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Ca²⁺-activated Cl⁻ current in retinal arteriolar smooth muscle

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Abstract

Purpose—To characterise the biophysical, pharmacological and functional properties of the Ca²⁺-activated Cl⁻ current in retinal arteriolar myocytes.

Methods—Whole-cell perforated patch-clamp recordings were made from myocytes within intact isolated arteriolar segments. Arteriolar tone was assessed using pressure myography.

Results—Depolarising voltage steps to -40 mV and greater activated an L-type Ca²⁺ current (I_{Ca(L)}) followed by a sustained current. On stepping back to -80 mV large tail currents were observed (I_{tail}). The sustained current and I_{tail} reversed close to 0 mV in symmetrical Cl⁻ concentrations. The ion selectivity sequence for I_{tail} was I⁻ > Cl⁻ > glucuronate. Outward I_{tail} was sensitive to the Cl⁻ channel blockers 9-anthracene-carboxylic acid (9-AC; 1mM), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS; 1mM) and disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS; 1mM), but only DIDS produced a substantial (78%) block of inward tail currents at -100mV. I_{tail} was decreased in magnitude when the normal bathing medium was substituted with Ca²⁺-free solution or if I_{Ca(L)} was inhibited by 1 μM nimodipine. 10 mM caffeine produced large transient currents that reversed close to the Cl⁻ equilibrium potential and were blocked by 1 mM DIDS or 100 μM tetracaine. DIDS had no effect on basal vascular tone in pressurised arterioles, but dramatically reduced the level of the vasoconstriction observed in the presence of 10 nM endothelin-1.

Conclusions—Retinal arteriolar myocytes have I_{Cl(Ca)} which may be activated by Ca²⁺ entry through L-type Ca²⁺ channels or Ca²⁺ release from intracellular stores. This current appears to contribute to agonist-induced retinal vasoconstriction.

Introduction

The regulatory control of retinal blood flow plays a crucial role in maintaining normal retinal function. Presently, our knowledge of the molecular mechanisms involved in controlling retinal blood flow remains incomplete, yet an improved understanding could lead to the development of new interventions aimed at restoring adequate tissue perfusion in ocular disease states such as diabetic retinopathy, hypertensive retinopathy and glaucoma. Arterioles form the main site of vascular resistance in the retinal microcirculation¹ and therefore the mechanisms that control smooth muscle tone in these vessels are largely responsible for regulating regional blood flow and capillary pressure in the retina. Arteriolar smooth muscle cells are known to express a variety of functional ion channels on their

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plasma membranes that are involved in setting the basal level of vascular tone and in mediating contractile responses to vasoactive agents². We have recently shown that retinal arteriolar myocytes possess large-conductance Ca^{2+} activated K^+ (BK) channels and $\text{K}_v1.5$ -containing voltage-activated K^+ channels that exert a vasodilatory influence on resting vascular tone³⁻⁵.

Ca^{2+} -activated Cl^- channels (Cl_{Ca}) have been identified in several types of vascular smooth muscle⁶⁻⁸, including choroidal arteriolar myocytes⁹. Because the Cl^- equilibrium potential is usually positive to the resting membrane potential, opening of Cl_{Ca} channels results in Cl^- efflux and membrane depolarisation¹⁰. In vascular myocytes, Cl_{Ca} channels are thought to contribute primarily to agonist-mediated responses¹¹. Agonists can produce vasoconstriction as a consequence of intracellular Ca^{2+} release acting directly on the contractile apparatus or indirectly by stimulating Cl_{Ca} channels, causing depolarization and the consequent opening of voltage-gated Ca^{2+} channels¹¹. Consistent with this idea, a number of studies have demonstrated that Cl^- channel blockers reduce the magnitude of agonist-induced contractions in the vasculature^{6,12-15}.

Initial work from our own laboratory has suggested that retinal arteriolar myocytes express Cl_{Ca} channels³, but their biophysical, pharmacological and functional properties have yet to be determined. The aim of the present study was, therefore, to identify and characterise the Ca^{2+} -activated Cl^- current in retinal arteriolar myocytes using the whole-cell perforated patch clamp technique and to assess their functional role in modulating basal and agonist-induced changes in retinal microvascular tone using pressure myography.

