choroid and sclera using an iris speculum. A quadrant of retina was placed on a hollowed glass slide containing Krebs solution plus 0.6% dialyzed albumin (BSA) kept at less than 4°C. Using a combination of transmitted light with a stereo microscope (Wild, M3Z, Heerbrugg, Switzerland), individual first-order retinal arteries (outside diameter 60 to 120 μm) were dissected from retinal tissue with a fire-polished micropipette. A segment of artery approximately 800 to 1500 μm long was selected; in most experiments, care was taken to include a side branch of outside diameter 15 to 30 μm, although in a few cases, a segment with no side branch was chosen. This arterial segment was then transferred to the incubator chamber.

Incubator Chamber
The incubator chamber (PDMI-2, Medical Systems, New York, NY) was mounted on the stage of an inverted microscope (Nikon Diaphot-TMD, Tokyo, Japan). Animals in Ophthalmic and Vision Research. The eyes were obtained either from a local abattoir immediately after slaughter or from animals under Halothane anesthesia in our own surgical facility. In both instances the eyes were enucleated and placed in a sealed bottle of oxygenated Krebs solution containing 0.6% dialyzed albumin (BSA) and kept on ice for transfer to the laboratory (10 to 45 minutes).

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FIGURE 1. Schematic of the configuration used in the pilot study of vessels with no side branches. The heights of the reservoirs $H_L$ and $H_R$ produce pressures $P_L$ and $P_R$ in the left-hand and right-hand pipettes.

The chamber contained 5 ml Krebs solution maintained at 37°C using a bipolar temperature controller (Model TC-202, Medical Systems). The incubating solution was equilibrated with 95% O₂, 5% CO₂ gas flowing over the surface of the chamber to maintain PO₂, PCO₂, and pH of the incubating solution. This was verified by occasional aspiration of samples for blood gas analysis (Ciba–Corning, Essex, UK).

Pilot Study

Initially, a modified form of the perfusion system of Duling and Rivers was set up as shown schematically in Figure 1. Pieces of vessel without any branches were selected, each end of the arterial segment was sealed into the specially made cannulae (described later), and hydrostatic pressure heads $H_R$ and $H_L$ were connected to the two ends, creating pipette pressures $P_R$ and $P_L$, respectively. Two configurations were used. $P_R > P_L$, in which case perfusate flowed from right to left with an unknown flow rate. Alternatively, the connection to the left-hand reservoir was closed, in which case the vessel was pressurized at a value equal to $P_R$ with no flow during equilibrium conditions. The effect on diameter of these two procedures for one vessel is shown in Figure 2, where both outside diameter in microns and luminal pressure are plotted as a function of time. Initially, a flow condition was used with $P_R > P_L$, and the vessel diameter was 104 μm. Then, when the vessel was pressurized at the same value (33 mm Hg), but with no flow, the diameter of the vessel dropped to 95 μm. Furthermore, when the $H_R$ pressure head was increased to 65 mm Hg, a clear myogenic constriction was seen, with a further decrease in diameter to 82 μm. On lowering the pressure once more, the vessel dilated back to 95 μm.

These data demonstrate two important points that were verified on many occasions. Firstly, a pressurized vessel with no flow exhibits a clear myogenic constriction, and, secondly, a vessel that is perfused but has a similar intraluminal pressure shows little myogenic activity and has a larger resting diameter. This flow dependency at low flow rates makes it particularly important that the perfusate flow rate should be a known and controlled parameter. We also reasoned that continuous perfusion is a more physiological condition, and consequently we...
FIGURE 3. Schematic of the cannulation and perfusion system for the isolated retinal arteries. Both ends of the vessel were cannulated and sealed by gentle squeezing the vessel wall between the inner and outer pipettes. Syringe pumps delivered perfusate at a controlled rate. Pressure transducers P1, P2, P3, and P4 measured the resulting pressures at the points indicated. Typically, the right-hand pipette system was used for the main perfusate (5 μl/min) and drug delivery, whereas the left-hand pipette was used to measure the intravascular pressure. The only perfusate outflow path (arrow) is through the side branch of the main vessel. Test agents were introduced into the perfusate stream through a sample injector valve, V1. The vessel was maintained in an incubation bath on the stage of an inverted microscope (M), and video images were output to a monitor and automated vessel diameter measuring system. All relevant parameters were recorded on a chart recorder, and data were streamed directly to computer disk when required.

devised a perfusion system in which the flow could be controlled and the pressures and the vessel diameter continuously monitored. Because this is a new technique, we describe it in some detail.

