The Role of K^+ and Cl^- Channels in the Regulation of Retinal Arteriolar Tone and Blood Flow

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PURPOSE. This study tested the role of K^+ and Cl^- channels in the regulation of retinal blood flow.

METHODS. Studies were carried out in adult Male Hooded Lister rats. Selectivity of ion-channel blockers was established using electrophysiological recordings from smooth muscle in isolated arterioles under voltage clamp conditions. Leukocyte velocity and retinal arteriolar diameter were measured in anesthetized animals using leukocyte fluorography and fluorescein angiography imaging with a confocal scanning laser ophthalmoscope. These values were used to estimate volumetric flow, which was compared between control conditions and following intravitreal injections of ion channel blockers, either alone or in combination with the potent vasoconstrictor Endothelin 1 (Et1).

RESULTS. Voltage-activated K^+ current (I_{Kv}) was inhibited by correolide, large conductance (BK) Ca^{2+} -activated K^+ current (I_{KCa}) by Penitrem A, and Ca^{2+} -activated Cl^- current (I_{ClCa}) by disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS). Intravitreal injections (10 μ L) of DIDS (estimated intraocular concentration 10 mM) increased flow by 22%, whereas the BK-blockers Penitrem A (1 μ M) and iberiotoxin (4 μ M), and the I_{Kv} -inhibitor correolide (40 μ M) all decreased resting flow by approximately 10%. Endothelin 1 (104 nM) reduced flow by approximately 65%. This effect was completely reversed by DIDS, but was unaffected by Penitrem A, iberiotoxin, or correolide.

CONCLUSIONS. These results suggest that Cl^- channels in retinal arteriolar smooth muscle limit resting blood flow and play an obligatory role in Et1 responses. K^+ -channel activity promotes basal flow but exerts little modifying effect on the Et1 response. Cl^- channels may be appropriate molecular targets in retinal pathologies characterized by increased Et1 activity and reduced blood flow.

Keywords: retinal blood flow, retinal arterioles, smooth muscle, ion channels

Regulation of retinal blood flow to meet local metabolic need is an important aspect of retinal homeostasis, directly analogous with neurovascular coupling within the brain and functional hyperemia in other tissues of the body.^{1,2} There is also accumulating evidence that loss of appropriate reflex changes in vascular resistance and blood flow is an early feature in a number of sight threatening pathologies, such as diabetic retinopathy, glaucoma, and AMD.^{3,4} Abnormalities in blood flow and its regulation may even play a pathogenic role in the natural evolution of these conditions.⁵ These observations emphasize the importance of understanding the physiological mechanisms responsible for the control of retinal blood flow because this may result in the identification of new therapeutic targets for early intervention in at risk individuals.^{6,7}

Despite its physiological and pathophysiological importance, the control of retinal blood flow is not well understood. Assuming both mean arterial blood pressure and IOP remain constant, changes in retinal flow must reflect changes in microvascular resistance. These result from contraction and relaxation of vascular smooth muscle and pericytes, which alter vessel diameter. Retinal arterioles have the greatest overall resistance as a vascular unit and a complete layer of smooth

muscle along their entire length.⁸ This makes them ideally suited to play an important role in the control of overall resistance, although capillary pericytes may also contribute.⁹ Studies using isolated arteriolar preparations from a range of tissues have provided considerable evidence that ion channels in the cell membrane of smooth muscle cells play an important role in regulating arteriolar tone, both under resting conditions and when exposed to vasoconstrictor and vasodilator agonists.¹⁰ A variety of Cl^- and K^+ channels modulate the membrane potential in smooth muscle, activating or deactivating voltage-operated Ca^{2+} channels. This, in turn, modulates intracellular $[Ca^{2+}]$, leading to myocyte contraction and relaxation, which result in vascular constriction or dilatation.^{11–14} We have previously used electrophysiological techniques to show that voltage sensitive K^+ channels (K_v), large conductance Ca^{2+} -sensitive K^+ channels (BK) and Ca^{2+} -sensitive Cl^- channels ($ClCa$) are all expressed in the smooth muscle of rat retinal arterioles.^{15–17} Inhibition of BK currents leads to increased constriction in pressurized arterioles, suggesting that these channels are involved in limiting development of myogenic tone.¹⁵ In contrast, inhibition of I_{ClCa} has no effect on arteriolar myogenic tone,¹⁶ but inhibits both the Endothelin

1 (Et1)-induced increases in arteriolar Ca^{2+} waves in vitro and the resulting constrictor response in vivo.¹⁸ This is consistent with the well-established role for I_{ClCa} in amplifying agonist-induced Ca^{2+} signals in smooth muscle through membrane depolarization and activation of Ca^{2+} channels.¹⁹

Although the use of isolated vessels allows maximal control of experimental conditions, the results obtained and interpretation placed on them require confirmation in vivo. Furthermore, vessel diameter is only important as a correlate of blood flow, the physiologically significant variable. We have, therefore, undertaken a series of experiments in which blood flow was measured directly in retinal arterioles in anesthetized rats. Pharmacologic agents were first assessed in vitro, using electrophysiological recording of the relevant membrane currents to demonstrate their specificity in the animal model used, and then injected intravitreally to assess the contribution of K^{+} and Cl^{-} channels both in the regulation of basal retinal flow and in the response to Et1. This approach also allowed the overall biological significance of these ion channels to be assessed, and suggests that Cl^{-} currents may play a more dominant role than K^{+} currents in regulating flow through the retinal vasculature, at least under the conditions of our experiments. This suggests that Cl^{-} channels may be appropriate molecular targets, suggesting novel therapeutic approaches to retinal pathologies characterized by abnormal flow and impaired microvascular reflexes.