Methods

Arteriole preparation

Animal use was performed in accordance with the guidelines of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and the United Kingdom Animals (Scientific Procedures) Act, 1986. Male Sprague-Dawley rats (300-450 g) were euthanized with CO_2 , and enucleated. Retinal arterioles devoid of surrounding neuropile were mechanically isolated in low Ca^{2+} Hanks' solution as previously described³⁻⁵. In brief, retinal segments were lightly triturated in a low Ca^{2+} Hanks' solution. The resulting suspension was centrifuged at 3000 rpm for 1 minute, the supernatant aspirated off and the tissue washed again with low Ca^{2+} medium. The suspension was then stored at 21°C until needed. Arteriolar segments remained useable for up to 10 h under these conditions.

Patch-Clamp Electrophysiology

Ionic currents were recorded from retinal arteriolar myocytes still embedded within their parent arterioles using the whole-cell perforated patch-clamp technique^{3-5,16}. Vessels were pipetted into a recording bath on the stage of a Nikon Eclipse TE300 inverted microscope. Isolated arterioles (200 μm -4000 μm length, 20-44 μm outer diameter) were anchored down with tungsten wire slips (50 μm diameter) and superfused for 20 minutes with an enzyme cocktail of collagenase 1A (0.1 mg/ml) and protease type XIV (0.01 mg/ml) to remove surface basal lamina, allowing gigaseal formation. This treatment also causes the smooth muscle and endothelial layers to separate⁴, and the smooth muscle cells become electrically isolated from each other⁵. Unless otherwise stated, arterioles were continuously superfused with normal Hanks' solution at 37°C during experimentation. Voltage-clamp recordings were performed using an Axopatch 1D amplifier (Axopatch-1D, Axon Instruments, US). Pipettes (1-2 M Ω) were filled with a Cs^+ -based solution containing amphotericin B as the perforating agent. To ensure complete block of contaminating currents through BK and K_v channels, 100 nM Penitrem A and 10 mM 4-aminopyridine (4AP) were added to the

external bathing medium³⁻⁵. After full perforation of the membrane patch had been achieved (usually 3-5 minutes), cell capacitance was determined from the time constant of a capacitance transient elicited by a 20 mV hyperpolarization from -60 mV with a sampling frequency of 20 kHz. Prior to experimentation, series resistance (15-20M Ω) and cell capacitance (7-18pF) were compensated by up to 80% using the circuitry of the amplifier. Online leak subtraction was carried out using a P/4 protocol. All data were corrected for liquid junction potentials (+2-3 mV for all bathing solutions used). Recordings were low pass filtered at 0.5 kHz and sampled at 2 kHz by a National Instruments PC1200 interface using custom software provided by J. Dempster (University of Strathclyde, UK). Drug solutions were delivered via a 7-way micro-manifold.

Pressure myography

Measurement of the diameter of intact pressurized retinal arteriole segments was performed as previously described³. Again, vessels were visualised in a recording bath using an inverted microscope. A tungsten wire slip (75 $\times$ 2000 μ m) was laid on one end of the vessel which provided anchoring and occluded of the distal open end. Vessels were cannulated with fine glass pipettes (tip diameters 3-10 μ m) held in a patch electrode holder and connected to a pressure transducer and water manometer. After equilibration in Hanks' solution for 30 minutes at 37°C, intravascular pressure was increased to 70mmHg. A section of vessel at least 150 μ m away from the cannula was viewed under a x40 NA 0.6 objective focused midway through its depth. The vessels were then treated with drug-containing Hanks' solutions and changes in the external diameter measured manually from saved video images.