Cannula and Perfusion System

General. The principle used in these experiments was that vessels with a side branch were cannulated at both ends and perfused through one end (proximal) with a flow of 5 μl/min in the orthograde direction, with the side branch acting as the exit route, as shown in Figure 3. This choice of 5 μl/min as a baseline perfusion flow rate was based on in vivo measurements using laser Doppler velocimetry. The other end (distal) was generally left with a small flow rate (0.3 μl/min). This residual flow helped ensure that drugs delivered from the other pipette did not collect in the distal end of the vessel. The vessel diameter to the right-hand side of the branch was monitored continuously. The diameter changes in response to intraluminal or extraluminal delivery of drugs were compared.

Cannula. Each cannula consisted of four concentric pipettes (Fig. 3). The outer or holding pipette A was shaped on its innermost surface to present two constrictions. The constriction closer to the open end of the pipette served as a surface against which the inner pipette B, the pipette that entered the vessel lumen, squeezed and sealed the vessel wall. The innermost constriction served to centralize the tip of pipette B and to separate it from the inner wall of A. Pipette D delivered the perfusate and IL drugs directly at the shoulder of the perfusing pipette B, close to the vessel to minimize the delay between injection and arrival of the drug in the vessel. Pipette C also delivered perfusate at a rate one fifth of that in D. This ensured that there was no back flow of drugs into the main body of B that could later serve as a pocket of contamination. The resistance of pipette B was minimized and offered less resistance to perfusate flow than that presented by a typical second-order branch.

Perfusion and Mounting System. Perfusion flow was delivered by a computer-controlled syringe pump (Model 22, Harvard Apparatus, South Natick, MA) containing one 1-ml and one 5-ml gas-tight syringe (Activon; Pennant Hills, New South Wales, Australia) connected to the C and D pipettes, respectively. A specially manufactured pipette holding system was developed that allowed relative movement between pipettes A and B while maintaining a pressure seal and minimal compliance. The whole assembly was mounted on a joystick-controlled XYZ micro drive (Fine Science Tools, Foster City, CA) and angled at 85° to the horizontal. An equivalent system of pipettes and manipulators was used for the left-hand side and perfused by a second, independently controlled pump.

Vessel Cannulation

The vessel was positioned horizontally in the incubation bath close to the bottom of the dish. By conven-
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Vessel diameter was measured by a second computer between pipettes A and B was achieved using a hydraulic micro drive. Pipette B was retracted into the cannula and gentle suction was applied to pipette A to draw the end of the vessel into the cavity between the two constrictions. Pipette B was then advanced into the vessel lumen such that it squeezed the vessel wall against the constriction. This procedure was performed while a continuous perfusion of 5 μl/min was flowing. It was found that this helped keep the vessel lumen open. Once this end of the vessel was sealed, the perfusate flow flushed out the red blood cells from the lumen of the vessel, and a similar procedure was repeated on the other end. Once both ends were cannulated, perfusion delivery at the distal end was reduced to a residual level, typically 0.3 μl/min. The vessel was left to stabilize for 30 minutes.