MATERIALS AND METHODS

All procedures complied with the United Kingdom Animals (Scientific Procedures) Act 1986, and with ARVO's Statement for the Use of Animals in Ophthalmic and Visual Research. In vivo experiments were carried out under general anesthesia (see below) followed by euthanasia without recovery.

Electrophysiological Recordings From Isolated Rat Retinal Arterioles

Arteriole isolation and the techniques used to make electrophysiological recordings have previously been described in detail.¹⁷ Male Hooded Lister rats (300–450 g; Harlan Laboratories, Wyton, England, UK) were euthanized with CO_2 . Retinas were dissected and mechanically triturated in low- Ca^{2+} Hanks' solution and pipetted into a glass bottomed organ bath. Arteriole segments devoid of neuropile (diameter 20–40 μm , length 50–500 μm) were visualized using an inverted microscope (Nikon Eclipse TE2000-S; Nikon Instruments, Kingston on Thames, UK) at $\times 40$ magnification, anchored in place using tungsten wire and superfused with normal Hanks' solution at 37°C . Partial digestion was carried out in situ with collagenase 1A (0.1 mg/mL; Sigma, Poole, UK) and protease type XIV (0.01 mg/mL; Sigma) in low Ca^{2+} Hanks solution. This removed the outer basal lamina and electrically uncoupled the smooth muscle cells from the underlying endothelium and each other.^{17,20}

Whole-cell electrophysiological recordings were made using the perforated patch clamp technique.²¹ Patch electrodes with a resistance of 0.5 to 1 M Ω were pulled using filamented borosilicate glass capillaries (inner diameter 1.17 mm; outer diameter 1.5 mm; Harvard Apparatus, Edenbridge, UK) and filled with a pipette solution containing 300 $\mu\text{g}/\text{mL}$ of amphotericin B (Sigma). Membrane currents were recorded using an Axopatch 1D patch-clamp amplifier (Axon Instruments, Aberdeenshire, UK) with voltage protocols and data acquisition controlled using WINWCP software (v.3.3.3; University of Strathclyde, Glasgow, UK). Current was low-pass filtered at 0.5 kHz and digitally sampled at 2 kHz. Liquid

junction potentials (< 2 mV) and up to 80% of the series resistance were compensated electronically. Currents were leak-subtracted offline and cell capacitance was used to determine current density (pA/pF).

In Vivo Imaging of Arteriolar Blood Flow

Retinal arteriolar blood flow was recorded using leukocyte fluorography and fluorescein angiography. Male Hooded Lister rats (320–450g) were anesthetized using an intraperitoneal injection of a solution containing ketamine (37.5 mg/mL; Fort Dodge Animal Health, Southampton, UK) and xylazine (5 mg/mL; KVP Pharma und Veterinar-Produkte GmbH, Kiel, Germany) at a dose of 0.35 mL per 100 g. Depth of anesthesia was assessed using corneal and foot pinch reflexes and top-up doses (0.1 mL per 100 g) introduced if needed. Rats were placed on a heat pad at 25°C (SnugglySafe; Lenric International, Littlehampton, UK) and rectal temperature monitored (digital thermometer model 77020; Medisana, Dusseldorf, Germany). The retina was imaged with a HRA2 confocal scanning laser ophthalmoscope (CSLO; Heidelberg Engineering, Heidelberg, Germany). Based on published keratometry results for the rat, a corneal curvature setting of 2.5 was used.²² Chin and forehead rests were removed and an adjustable animal stage added. Images were recorded in high-speed mode (8.8 frames/s) with a 30° field of view. Acridine orange (4.14 mM in PBS; Molecular Probes, Leiden, The Netherlands) was injected into the peritoneum at 0.125 mL per 100 g body weight. Acridine fluoresces when bound to DNA, and so selectively labels leukocytes in the blood.²³ Leukocyte transit velocity along individual vessels was estimated from sequential confocal images (488-nm excitation, emitted light filtered using a 500-nm long-pass filter). Arterioles were easily identifiable by the direction of leukocyte travel, which was away from the optic disk (Fig. 1A). Vessels in the superior hemisphere of the retina, where flow was oppositely directed from the confocal raster, were selected for video acquisition, as this maximized the probability of imaging a labelled cell in successive frames. Once leukocyte fluorography was completed, 10% sodium fluorescein (0.025 mL per 100 g body weight; Martindale Pharmaceuticals, Essex, UK) dissolved in sterile water (Braun, Melsungen, Germany) was injected into the tail vein of the rat. Single images were captured to allow vessel diameter to be accurately estimated (Fig. 1B).