Solutions

Hanks' solution contained (in mM) 140, NaCl; 6, KCl; 5, D-glucose; 2, CaCl₂; 1.3, MgCl₂; 10, HEPES (pH 7.4 with NaOH). Low Ca²⁺ Hanks' differed only in that it contained 0.1 mM CaCl₂. For patch clamp experiments the internal solution was composed of (in mM): 138 CsCl; 1 MgCl₂; 0.5 EGTA; 0.2 CaCl₂; 10 HEPES (pH adjusted to 7.2 using CsOH). 300 μ g/ml of amphotericin B was added to the internal solution. For ion substitution experiments, 86 mM NaCl from the Hanks' solution was replaced with either equimolar NaI or NaGlucuronate. For Ca²⁺-free solution, Ca²⁺ was omitted from the external medium and 10 μ M EGTA was added.

4-aminopyridine (4-AP), amphotericin B, 9-anthracene carboxylic acid (9-AC), caffeine, collagenase 1A, disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), penitrem A, protease type XIV, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) and tetracaine were purchased from Sigma (Poole, UK). Nimodipine was obtained from Alexis Biochemicals (Nottingham, UK) and endothelin-1 (Et-1) was from Tocris (Bristol, UK).

Data analysis

Currents were normalized to cell capacitance to obtain current densities (pA/pF). Data are reported as the mean $\pm$ S.E.M, and n denotes the number of vessels from which recordings were made. Unless otherwise indicated, significant differences between control and experimental treatments were determined using the paired t-test. P values <0.05 were considered significant. The following labelling convention has been used to indicate the statistical significance of differences between control and test data in all figures: no asterisk, not significant; *, P<0.05; **, P<0.01; ***, P<0.001.

The permeability of I⁻ and glucuronate relative to Cl⁻ was calculated using the Goldman-Hodgkin-Katz equation^{17,18}:

$$P_{X^-}/P_{Cl^-} = ([Cl^-]_i \exp(\Delta E_{rev} F/RT) - [Cl^-]_{res})/[X^-]_o \quad (1)$$

Where $[X^-]_o$ is the concentration of the substituted ion (I^- or glucuronate), $[Cl^-]_i$ and $[Cl^-]_{res}$ are the intracellular and 'residual' extracellular concentrations of Cl^- respectively and ΔE_{rev} is the shift in reversal potential upon ion substitution. F , R and T have their usual meanings.

Results

Depolarisation-evoked Cl^- current

In the presence of K^+ channel inhibitors, retinal arteriolar myocytes were held at a membrane potential of -80 mV and a series of 1 s voltage pulses were applied, incrementing in amplitude in 20 mV steps between -100 mV and $+100$ mV. This protocol evoked a family of inward, outward and tail (I_{tail}) currents as shown in Fig 1Ai. At negative potentials an early inward current was followed by a sustained late current that was maintained throughout the voltage step. The early current, measured within the first 50 ms of the test pulse, activated at -40 mV, peaked at -20 mV and reversed at $+40$ mV (Fig 1Aii). The late current, measured at the end of the 1 s test pulse, had a similar current-voltage (I - V) profile (Fig 1Aii), but reversed close to 0 mV. The difference in the reversal potentials (E_{rev}) for the two currents suggests that they are carried by different ions. We have previously characterised the early current as an L-type Ca^{2+} current ($I_{Ca(L)}$)¹⁹. The E_{rev} for the late current is close to the calculated value for the Cl^- equilibrium potential ($E_{Cl} = +2.2$ mV), suggesting that this may be a Cl^- current.

I_{tail} reflects the continued activation of channels beyond the duration of the voltage pulse²⁰. To investigate the relationship between I_{tail} and the two preceding currents further experiments were performed. An initial conditioning step (250 ms) was fixed at 0 mV, close to the reversal potential of the late current, and subsequent steps were applied between -100 to $+100$ mV in 20 mV intervals. The resultant I_{tail} was inward at negative voltages and outward at positive voltages (Fig 1Bi) and had an approximately linear I - V relationship (Fig 1Bii). I_{tail} declined rapidly at negative voltages (τ for decay of I_{tail} at -60 mV was 45.31 ± 4.52 ms; $n=8$), but was sustained during positive test steps (Fig 1Bi). I_{tail} reversed at 0 mV, suggesting that I_{tail} represents persistent activation of the channels underlying the late current. Because the late current and I_{tail} appear to be mediated by the same population of channels, I_{tail} became the primary focus during experiments to characterise these currents as I_{tail} was larger in amplitude and could be evaluated across a wider voltage range (i.e. -100 to $+100$ mV as compared to -40 to $+100$ mV).