Experiment Control and Diameter and Pressure Measurement

All of the data recording and much of the instrument control was under computer control. The software was developed using the graphical programming language LabView (National Instruments, Austin, TX) and run on a 486DX PC with the appropriate serial and IEEE communications cards added. The inverted microscope image of the vessel was captured with a CCD camera (MW-BL602, Panasonic, Tokyo, Japan), was displayed on a color video monitor (Trinitron; Sony, Osaka, Japan), and could be recorded using a video-cassette recorder (VO-9850P, Sony; Tokyo, Japan). Vessel diameter was measured by a second computer (386SX) using a commercially produced software package and frame grabbing card (DIAMTRAK, Dinda; Ringwood, Victoria, Australia), which produced an analog output proportional to vessel diameter. The pressures P1, P2, P3, and P4 (Fig. 3) were measured by conventional transducers (Cobe, Arvada, Colorado), each connected to a bridge amplifier (5B38-02, Analog Devices; Norwood, MA), and the data were reproduced on the computer screen and streamed directly to a spreadsheet file as required.

Intraluminal and Extraluminal Drug Delivery

Drugs to be delivered intraluminally were administered as a 5-μl bolus into the perfusate stream through the sample injector valves. The intrinsic design of this injector system permitted inclusion of the drug bolus without any pressure artifact or problems with air bubbles. A built-in switch allowed a signal to be generated to indicate loading and injection phases of the procedure, and this signal was recorded by the chart recorder and the computer. At a typical perfusion rate of 5 μl/min, the drug arrived at the vessel approximately 90 seconds after injection. This was determined by visual observation of dye bolii. Spreading of the original bolus in transit results in a slight dilution of the drug, but this is small compared to the log unit increments in concentration. The size, and hence the duration of the bolus, was sufficient for the vasoactive response to stabilize. Extraluminal drug delivery was achieved by direct pipetting into the incubating solution to achieve the required concentration. The bathing solution was flushed frequently.

Blood Retinal Barrier

The retinal circulation normally has a tight blood retinal barrier. Sodium fluorescein (0.05%) was injected intraluminally in the manner described above. Using the epifluorescence attachment (TMD-EF, Nikon), the sodium fluorescein could be seen arriving at the vessel, and we looked carefully for leakage through the vessel wall and for the presence of fluorescein within or between the mural cells of the vessel.

Solutions

Vessels were usually bathed and perfused with normal Krebs solution with the following composition: NaCl 119 mM, KCl 4.6 mM, CaCl2 1.5 mM, MgCl2 1.2 mM, NaHCO3 15 mM, NaH2PO4 1.2 mM, Glucose 6 mM. High potassium solution (124 mM K+) was produced by equimolar substitution of Na+ with K+. Solutions were equilibrated with 95% O2, 5% CO2.

Agonists

A agonists, (+−)-noradrenaline HCl (NA) and (−)-adrenaline (A) were obtained from Sigma Chemicals (St. Louis, MO) and dissolved in 0.9% NaCl. Stock solutions of all drugs were stored at −70°C, and fresh dilutions were made daily.

Experimental Protocol

After an equilibration time of 30 minutes, three repeated IL injections of 124-mM K+ were performed to test for vessel viability and stability. After successful completion of this stage, frequently, but not always, a 10−6 M Ach dilatation response was checked to ensure viability of the endothelial cells. Then IL dose re-
sponse curves were measured for the appropriate agonist. For each IL injection, the valve loop was loaded with the 5 µl of the appropriate concentration. The 5-µl bolus was switched into the perfusion line, passing through the pipette into the artery. As soon as the response stabilized, replacement of the incubating solution was begun to ensure that drug concentrations in the bath remained low. After at least 10 minutes, the time required for the vessel to return to its preactivated state, the process was repeated with a higher concentration of intraluminal drug. Dose concentration was usually increased in log units from 10^{-10} to 10^{-5} M. Before and after each type of agonist, the valve was flushed and loaded with Krebs solution, and a control IL injection was performed. A positive control response meant that further flushing of the valve was required. Then, cumulative EL dose response curves were measured by adding a stepwise increasing dose to the incubating solution, whereas the vessel was perfused IL by Krebs solution. On some occasions, as a direct confirmation of IL and EL agonist sensitivities, the agonist was first applied EL and left in the bath, and the same dose was perfused intraluminally; comparisons of diameter changes on the proximal and distal sides of the branch vessel were made (see later). Any vessel in which the diameter did not return to within 10% of baseline after exposure to an agonist was rejected.