Image Analysis

Each pixel in high-resolution HRA2 images of the rat retina equates to 1.15 μm .²⁴ High-speed images obtained during leukocyte fluorography have one-half this resolution (i.e., 2.30 $\mu\text{m}/\text{pixel}$). These values were used to estimate single arteriole blood flow (SABF) from leukocyte fluorography and fluorescein angiograms for the same vessels. Each time series of consecutive confocal images acquired during fluorography were saved as audio video interleave files and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). A plug-in was customized to calculate the distance traversed by any labelled cell between consecutive frames. These distances were converted to transit velocities using a factor of 2.30 $\mu\text{m}/\text{pixel}$ and an inter-image interval of 0.11 seconds (8.8 frames/s). The diameters of the relevant arterioles were determined from high-resolution fluorescein angiograms. These were saved as bitmaps and analyzed using custom software using the Image Processing Toolbox in Matlab (The Mathworks, Natick, MA, USA). This calculated the average diameter (d) for any selected arteriole. Leukocyte transit velocity (v) was converted to SABF using this diameter and a presumed circular cross-section ($\text{SABF} = v \cdot \text{Cross Sectional Area} = v \cdot \pi r^2 = \frac{1}{4} \cdot v \cdot \pi d^2$). This assumes

FIGURE 1. Measurement of retinal blood flow using leukocyte fluorography and fluorescein angiography in the rat retina. (A) Three consecutive frames tracking the movement of a fluorescently labelled leukocyte (white arrows) through a retinal arteriole. (B) A subsequent fluorescein angiogram of the same region of the retina. Leukocyte transit velocity (v) and average arteriole diameter (d) were used to calculate SABF ($\text{SABF} = v \cdot \pi d^2 / 4$). Scale bars: 100 μm .

that flow is not turbulent and that v is a reasonable measure of the spatially averaged blood velocity. Individual observations will over- or underestimate flow, depending on how close a specific cell is to the vessel midline, but these errors will tend to average out in cumulative data sets, allowing meaningful comparisons between different experimental conditions (see Discussion). This technique produced results with low levels of variability, with an SEM under control conditions (no intravitreal injection) of 0.2 nL s^{-1} , less than 2% of the mean volumetric flow and even at the lowest flow rates, the SEM was still less than 5% of the mean (Tables 1, 2).

Intravitreal Injections

The effects of drugs were assessed following intravitreal injections. Stock solutions were diluted in Hanks' solution and injected at the following concentrations: 260 nM Et1 (Tocris, Abingdon, UK); 25 mM disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS; Sigma), 2.5 μM Penitrem A (Sigma); 10 μM iberiotoxin (Tocris); 100 μM correolide (gift from Maria Garcia and Jianming Bao of Merck Research Laboratories, Rahway, NJ, USA). Using a 29-gauge insulin syringe (Kendall monoject; Covidien, Dublin, Ireland), 10 μL were administered while viewing the eye through a surgical microscope (Zeiss OPH1 1-H; Carl Zeiss, Cambridge, UK). The intraocular drug concentration (see Results) was estimated

assuming equilibration into a vitreal volume of 15 μL (total volume, 25 μL).²⁵ High intravitreal concentrations (~ 10 times those typical *in vitro*) were used since rapid clearance via the anterior segment may greatly reduce the effective concentration at the retina.²⁶ Observations were made at least 15 minutes after injection.

Solutions and Drugs

The Hanks' solution used had the following composition: NaCl, 140 mM; KCl, 6 mM; D-glucose, 5 mM; CaCl_2 , 2 mM; MgCl_2 , 1.3 mM; HEPES, 10 mM (Sigma); pH set to 7.4 with NaOH. The low- Ca^{2+} solution used for vessel isolation differed only in that it contained 0.1 mM CaCl_2 . For electrophysiological experiments, either a K^+ -based (KCl , 138 mM; MgCl_2 , 1 mM; EGTA 0.5 mM; CaCl_2 0.2 mM; HEPES, 10 mM; pH adjusted to 7.2 using KOH: free $\text{Ca}^{2+} \sim 100 \text{ nM}$; Maxchelator, Stanford University, Stanford, CA), or a Cs^+ -based (in mM: CsCl, 138; MgCl_2 , 1; EGTA 0.5; CaCl_2 0.2; HEPES, 10; pH adjusted to 7.2 pH using CsOH) pipette solution was used. In each case, 300 $\mu\text{g/mL}$ of amphotericin B was added to the pipette solution.

Acridine orange, 4-aminopyridine (4AP; Sigma), amphotericin B, collagenase 1A, DIDS, Penitrem A, protease type XIV, Et1, and correolide were dissolved directly in Hanks' solution and applied in prewarmed bath solution to isolated arterioles or injected intravitreally as described above.