To test whether the late current and I_{tail} are Cl^- currents, ion substitution experiments were performed. Cl^- channels in other tissues are known to be relatively impermeable to the anion glucuronate, but have a higher permeability to I^- than to Cl^- ²¹. Using a 500 ms duration ramp between $+100$ and -100 mV, applied after a 500 ms conditioning step to 0 mV, the effect of equimolar replacement of 86 mM external Cl^- with glucuronate or I^- could be rapidly assessed. The use of a negative-going ramp protocol reduced the impact of the decay of I_{tail} seen at negative potentials on the amplitude of the current. In the representative trace shown in Fig. 2Ai, E_{rev} shifted from $+2.2$ to $+12.2$ mV when 86 mM external Cl^- was replaced with equimolar glucuronate. On average E_{rev} was shifted by 12.5 mV ($E_{rev} Cl^- = +3.9 \pm 2.1$ mV vs. $E_{rev} glucuronate = +16.4 \pm 2.5$ mV, $n=8$, $P=0.0004$) and the amplitude of outward I_{tail} was substantially reduced (Fig. 2Aii; Cl^- , 32.49 ± 8.13 pA/pF vs. glucuronate, 6.18 ± 1.62 pA/pF at $+80$ mV, $n=8$, $P=0.02$). Using equation 1, relative permeabilities for Cl^- and glucuronate were calculated as 1:0.25. As shown in Fig 2Bi, substitution with I^- resulted in a negative shift in E_{rev} . In four cells, E_{rev} shifted by 25.1 mV in a negative direction ($E_{rev} Cl^- = +0.9 \pm 6.2$ mV vs $E_{rev} I^- = -24.2 \pm 6.2$ mV; $P=0.002$), and based upon

this shift, relative permeabilities for Cl^- and I^- were calculated as 1:3.4. An attendant increase in the amplitude of both I_{tail} (Fig 2Bi,ii; Cl^- , 34.89 ± 13.70 pA/pF vs. I^- , 73.91 ± 24.79 pA/pF at +80 mV; $P=0.04$) and the late current (Fig 2Bi; Cl^- , -0.14 ± 0.36 pA/pF vs. I^- , 13.11 ± 3.13 pA/pF at 0 mV; $P=0.03$) was also apparent. These findings are consistent with the suggestion that both currents result from activation of the same channel proteins. Taken as a whole, the selectivity sequence of $\text{I}^- > \text{Cl}^- > \text{glucuronate}$ strongly suggests that the late current and I_{tail} are carried through Cl^- channels.