RESULTS

Histology and Visualization of Perfused Vessel

Figure 4A is a light micrograph of a pig retinal section containing a retinal artery. Perfusion fixation was performed at close to in vivo pressure. The retinal artery (4 to 5 mm peripheral to the disk edge) lies close to the internal limiting membrane and protrudes into the vitreous body; endothelial cells and smooth muscle cells are clearly visible. Figure 4B is an electron micrograph of a small section of the same arterial wall. The endothelial cell (E) is supported by a peripherally located basement membrane (*). External to this basement membrane is one smooth muscle cell of the media (SM) and its nucleus. Also present is the muscle cell process (P) of another smooth muscle cell. Other similar sections showed that the arterial media consists of no more than two cell bodies and a few cell processes at any location. Each smooth muscle cell is surrounded not only by its own plasma membrane but also by a basement membrane that often connects with the endothelial basement membrane of similar structure and width. External to the smooth muscle cells are loosely arranged collagen fibers that make up the meager adventitia (A).

Figure 5A is a photograph of a perfused retinal artery in situ in the incubation chamber, showing the cannulation pipette at the proximal end. The artery has an external diameter of 83.3 µm and a side branch of diameter 16.7 µm. A magnified photograph of the same vessel in a different focal plane is shown in Figure 5B. Note the thin muscle (media) endothelial cell layer, and the clear outer edge free of neural and glial tissue. With still higher magnification, it is possible to distinguish the intact endothelial and smooth muscle cells by changing the focal plane of the inverted microscope. The average diameter of arteries used in this study was 90.4 ± 2.7 µm (n = 42).

Effects of Perfusion Pressure and Flow on the Vessel

The effect of increasing and then decreasing flow and hence intravascular pressure over a wide range is demonstrated in the left and right panels of Figure 6, respectively, for a vessel with a small-diameter side branch, so that lumen pressures are relatively high. In the bottom panel are the stepwise increases in flow from 1 µl/min to 10 µl/min. The two traces in the middle panel are the pressure values P2 (upper) and P4 (lower). P4 represents the IL pressure at the distal end of the vessel. P4 increases stepwise with flow. Vessel outside diameter (OD, top panel) increases with pressure and flow from 97 µm to 112 µm. With stepwise reduction in flow, diameter and pressure return to similar values. The diameter of the main vessel is not greatly influenced by changes in intraluminal pressure or flow over what one might expect to be the normal physiological range.

However, luminal flow and pressure were important parameters in determining the magnitude of drug responses. Figure 7 shows outside diameter changes in response to repeated injections of 10^{-5} M noradrenaline (arrow) IL (upper panel) as luminal pressure/flow was increased (lower panel). The vessel constricts from 108 µm to 95 µm at a pressure of 35 mm Hg (A), with an accompanying small increase in pressure caused by an increase in branch resistance. For increasing values of pressure and flow, the noradrenaline response decreases (B) and is then lost altogether between 65 (C) and 110 (D) mm Hg. Returning to lower values of pressure and flow allows the vessel to constrict once more in response to noradrenaline (E). If the luminal pressure was left high for extended periods, vasoactivity did not recover on return to normal pressures.