TABLE 1. Effects of Intravitreal Injections (10 μL) of Ion Channel Blockers on Basal Arteriolar Blood Flow in Single Retinal Arterioles of Anesthetized Hooded Lister Rats (Mean $\pm$ SEM)

Treatment, Estimated Intraocular Concentration	N Observations	Retinal Arteriole Leukocyte Velocity, mm s^{-1}	Retinal Arteriole Diameter, μm	Retinal Arteriole Blood Flow, nL s^{-1}
Control (no injection)§	19 arterioles 9 animals	10.3 ± 0.1	38.9 ± 0.3	12.3 ± 0.2
Hanks' only§	13 arterioles 8 animals	10.4 ± 0.1	39.6 ± 0.4	12.9 ± 0.3
DIDS (10 mM)	17 arterioles 9 animals	10.6 ± 0.2	$43.1 \pm 0.4^\ddagger$	$15.7 \pm 0.4^\ddagger$
Penitrem A (1 μM)	30 arterioles 11 animals	$9.6 \pm 0.1^\ddagger$	37.6 ± 0.5	$11.3 \pm 0.3^\dagger$
Iberiotoxin (4 μM)	18 arterioles 8 animals	10.1 ± 0.1	36.7 ± 0.7	$11.2 \pm 0.4^*$
Correolide (40 μM)	15 arterioles 6 animals	$9.5 \pm 0.2^\ddagger$	39.7 ± 0.4	$11.7 \pm 0.3^\dagger$

Post hoc analysis: * $P < 0.05$, $^\dagger P < 0.01$, and $^\ddagger P < 0.001$ versus Hanks' only.¹⁸

§ Summary diameter data for these experiments reflects a subset from previously published results.¹⁸

TABLE 2. Effects of Intravitreal Injections (10 μ L) of Et1 (Final Intraocular Concentration 104 nM) in the Presence or Absence of Ion Channel Blockers on Blood Flow in Single Retinal Arterioles of Anesthetized Hooded Lister Rats (Mean $\pm$ SEM)

Treatment, Estimated Intraocular Concentration	N Observations	Retinal Arteriole Leukocyte Velocity, mm s ⁻¹	Retinal Arteriole Diameter, μ m	Retinal Arteriole Blood Flow, nl s ⁻¹
Hanks' only§,	13 arterioles 8 animals	10.4 $\pm$ 0.1	39.6 $\pm$ 0.4	12.9 $\pm$ 0.3‡
Et1 only	14 arterioles 8 animals	7.6 $\pm$ 0.2*	27.4 $\pm$ 0.2*	4.6 $\pm$ 0.2*
Et1 + DIDS (10 mM)	16 arterioles 8 animals	10.6 $\pm$ 0.1‡	41.75 $\pm$ 0.4‡	14.7 $\pm$ 0.3‡
Et1 + Penitrem A (1 μ M)	20 arterioles 9 animals	7.8 $\pm$ 0.2*	29.6 $\pm$ 0.2*‡	5.4 $\pm$ 0.1*
Et1 + Iberiotoxin (4 μ M)	30 arterioles 10 animals	8.5 $\pm$ 0.1*†	27.9 $\pm$ 0.3*	5.3 $\pm$ 0.1*
Et1 + Correolide (40 μ M)	11 arterioles 6 animals	7.0 $\pm$ 0.2*	30.9 $\pm$ 0.3*‡	5.4 $\pm$ 0.1*

Post hoc analysis: * $P < 0.001$ versus Hanks' only, and † $P < 0.05$ and ‡ $P < 0.001$ versus Et1 only.

§ Same dataset as presented in Table 1.

|| Summary diameter data for these experiments reflects a subset from previously published results.¹⁸

Statistical Analysis

Data were plotted as mean $\pm$ SEM and were analyzed using GraphPad Prism V3 (GraphPad Software, San Diego, CA). Data were asymmetrically distributed, so the statistical significance of apparent differences was tested using nonparametric one-way ANOVA (Kruskal-Wallis test) with Dunn's post hoc test to correct for multiple comparisons. In all cases, the acceptable significance level was set at 0.05.

RESULTS

Pharmacological Isolation of K⁺ and Cl⁻ Currents in Retinal Arterioles From Hooded Lister Rats

To validate the use of pharmacologic blockers for the inhibition of ion currents in vivo, it was first necessary to establish their actions electrophysiologically in the animal model used. Retinal arterioles were isolated from Hooded Lister rats and electrophysiological recordings were made under voltage clamp conditions. Brief enzyme digestion electrically isolated individual myocytes from neighboring endothelial and smooth muscle cells.¹⁷ Different conditions and drug combinations were then used to isolate specific currents and demonstrate that these could be blocked using relevant inhibitors. Using a K⁺-based pipette solution with 100 nM Penitrem A and 1 mM DIDS in the external solution to block large conductance (BK) Ca²⁺-activated K⁺ channels and Ca²⁺-activated Cl⁻ channels, respectively, depolarizing steps positive to -50 mV evoked a rapidly inactivating outward current that peaked within 10 ms (Fig. 2Ai). This resembles the A-type K_v current (I_{Kv}) previously observed in Sprague Dawley retinal arteriolar smooth muscle cells, which we have shown to be inhibited both by 4AP, a classical A-type inhibitor, and correolide, a selective Kv1 blocker.^{17,20} The A-type K_v current in retinal arteriolar smooth muscle Hooded Lister rats was almost completely abolished by correolide at all potentials tested (Fig. 2Aii). When the same voltage protocol was applied with 10 mM 4AP and 1 mM DIDS in the external solution to block I_{Kv} and I_{ClCa}, respectively, a noisy outward current typical of BK channel activation was uncovered (Fig. 2Bi). This was inhibited by 100 nM Penitrem A (Fig. 2Bi-2Bii).