In a further series of related experiments, the effects of three classical Cl^- channel inhibitors, 9-AC, SITS and DIDS were tested on I_{tail} . 9-AC preferentially inhibited outward I_{tail} (Fig 3A,C) with an $82 \pm 6\%$ block at +100 mV (control, 49.34 ± 10.93 pA/pF vs. 9-AC, 10.06 ± 5.12 pA/pF; $n=5$; $P=0.001$) as opposed to a $25 \pm 10\%$ inhibition of inward I_{tail} at -100 mV (control, -56.66 ± 7.08 pA/pF vs. 9-AC, -39.59 ± 2.82 pA/pF; $n=5$; $P=0.05$). Similarly, 1mM SITS also displayed a voltage dependent block with $81 \pm 16\%$ inhibition of I_{tail} at +100 mV (control, 39.34 ± 10.74 pA/pF vs. SITS, 3.99 ± 3.75 pA/pF; $n=6$; $P=0.04$) but a non-significant reduction of $31 \pm 15\%$ at -100 mV (control, -60.69 ± 15.19 pA/pF vs. SITS, -35.03 ± 8.74 pA/pF; $n=6$; $P>0.05$). 1mM DIDS on the other hand blocked both outward and inward I_{tail} to a similar degree (Fig. 3B,C) with the currents at +100mV and -100mV reduced on average by $92 \pm 9\%$ and $78 \pm 4\%$, respectively (+100mV control, 39.78 ± 9.97 vs. DIDS, 3.29 ± 3.18 pA/pF; -100 mV control, -68.0 ± 12.18 pA/pF vs. DIDS, -16.65 ± 5.32 pA/pF; $n=5$; $P=0.002$ and $P=0.02$, respectively). A lower concentration of DIDS (10 μM) did, however, exhibit a more pronounced voltage dependence of block with $57 \pm 8\%$ inhibition at +100 mV and non-significant block ($28 \pm 12\%$) at -100 mV (+100 mV control, 46.56 ± 9.40 vs. DIDS, 23.20 ± 6.83 pA/pF; -100 mV control, -22.97 ± 4.65 pA/pF vs. DIDS, -15.44 ± 3.61 pA/pF; $n=7$; $P=0.002$ and $P>0.05$, respectively). Neither 9-AC, SITS nor DIDS had any discernible effect on $I_{\text{Ca,L}}$ ($p>0.05$ in all cases). These pharmacological data further substantiate the view that depolarisation evokes a Cl^- conductance in retinal arteriolar myocytes.

Ca^{2+} -dependence of the Cl^- current

To explore the Ca^{2+} -dependence of the depolarisation evoked Cl^- current, we compared I_{tail} before and after lowering of the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) as well as investigating the effects of the $I_{\text{Ca(L)}}$ inhibitor, nimodipine. As shown in Fig 4Ai, both $I_{\text{Ca(L)}}$ and I_{tail} were decreased when the normal bathing medium was substituted with Ca^{2+} -free solution (no added Ca^{2+} plus 10 μM EGTA). In 6 vessels, I_{tail} was reduced on average by $\sim 75\%$ at -100 and +100 mV (Fig 4Aii). Bath application of 1 μM nimodipine markedly reduced both $I_{\text{Ca(L)}}$ and I_{tail} (Fig 4B). These results suggest that the depolarisation evoked Cl^- current is dependent on the influx of Ca^{2+} through L-type Ca^{2+} channels.

To determine whether Ca^{2+} release from intracellular stores was also capable of activating the Cl^- current, we tested the effects of caffeine at several different voltages between -80 to +80 mV. In retinal arteriolar myocytes, 10 mM caffeine evokes rapid global Ca^{2+} transients which reflect Ca^{2+} release from intracellular ryanodine-sensitive Ca^{2+} stores²³. Administration of 10 mM caffeine resulted in a transient inward current at negative voltages, little or no current at 0 mV, and a transient outward current at positive voltages (Fig 5Ai). The I - V relationship for the caffeine-induced current was approximately linear and reversed close to 0mV (Fig 5Aii), consistent with a Cl^- current. In addition, the current was inhibited by $\sim 90\%$ in the presence of 1 mM DIDS (Fig 5B). To exclude the possibility that caffeine activates the Cl^- channels through a Ca^{2+} -independent mechanism, we blocked caffeine-induced Ca^{2+} release using 100 μM tetracaine. Tetracaine totally abrogated the currents measured at -40 mV (Fig 5C), suggesting that caffeine activates the Cl^- channels by raising cytosolic Ca^{2+} .