Drug Responses

A raw dose response sequence for IL and EL delivery of noradrenaline is presented in Figure 8 for a vessel of external resting diameter 105 µm. The top panel shows the diameter response for IL delivery. There is a control injection causing no change in arterial
diameter, followed by increasing noradrenaline doses of $10^{-8}$ to $10^{-5}$ M producing an increasingly large contraction. This was then followed by a control injection. At $10^{-3}$ M, the contraction was 22% of resting external diameter. Recovery times were considerably longer for the higher concentrations, and it was necessary to ensure a prompt bath washout of any accumulated drug in the bathing solution to guarantee a return to baseline. For cumulative EL delivery (lower panel), it is clear that all contractions are smaller, with the contraction at $10^{-6}$ M only 9.4% of the original external diameter. Results from several such experiments are averaged and plotted in Figure 9, which shows the mean ± SEM, $n = 7$ to 13, dose response curves for IL (open circles) and EL (filled circles) noradrenaline. They have similar thresholds between $10^{-9}$ and $10^{-8}$ M and rise to saturate at $10^{-4}$ M, but the IL response is significantly larger (Student’s t-test, $P < 0.05$) at concentrations of $10^{-6}$ M and greater. Plateau values were 17.8% ± 1.9% IL and 6.8% ± 1.1% EL.

The possibility that this differential response was caused by the cumulative nature of the EL drug delivery compared with the transient nature of the IL delivery was ruled out as follows. A single EL dose of $10^{-5}$ M noradrenaline was administered, and the response was allowed to plateau. At this stage, the same concentration of IL noradrenaline was administered. The IL dose always caused a further contraction in addition to the sustained EL induced contraction. A second possible explanation for the smaller EL responses is that the flowing perfusate continuously flushed away the EL delivered drug so that its effective concentration at the smooth muscle cell was reduced. To investigate this possibility, the vessel diameter responses were measured on both the distal and proximal sides of the branch point after the drug was added to the bath (EL) with no flow in the distal end so there could be...
FIGURE 5. (A) Photograph of a perfused retinal artery taken through the inverted microscope. Note the cannulation pipette and the side branch (15 μm in diameter) through which the perfusate exits (×120). (B) High-power photograph of the same artery to show the mural cells in focus (see Results) (original magnification, ×480).

no "flushing” effect in this region (see Fig. 3). In this situation, both segments contracted a similar amount, with the proximal end (with flow) contracting slightly less than the distal end (no flow). These contractions were much smaller than those produced by subsequent IL application in the proximal end. Thus, we conclude that any removal of the EL-delivered drug by the IL perfusate flow does not account for the difference in response between IL and EL applied agonists.

Adrenaline shows an IL–EL response pattern similar to that observed for noradrenaline. Raw data sets for IL (top panel) and EL (bottom panel) adrenaline delivery for a 92-μm vessel are shown in Figure 10. The contraction at 10⁻⁵-M IL was 26% of the original diameter, whereas the contraction with 10⁻⁵-M EL delivery was only 8%. The averaged dose response curves (n = 7 to 13) shown in Figure 11 tell the same story, with IL delivery producing significantly larger responses than EL delivery (P < 0.05) at concentrations of 10⁻⁵ to 10⁻³ M. For adrenaline, the IL responses

FIGURE 6. Graph of perfusion flow (bottom panel), P2 mm Hg (upper trace), and P4 mm Hg (lower trace) in the middle panel, and outside arterial diameter (OD μm, top panel) as a function of time in minutes.

FIGURE 7. Contraction responses (OD μm, upper panel) to single intraluminal doses of 10⁻⁵ M noradrenaline (arrow), as luminal pressure (PP mm Hg, lower panel) is increased and then returned to control value. PP = Perfusion pressure.
have a lower threshold \((10^{-8} \text{ M})\) than do the EL responses \((10^{-7} \text{ M})\). The maximum percentage constrictions, 19.0% ± 2.1% IL and 8.4% ± 1.5% EL with adrenaline, are not significantly different \((P > 0.05)\) from those for noradrenaline.

K⁺-induced contractions also caused differential responses for IL and EL application. Intraluminal injection of 124-mM K⁺ caused an average contraction of 19.0% ± 1.6% \((n = 21)\) of the vessel's resting diameter, whereas EL application of the same concentration caused only 5.0% ± 1.0% \((n = 13)\). These contraction percentages are significantly different \((P < 0.001)\).