Ca²⁺-activated Cl⁻ currents (I_{ClCa}) were isolated electrophysiologically using a combination of a Cs⁺-based pipette

solution and externally applied 100 nM Penitrem A and 10 mM 4AP to inhibit K⁺ currents. A 250 ms conditioning step to 0 mV activated an inward Ca²⁺ current and subsequent steps to voltages ranging from -100 to +100 mV resulted in inward and outward 'tail' currents (I_{tail}; Fig. 2Ci). The amplitude of I_{tail} was measured after the brief capacitive transient, approximately 25 ms after the start of the test step. The I-V relationship was linear and reversed close to the calculated Cl⁻ equilibrium potential (+2.2 mV; Fig. 2Cii). This current was significantly inhibited by 1 mM DIDS (Figs. 2Ci, 2Cii) and the block showed relatively little voltage dependence, with 65% inhibition at -80 mV and 68% inhibition at +80 mV. This indicates that DIDS is an appropriate drug to use for the assessment of the functional significance of I_{ClCa} in arteriolar regulation in vivo, since the resting membrane potential in vascular smooth muscle is likely to be negative.^{27,28} DIDS had no effect on the inward I_{Ca} elicited during the conditioning step to 0 mV, with mean current densities of 2.04 $\pm$ 0.53 pA/pF under control conditions and 2.39 $\pm$ 0.79 pA/pF in the presence of DIDS ($n = 5$).

Further control experiments were carried out to test whether DIDS affected K_v and BK currents in our preparation. K⁺-based pipette solutions were used with depolarizing steps to 0 mV (Fig. 3). This is close to the theoretical E_{Cl} and should minimize any current contamination by I_{ClCa}. When 1 mM DIDS was applied in the presence of 100 nM Penitrem A (to block BK channels) it had no effect on peak K_v currents, with mean current densities of 10.5 $\pm$ 4.2 pA/pF and 10.0 $\pm$ 3.9 pA/pF in the absence and presence of DIDS, respectively ($n = 6$). Similarly, DIDS had no effect on BK currents recorded in the presence of a K_v blocker (10 mM 4AP), with mean values of 18.2 $\pm$ 4.4 pA/pF and 18.6 $\pm$ 4.4 pA/pF in the absence and presence of DIDS, respectively ($n = 6$). These results suggest that DIDS selectively blocks I_{ClCa} in retinal arteriolar myocytes from Hooded Lister rats.

Role of Ion Channels in Control of Retinal Arteriolar Blood Flow In Vivo

The importance of K⁺ and Cl⁻ channels in retinal blood flow regulation was tested directly using leukocyte fluorography and fluorescein angiography to estimate volumetric flow in single arterioles in anesthetized Hooded Lister rats (see Materials and Methods). Ion channel blockers dissolved in Hanks' solution were injected intravitreally and leukocyte

FIGURE 2. Pharmacologic inhibition of specific ion channels in Hooded Lister retinal arteriolar smooth muscle. (**A_i**) Voltage operated K^+ currents recorded using K^+ -based pipette solution in the presence of 100 nM Penitrem A and 1 mM DIDS. Membrane potential (V_m) was stepped from -100 to $+100$ mV in 20 mV steps (*upper panel*). Currents were recorded from the same cells under control conditions and in the presence of correolide (10 μ M). (**A_{ii}**) Membrane current was expressed as pA/pF and summarized for six cells (mean $\pm$ SEM) before (*filled symbols*) and during (*open symbols*) superfusion with correolide. (**B_i**) Ca^{2+} -activated K^+ currents recorded in the presence of 10 mM 4AP and 1 mM DIDS using the same pipette solution and voltage protocol as in (**A**). Control currents are compared with those recorded from the same cell in the presence of Penitrem A (100 nM). (**B_{ii}**) Summary data for the peak outward current under control conditions (*solid symbols*) and during exposure to Penitrem A ($n = 6$). (**C_i**) Ca^{2+} -activated Cl^- currents recorded using a Cs^+ -based pipette solution and in the presence of 100 nM Penitrem A and 10 mM 4AP to block K^+ currents. A 250 ms conditioning depolarization to 0 from -80 mV elicited an inward Ca^{2+} current. Subsequent test steps (ranging from -100 to 100 mV in 20 mV steps) revealed a series of tail currents, which were largely inhibited by DIDS (1 mM). (**C_{ii}**) Summary data for the tail current density measured immediately after the capacitive transient due to the test step. Control currents (*solid symbols*) are compared with currents recorded from the same cells in the presence of 1 mM DIDS (*open symbols*). (In all summary data, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.)