Physiological Significance

The results from the experiments above suggest that retinal arteriolar myocytes possess a Ca^{2+} -activated Cl^- current ($I_{\text{Cl}(\text{Ca})}$) that may be activated by Ca^{2+} influx through L-type Ca^{2+} channels or Ca^{2+} release from the sarcoplasmic reticulum. A further series of experiments were designed to establish the role of $I_{\text{Cl}(\text{Ca})}$ in regulating retinal arteriolar tone. Retinal arteriole segments were cannulated and pressurized to 70 mmHg and the effect of blocking $I_{\text{Cl}(\text{Ca})}$ on the external diameter of the vessels was examined. 1mM DIDS had no effect on basal vascular tone (external diameters were 36.2 ± 2.4 and $36.0 \pm 2.3 \mu\text{m}$ before and after 5-min application of DIDS, respectively; $n=8$; $P>0.05$). Ca^{2+} -activated Cl^- channels have been shown to be activated by a wide range of agonists that raise intracellular Ca^{2+} ¹¹, and we have recently shown that the vasoconstrictor peptide, endothelin-1 (Et-1), stimulates regenerative transient depolarisations in retinal arteriolar myocytes and that these depolarisations are rapidly blocked by application of a Cl^- channel inhibitor²⁴. To examine the possible involvement of $I_{\text{Cl}(\text{Ca})}$ in modulating Et-1-induced retinal arteriolar vasoconstriction, pressurized vessels were exposed to either Et-1 alone or Et-1 with 1 mM DIDS. Co-application of DIDS dramatically reduced the level of the vasoconstriction observed with 10 nM Et-1 (Fig 6). These findings suggest that activation $I_{\text{Cl}(\text{Ca})}$ represents a key signalling component regulating Et-1 induced vasoconstriction in the retina.

Discussion

The present study is the first to characterize $I_{\text{Cl}(\text{Ca})}$ in the retinal microvasculature. We studied the biophysical and pharmacological properties of $I_{\text{Cl}(\text{Ca})}$ in retinal arteriolar myocytes by focusing mainly on I_{tail} . Our data are consistent with I_{tail} being mediated by $\text{Cl}(\text{Ca})$ channels as: (i) it reversed close to E_{Cl} ; (ii) the ion selectivity sequence was $\text{I} > \text{Cl} > \text{glucuronate}$, which is similar to $I_{\text{Cl}(\text{Ca})}$ in other tissues²⁵; (iii) it was reduced by the classical Cl^- channel inhibitors, 9AC, SITS and DIDS; (iv) it was blocked if normal Hanks' was substituted with Ca^{2+} -free solution or if $\text{I}_{\text{Ca}(\text{L})}$ was inhibited by nimodipine; and (v) it could be activated by release of Ca^{2+} from caffeine-sensitive Ca^{2+} stores. We also examined the effects of DIDS on basal vascular tone and vasoconstriction induced by Et-1 in isolated, pressurized retinal arterioles. Inhibition of $I_{\text{Cl}(\text{Ca})}$ by DIDS had no effect on basal vascular tone, but did substantially reduce the vasoconstrictor response to Et-1.

The electrophysiological features of $I_{\text{Cl}(\text{Ca})}$ in retinal arteriolar myocytes resemble those reported for $I_{\text{Cl}(\text{Ca})}$ in large vessel smooth muscle in other vascular beds. In portal vein, coronary and pulmonary artery myocytes, $I_{\text{Cl}(\text{Ca})}$ activates slowly upon depolarization, is maintained throughout the voltage step, and exhibits long tail currents upon repolarisation^{8,20,26,27}. In addition, in rabbit portal vein, I_{tail} decays exponentially at potentials negative to -20 mV, but is evident as a sustained current at more positive voltages²⁰. It has been postulated that at negative voltages the decline of I_{tail} is determined by the slow deactivation kinetics of the $\text{Cl}(\text{Ca})$ channels, while at more positive voltages I_{tail} may be sustained due to persistent influx of Ca^{2+} through non-inactivating voltage-dependent Ca^{2+} channels²⁰. The only apparent discrepancy in the electrophysiological properties of the $\text{Cl}(\text{Ca})$ channels in retinal arteriolar myocytes compared to those in other vascular tissues lies in their rate of deactivation. At -60 mV, I_{tail} decayed with a mono-exponential time constant of ~45 ms, which is considerably faster than rate of decline of I_{tail} seen in coronary artery and portal vein myocytes, where equivalent values are ~160ms and ~85ms, respectively^{8,20}. While the exact reasons for this difference remain unclear, it is known that the deactivation kinetics of $I_{\text{Cl}(\text{Ca})}$ in vascular smooth muscle can be modified by external anions and the phosphorylation and redox status of the channels²⁸⁻³⁰.