**Blood Retinal Barrier**

Using IL sodium fluorescein with fluorescent microscopy, visualization of the perfusion of a fluorescent marker through the vessel lumen was observed without leakage from either cannulation point. This indicated a tight cannulation with all perfusate and drugs passing through the vessel lumen. Furthermore, movement of the marker across the vessel wall or between endothelial cells to smooth muscle cells was never observed. Therefore, an intact blood retinal barrier to sodium fluorescein can be assumed to exist in this retinal artery preparation.

**DISCUSSION**

This technique of continuous IL perfusion confers several important advantages over the more commonly used ring segment preparation that we and others have previously used to investigate the vasoactivity of ocular arteries. A perfused artery corresponds more closely to the in vivo situation, allows differentiation of IL and EL responses, may cause less endothelial cell damage, especially for small vessel of outside diameter less than 100 μm, and is more readily applied to smaller vessels such as the human or porcine retinal arteries and arterioles. Moreover, the use of a controlled perfusion technique means that the flow rate is known, and the intraluminal pressure and vessel diameter can be measured. Drug-induced changes in this pressure provide information about the combined vascular resistance of the main vessel.
Interestingly, our comparison of pressurized vessels with no flow and pressurized vessels with flow uncovered a strong myogenic contraction with increasing pressure for vessels in which there was no flow. However, with flow, but at the same pressure, the vessel dilates. Bevan et al. and Kuo et al. also observed flow-induced dilatation in isolated vessels. This effect is currently the subject of further investigations in which the effects of flow and intraluminal pressure can be studied independently. It seems likely that vessel diameter reflects the balance of opposing forces generated by mechanisms that are not well understood. Direct extrapolation of these in vitro findings to the in vivo situation should be resisted because the balance of competing vasoactive mechanisms and the additional systemic influences are too complicated to predict.

Under our normal experimental conditions where flow was present, the arterial diameter was relatively unaffected by incremental increases in flow (Fig. 6). The size of the side branch diameter determined the resultant intraluminal pressure. A small-diameter side branch caused high IL pressure at constant flow, whereas a large-diameter side branch resulted in low IL pressure.

We conclude that the loss of agonist response at high pressures and flows (Fig. 7) was a pressure effect rather than a flow effect. This conclusion is based on observations from vessels of similar outside diameter but with different diameter side branches. All vessels were subjected to the same constant flow, but those with small diameter side branches naturally had higher intraluminal pressure in the main vessel than those with larger side branches. The vessels with higher pressures did not respond to noradrenaline, whereas those with larger side branches and lower intravascular pressures did respond.

In parallel with these quantitative measurements, we were able to visualize clearly and observe the smooth muscle and endothelial cells lining the lumen through the microscope and video camera, because of the thin nature of the vessel walls and the minimal adventitia (Figs. 4, 5). With change of focus, individual smooth muscle cells and endothelial cells could be observed. During contraction, the wall thickness was seen to increase, leading to a proportionally larger decrease in luminal diameter than that monitored by outside diameter measurements.

With experience, it was possible to judge from the visual appearance of the vessel the viability of vasoactive responses. Occasional problems with air emboli in a few experiments not included in these data revealed dramatic changes in cellular appearance, a period of vasospasm, and visible detachment of endothelial cells.

With this continuous visualization, it became apparent when there was some damage to the preparation as the endothelial cells changed appearance, and in extreme cases detached from the smooth muscle cells. These changes in appearance were confirmed by loss of vasoactivity. Thus, in conclusion, we believe that, by using this side branch preparation and associated monitoring techniques, we will be able to separate vasoactive responses from side branches and from the main vessels in future studies.