velocity and arteriolar diameter measured approximately 15 minutes after injection (Fig. 4; Table 1). Arteriolar blood flow was estimated from these values (Table 1). Arterioles in noninjected controls and in eyes injected with Hanks' alone (10 μ L) exhibited similar velocities, diameters, and flow (Table 1). Flow rate increased by over 22% following DIDS injections, reflecting an increase in arteriolar diameter (vasodilatation). In contrast, inhibitors of both large conductance Ca^{2+} -activated K^+ channels (Penitrem A and iberiotoxin) and $Kv1$ voltage-operated K^+ channels (correolide) reduced flow by approximately 10%. This reflected changes in both velocity and diameter, but only the former reached statistical significance and then only for Penitrem A and correolide. Taken together, these data suggest that Cl_{Ca} , BK, and K_v channel activity all play a role in determining retinal blood flow under resting conditions.

Ion channel activation may also play a role in vascular responses to constrictor agonists. We have previously shown that intravitreal injection of DIDS abolishes the vasoconstrictor effect of Et1 on retinal arterioles.¹⁸ Using leukocyte fluorography in combination with fluorescein angiography in the current study allowed us to directly assess the importance of Cl^- and K^+ channels in terms of the most physiologically relevant response to Et1 signaling (i.e., changes in retinal blood flow). Comparisons were made between animals injected with Et1 in combination with one of the ion channel blockers described above and those injected with Et1 or Hanks' alone (Fig. 5; Table 2). Intravitreal injection of Et1 decreased retinal arteriolar blood flow by nearly 65% compared with that observed in eyes injected with Hanks' alone, with significant reductions in both leukocyte velocity and arteriole diameter. Inhibition of Cl^- channels with DIDS completely reversed these effects, returning blood flow to control levels (Table 2).

FIGURE 3. Under the experimental conditions used, DIDS had no effect on IK_v , IBK, or ICa. **(Ai)** K_v current was recorded during a 250 ms depolarizing step from -80 to 0 mV (close to E_{Cl}) in the presence of 100 nM Penitrem A (black trace). Addition of 1 mM DIDS had no effect on this current (red trace). **(Aii)** Summary data for the peak outward K_v current in the absence (control) and presence of DIDS for six such experiments (mean $\pm$ SEM). **(Bi)** A depolarizing step from -80 to 0 mV recorded in the presence of 10 μ M correolide evoked a brief inward Ca^{2+} current followed by a sustained outward BK current (black trace). These currents were unaffected by addition of 1 mM DIDS (red trace). **(Bii)** Summary data for the peak outward BK current in the absence (control) and presence of DIDS for six such experiments (mean $\pm$ SEM).

K^+ channel blockers had variable effects on leukocyte velocity and arteriolar diameter, but no significant effect on arteriolar flow relative to that observed in the presence of Et1 alone (Table 2).

DISCUSSION

The core objective in this study was to directly assess the role of arteriolar ion channels in the control of retinal vascular resistance and blood flow. Arteriolar blood flow was estimated by measuring the transit velocity of leukocytes followed by accurate diameter measurement for the same vessel using

angiography. Techniques based on the assessment of leukocyte velocity in different elements of the retinal circulation have been used extensively before. These often involve visualization of fluorescently-tagged white cells using scanning laser ophthalmoscopy.^{28–32} A more subjective assessment of leukocyte velocity may also be obtained using the blue field simulation technique.^{33–39} In our study, the confocal scanning laser ophthalmoscope was used to record the transit of acridine orange-labeled leukocytes through arterioles (Fig. 1). Labeled leukocytes were easily identified and tracked in consecutive images, allowing transit velocity to be calculated. This was converted to volumetric flow using the vessel

FIGURE 4. Effect of intravitreal injection of selective ion channel blockers on basal blood flow through individual retinal arterioles. Each column represents the mean ($\pm$ SEM) blood flow under the conditions indicated. The estimated final drug concentration and the number of arterioles and animals observed in each case are summarized in Table 1 (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus injection of Hanks' alone).

FIGURE 5. Effect of intravitreal injection of Et1 on blood flow through retinal arterioles in the absence and presence of specific ion channel blockers. Each column represents the mean ($\pm$ SEM) blood flow under the conditions indicated. The estimated final drug concentration and the number of arterioles and animals observed in each case are summarized in Table 2 (*** $P < 0.001$ versus Hanks' alone; ### $P < 0.001$ versus Et1 alone).

diameter, as assessed using fluorescein angiography, to calculate cross-sectional area (Fig. 1).