The anion permeability of $\text{Cl}(\text{Ca})$ channels has been widely tested (for review, see¹¹). $\text{Cl}(\text{Ca})$ channels are known to be less permeable to large organic anions such as glucuronate,

glutamate and isothionate (permeability relative to Cl^- of ~ 0.1 - $0.3^{9,27,31}$). Consistent with this, in retinal arteriolar myocytes, the calculated permeability for glucuronate was 0.25 times that of Cl^- . Replacement of Cl^- with I^- , an anion that is known to be more permeant through $\text{Cl}_{(\text{Ca})}$ channels^{11,21}, resulted in a relative permeability value of 3.4. This is in close agreement with values reported for $\text{Cl}_{(\text{Ca})}$ channels in lymphatic smooth muscle (3.2^{32}), *Xenopus* oocytes (3.6^{33}), portal vein myocytes (4.1^{34}) and ear artery smooth muscle (4.7^6). These data indicate that the permeability properties of $\text{Cl}_{(\text{Ca})}$ channels in retinal arteriolar smooth muscle are similar to those previously reported in other vascular myocytes.

To assess the physiological significance of $\text{I}_{\text{Cl}(\text{Ca})}$ in retinal arteriolar smooth muscle cells we tested the effects DIDS on basal and Et-1-evoked changes in retinal arteriolar tone. We chose DIDS, rather than 9AC or SITS, for these experiments because we found that this drug, at a concentration of 1mM, was more effective at blocking $\text{I}_{\text{Cl}(\text{Ca})}$ at negative membrane potentials close to the resting membrane potential of retinal arteriolar smooth muscle cells (~ -40 to -50 mV²⁴). Our results showed that DIDS had no effect on the diameter of pressurized retinal arterioles, indicating that $\text{Cl}_{(\text{Ca})}$ channels do not appear to contribute to the control of basal vascular tone in these vessels, at least under in vitro conditions. Similarly, $\text{I}_{\text{Cl}(\text{Ca})}$ is not believed to participate in modulating resting vascular tone in cerebral and renal resistance vessels^{35,36}. In contrast, several studies have suggested that stimulation of $\text{I}_{\text{Cl}(\text{Ca})}$, which leads to membrane depolarization and the opening of voltage-gated Ca^{2+} channels, plays a pivotal role in agonist-induced contractile responses in various vascular tissues¹²⁻¹⁵. Evidence from the present work indicates similar findings in retinal arterioles since DIDS substantially attenuated the vasoconstrictor action of Et-1. A major issue that has hampered research into the functional significance of $\text{Cl}_{(\text{Ca})}$ channels in the vasculature has been the poor selectivity of available antagonists. Blockers of $\text{Cl}_{(\text{Ca})}$ channels, including DIDS, also inhibit other types of Cl^- currents e.g. swelling- and voltage-activated Cl^- currents^{37,38}. Moreover, several inhibitors of $\text{I}_{\text{Cl}(\text{Ca})}$ have also been shown modify $\text{I}_{\text{Ca}(\text{L})}$ ^{39,40}. Our electrophysiological data showed that DIDS had no effect on $\text{I}_{\text{Ca}(\text{L})}$ in retinal arteriolar myocytes at the same concentration used in the mechanical studies. However, we cannot fully discount the possibility that the effects of DIDS on the Et-1-induced vasoconstriction may have been due to blockade of Cl^- conductances other than $\text{I}_{\text{Cl}(\text{Ca})}$.

In conclusion, this study has clearly demonstrated the presence of a Ca^{2+} -activated Cl^- current in retinal arteriolar smooth muscle cells with characteristics similar to those reported in large vessel smooth muscle. $\text{I}_{\text{Cl}(\text{Ca})}$ does not appear to contribute to resting vascular tone in vitro, but does appear to play a prominent role in the constriction of retinal arterioles by Et-1. Future research should now be directed towards understanding the involvement of $\text{I}_{\text{Cl}(\text{Ca})}$ in the regulation of retinal haemodynamics in vivo under both normal and pathological conditions.