Using this technique, we provide the first evidence of differential responses of the retinal artery to IL and EL delivery of K⁺, noradrenaline, and adrenaline in...
we can state with confidence that the IL-EL differences were both refuted experimentally (see Results). Thus, EL demonstrated in brain vessels, where the basilar artery small intracerebral arteries had stronger EL responses to adenosine and its analogs.13 In the latter study, this asymmetry between EL and IL was found to be independent of the vascular endothelial cells. Intraluminal responses have also been shown to be larger than EL in the tail artery16 and the mesentery,17 where again the asymmetry was shown not to depend on the endothelial cells. However, in the cheek pouch,18,19 EL responses exceeded IL, and this difference was enhanced further down the vascular bed. In this case, the difference was ascribed to the solubility of the tested molecule in water, because lipid-soluble molecules gave similar responses to EL and IL application. Finally, in the carotid artery,20 there was again a difference depending on which molecules were tested, with IL > EL for purinergic agonists but EL > IL for phenylephrine and 5-HT. Thus, the asymmetry appears to be due to multiple factors and requires further investigation in the retinal artery.

In our study, the threshold responses to intraluminal and extraluminal A and NA were similar and between $10^{-7}$ and $10^{-6}$ M. The maximal contraction produced by these adrenergic agonists and 124-mM K$^+$ was mostly less than 25%. In the cerebral circulation there is also evidence for small responses to adrenergic agonists in small pial vessels (60-μm diameter),32 where relaxing factors released from endothelial cells have been shown to reduce the response to NA. In the retina, Forster et al.33 have demonstrated the presence of few α1 and α2 binding sites in homogenates of bovine retinal arteries and veins, although these sites do have a high affinity at nM concentrations. This lack of sites agrees with the weak contractile responses we have observed. In bovine retinal artery ring segments, the response to adrenergic agonists was weak and had a high threshold (compared with the PGF$_2$α-induced contraction).9 Because blood levels of adrenergic agonists are in the nM range, it would appear unlikely that adrenergic agonists are important factors for the control of the resistance of first-order arteries of the retinal circulation. In lieu of autonomic control of these arteries, therefore, one can speculate that metabolic status signalers released from retinal neural tissue may be responsible for much of their vasoactivity, and the Müller cells and astrocytes that surround the retinal vasculature may mediate these responses directly on to the muscle cells of the arterial wall. On the IL side, however, the endothelial cells may mediate blood-borne factors. It is perhaps relevant that the first-order retinal artery has only 1 to 2 layers of smooth muscle cells with virtually no adventitia, so that it is a simple system in which the interaction between endothelial cells, smooth muscle cells, and glial and neural cells can be studied.

Spontaneous vasomotion was sometimes observed in the retinal artery, and its magnitude was enhanced after exposure to NA. This agrees with data from cerebral arteries, in which vasomotion at 4 cyc/minute was common, and which was also potentiated by NA. In the cerebral arteries, vasomotion was abolished by el-

FIGURE II. Average percentage contraction for increasing log ([M]) concentrations of adrenaline for intraluminal (open circles) and extraluminal (filled circles) application. n = 7 to 13.
ther low or high pressures, leading the authors to conclude that vasomotion is a normal property of cerebral arteries.

In conclusion, we have developed a sophisticated and technically demanding technique that has demonstrated that heterogeneity is an important factor in the retinal circulation between intraluminal and extraluminal responses. It remains to be seen whether this polarity of response is general for all drugs or whether some are more active when applied extraluminally. One might predict that vasoactive agents released as metabolic signalers between tissue and the retinal vessels will be most effective on the EL side, but that remains to be determined. The data presented here demonstrate that this technique is capable of providing reliable and reproducible data and that it has considerable potential to enhance our understanding of retinal vascular control. Indeed, it is already clear that the first-order retinal circulation differs substantially from the ophthalmic artery in its pharmacologic responses, and it is likely that this heterogeneity in response continues down the vascular pathway. Thus, for a complete understanding of the sites and actions of drugs in the retinal circulation in health and disease, it is essential that all regions of the vasculature from conduit artery to capillaries and veins be studied. Moreover, the perfused retinal artery offers an exciting system to study more fundamental questions interrelating smooth muscle and endothelial cell function for vascular physiologists, because it is a vessel with a simple wall structure, only 1 to 2 layers of smooth muscle cells, an endothelial cell layer, and no confounding autonomic nerve endings.

Key Words
vasoactivity, perfused artery, pig, retina, agonists

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