The assumptions underlying this approach are that arterioles are circular in cross-section, flow is nonturbulent and leukocyte velocity is representative of the spatially-averaged blood velocity. There was no evidence of turbulence in our recordings, and leukocytes maintained a constant position relative to the width of any given vessel in consecutive images (Fig. 1). Previous studies have reported a parabolic blood velocity profile in retinal vessels with a diameter in excess of 100 μm , as predicted for laminar flow conditions.³⁰ The velocity profile is flattened in smaller retinal vessels, however, and the maximum (midline) velocity may be as little as 30% to 40% greater than the spatially averaged velocity.³¹ This helps limit the error in using leukocyte velocity as a measure of mean blood velocity, especially since each white cell occupies a substantial fraction of the width of these small vessels. Blunting of the velocity profile also introduces errors into other more widely used techniques of retinal flow determination, such as bidirectional laser Doppler flowmetry, which estimates the maximum (midline) erythrocyte velocity (v_{max}). Velocity is converted to flow using the equation:

$$\begin{aligned}\text{Flow} &= v_{\text{mean}} \text{ Cross Sectional Area} \\ &= \frac{1}{2} \cdot v_{\text{max}} \text{ Cross Sectional Area} \quad (1)\end{aligned}$$

The relationship $v_{\text{mean}} = \frac{1}{2} \cdot v_{\text{max}}$ is based on the assumption of laminar flow with a parabolic velocity profile.³² With a blunted velocity profile, however, this formula will systematically underestimate the actual flow. Use of leukocyte fluorography also has limitations in this regard since the measured velocity will depend on the position of any given cell relative to the vessel midline. Individual measures will under- or overestimate cell velocity but these errors will tend to average with repeated measurements. It might also be argued that the larger leukocytes will travel less quickly than erythrocytes and so underestimate flow. Leukocyte velocity has been reported to be lower than erythrocyte velocity in retinal capillaries.³³ In a study comparing red and white cell velocity in small arterioles in the hamster cheek pouch, however, no difference was seen.³⁴ Leucocyte margination, rolling, and adhesion were not observed in arterioles in this study.

Fluorescein angiography increases image contrast, allowing vessel diameter to be assessed more accurately than would be possible using confocal imaging alone.³¹ Possible changes in the diameter of the arterioles between measurement of the leukocyte velocity and subsequent measurement of vessel diameter represent another potential source of error. Diameter was not observed to change from image to image during angiography but the fact that cell velocity and diameter were not measured simultaneously is a weakness of the method. Despite these shortcomings, the technique produced flow values that are consistent with results from more established approaches. Although flow calculated from any one observation in any given arteriole cannot be extrapolated to assess total retinal flow, calculations suggest the values obtained are credible.²⁹ Given that there are four to six distributing arterioles in the Hooded Lister rat retina, an average single arteriole flow rate of 12.3 nL/s (Table 1) equates to a total retinal blood flow in the range of 49 to 74 nL/s or 2.94 to 4.44 $\mu\text{L}/\text{min}$. This is comparable with data from a recently published study based on Doppler optical coherence tomography, which reported total retinal arterial blood flow with a time average of approximately 6 $\mu\text{L}/\text{min}$ in Sprague-Dawley rats anesthetized with isoflurane and xylazine, and approximately 3.5 $\mu\text{L}/\text{min}$ with ketamine and xylazine, as used in the experiments reported here.³⁵

Previous reports on the roles of ion channels expressed in retinal vascular smooth muscle have relied almost exclusively on data from *in vitro* experiments using isolated vessels or myocytes.^{15–17,36,37} Few, if any, studies have attempted to test the functional significance of these ion channels *in vivo*. In the current work, the effects of intravitreal injection of pharmacologic channel blockers on retinal blood flow were directly investigated in anesthetized animals. One criticism of this approach is that such injections might alter IOP, and so affect perfusion pressure and flow independently of vascular resistance. Although pressures were not measured directly, control injections of the diluent (Hanks' solution) had no effect on flow, suggesting that mechanically-induced changes in IOP were not responsible for changes in flow. This does not, however, preclude the possibility that injected drugs may affect IOP.³⁸ Any differences in systemic blood pressure between experimental groups (e.g., due to varying levels of anesthesia) might also alter blood flow. Although it seems unlikely that intravitreal injections of specific agents would result in consistent changes in total peripheral vascular resistance or depth of anesthesia required to explain the results reported here, measurement of both IOP and arterial blood pressure would have allowed such concerns to be addressed directly.

Inhibition of I_{ClCa} with intravitreal injection of DIDS increased average retinal flow by 23% under basal conditions (Table 1; Fig. 4). This was associated with dilatation of the arteriole and is consistent with a model in which the DIDS-sensitive inward current recorded at all negative potentials (Fig. 2) plays a significant role in setting resting membrane potential and tone in retinal arteriolar smooth muscle *in vivo*. The results of the current experiment appear to conflict with our previous observation that DIDS did not affect basal tone in pressurized retinal arterioles under conditions of myogenic tone *ex vivo*, although DIDS has been shown to relax pressurized cerebral arteries by other groups.^{16,39} We have previously reported, however, that DIDS inhibits the constrictor effect of Et1 in retinal arterioles both *in vitro* and *in vivo*.^{16,18} We now show that the effects of exogenous Et1 on retinal blood flow are also reversed by DIDS (Table 2; Fig. 5). This is consistent with a range of studies suggesting that I_{ClCa} , which depolarizes smooth muscle cells and activates Ca^{2+} channels, may play an important role in agonist-induced vasoconstriction in a variety of tissues.^{40–42} Blockade of L-type voltage-operated Ca^{2+} channels dilates pressurized retinal arterioles *ex vivo* and these are likely to act as voltage sensors transducing changes in membrane potential to control vascular resistance and flow.⁴³ It may be that the pronounced effects of DIDS on basal blood flow *in vivo* reflect inhibition of I_{ClCa} activated by intracellular Ca^{2+} release in response to endogenous production of Et1, although many other vasoactive substances are known to be released by endothelial and glial cells and may play a similar role.^{4,7,44}

One factor complicating the interpretation of responses to blockers of Cl_{Ca} channels, including DIDS, is their poor selectivity. Electrophysiological recordings showed that DIDS did not affect K_v , BK, or Ca currents in retinal vascular smooth muscle (Fig. 3). These experiments were carried out using 1 mM DIDS, however, while the estimated intravitreal concentration after injection was 10 mM. High drug concentrations were used to circumvent possible problems due to rapid clearance via the anterior segment, but the concentration in the region of the inner retinal vessels is unknown.²⁶ Swelling- and voltage-activated Cl^- currents can be inhibited by DIDS, and we cannot exclude the possibility that these are involved in Et1 responses.^{45,46} The observed effects of blockers on blood flow may also reflect indirect actions on the vasculature secondary to inhibition of ion channels in other retinal cells.⁴⁷

Activation of chloride currents reduces light responses in cones and contributes to γ -amino butyric acid and glycine-mediated inhibition of bipolar cells by amacrine inputs.^{48,49} Inhibition of such currents might be expected to increase neuronal activity, and so could indirectly account for the increased flow seen, at least under basal conditions (Fig. 4). It seems less likely, however, that this explains the reversal of Et1-induced constriction by DIDS (Table 2; Fig. 5).

The effects of K^+ -channel blockers were more variable and less easily interpreted.⁴³ Inhibition of voltage-operated K^+ channels with correolide and BK Ca^{2+} -activated K^+ channels with either Penitrem A or iberiotoxin resulted in decreased basal blood flow (Table 1 and Fig. 4), as predicted from the vasodilator action of K^+ channels.^{12,15,20,50} The effects were not dramatic, however, with only a 11% to 12% fall in each case. Mean arteriolar diameter was not altered by any of the drugs used, whereas Penitrem A and correolide reduced leukocyte velocity. This suggests their action was downstream of the arterioles. Studies have shown that retinal pericytes express a range of K^+ currents, including BK and K_v , and blocking these may lead to pericyte contraction and increased capillary resistance.⁵¹ Indirect effects secondary to actions on nonvascular elements of the retina must also be considered. Inhibition of neuronal K^+ channels, (e.g., in ganglion cells), however, would be expected to increase excitability, favoring dilatation and increased flow, and so is unlikely to explain the decrease seen (Fig. 4).⁵² In cerebral vessels, K^+ efflux into the perivascular space via glial BK channels contributes to neurovascular coupling.⁵³ Blockade of tonic activity in such channels could, therefore, lead to vascular constriction. No reduction in arteriolar diameter was seen but pericyte-mediated responses cannot be ruled out. There is, however, little evidence to date that BK signaling plays an important role in retinal gliovascular signaling.⁴ Surprisingly, injection of Penitrem A and correolide along with Et1 produced increased arteriolar diameter relative to Et1 alone (Table 2; Fig. 5). This is difficult to explain, since inhibition of polarizing K^+ currents would be expected to lead to vascular constriction, rather than dilatation. As already discussed, indirect actions (e.g., secondary to increased neuronal or glial activity) may be possibilities, but it remains difficult to explain why this should only be observed in the presence of Et1. The changes in diameter seen were small and we have previously reported a larger dataset showing that Penitrem A had no effect on arteriolar diameter in the presence of Et1.¹⁸

Blockade of K^+ channels had no effect on blood flow in the presence of Et1, suggesting that they play little functional role in limiting Et1-induced changes in total retinal resistance. The failure of K^+ blockers to increase arteriolar diameter suggests that relatively few K^+ channels are activated in arteriolar smooth muscle, either under basal conditions, or in the presence of Et1. Studies on an isolated microcirculation preparation from Long Evans rats report a mean resting potential in arteriolar smooth muscle of -45 mV.²⁷ At this potential, the K^+ current available to be blocked would be very small (Fig. 2). The membrane potential in vivo would be expected to be depolarized by stretch, an important mechanism in the generation of myogenic tone.²⁸ Other, less predictable influences are also at play in the intact circulation, including endothelial and neurovascular coupling mechanisms.^{4,44}

Overall, these in vivo studies provide evidence that K^+ channels play a limited role in regulating basal retinal blood flow, possibly through a dilator influence in capillary pericytes, but do not appreciably alter changes in flow due to Et1. In contrast, I_{CaCa} plays a quantitatively larger role in regulating resting retinal flow and appears to be crucial in mediating Et1's constrictor action. This may be of relevance to our under-

standing not just of the physiological control of blood flow, but also to the potential pathophysiological role of Et1 in microvascular diseases such as diabetic retinopathy.^{54,55} Ca^{2+} -activated Cl^- channels may represent novel therapeutic targets in ocular disease characterized by abnormal flow and impaired microvascular reflexes.

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