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Figure 1.

Early, late and tail currents elicited by step depolarisations in retinal arteriolar myocytes. Ai. Representative current records showing early, late and tail currents evoked by a 1 s voltage step protocol (inset) applied from a hold potential of -80 mV to voltages between -100 and +100 mV in 20 mV increments. Aii. Average early and late current densities plotted against test voltage (n=9). Bi. Tail currents elicited by test steps to voltages between -100 and +100 mV in 20 mV increments (inset) applied after a 250 ms conditioning step from -80 to 0 mV. Bii. Average tail current densities (measured 10-20 ms after the start of the test step) plotted against the tail step voltage (n=8).

Figure 2.

Effect of substitution of external Cl^- on I_{tail} . Ai. Representative current traces elicited by a 500 ms voltage ramp protocol (inset) from +100 to -100 mV applied following a 500 ms conditioning step to 0 mV recorded in normal Hanks' solution and in glucuronate-substituted Hanks'. Arrows indicate reversal potentials. Bi. Original current traces recorded in normal Hanks' solution and in I^- -substituted Hanks'. A&Bii. Average current densities at +80 mV in Cl^- only versus (A) glucuronate- and (B) I^- -containing solutions ($n=8$ and 4, respectively).

Figure 3.

Pharmacological analysis of I_{tail} . A,B&Ci I_{tail} elicited by alternating test steps to -100 and +100 mV applied after a conditioning step to 0 mV in normal Hanks' solution (control) versus (A) 1 mM 9-AC, (B) 1 mM SITS and (C) 1 mM DIDS. A,B&Cii Average current densities measured in 20 mV intervals between -100 and +100 mV in the normal Hanks' solution versus (A) 1 mM 9-AC, (B) 1 mM SITS and (C) 1 mM DIDS plotted against the test step voltage ($n = 5, 6$ and 5 , respectively) D. Percentage inhibition of I_{tail} by 9-AC, SITS and DIDS at different voltages calculated from the data in A,B&Cii.

Figure 4.

Dependence of I_{tail} on external Ca^{2+} . Ai. I_{tail} elicited by alternating test steps to -100 and +100 mV (inset) applied after a conditioning step to 0 mV in normal Hanks' solution (control) and Ca^{2+} -free Hanks' ($0Ca^{2+}_o$). Bi. I_{tail} elicited in normal Hanks' solution (control) and the L-type Ca^{2+} channel inhibitor, nimodipine (1 μM). Nimodipine reduced $I_{Ca(L)}$ by 85% and I_{tail} by 81% and 86% at -100 and +100 mV, respectively. A&Bii. Average current densities measured at -100 and +100 mV in the normal Hanks' solution versus (A) Ca^{2+} -free Hanks' and (B) 1 μM nimodipine (nim; n=6 and 10, respectively).

Figure 5.

Activation of $I_{Cl(Ca)}$ by Ca^{2+} release from intracellular Ca^{2+} stores. A. Original traces showing caffeine-induced currents recorded at holding potentials of -80 to +80 mV in 40 mV intervals. Aii Mean $\pm$ SEM of caffeine-induced current densities (measured as peak current versus baseline at each holding potential) (n=11). B. Caffeine-induced currents elicited at a holding potential of -40 mV before (i) and during (ii) application of 1 mM DIDS. Biii Average caffeine-induced current densities recorded at -40 mV before and during application of DIDS (n=6). C. Caffeine-induced currents elicited at a holding potential of -40 mV before (i) and during (ii) application of 100 μ M tetracaine. Ciii. Average caffeine-

induced current densities recorded at -40 mV before and during application of tetracaine(n=6).

Figure 6.

Effect of DIDS on the Et-1-induced changes in retinal arteriolar tone. A Photomicrographs of a pressurized (70mmHg) retinal arteriole before (i) and 40 s after application 10 nM Et-1 (ii). B Photographs of a different vessel before (i) and 40 s after application 10 nM Et-1 and 1 mM DIDS. Scale bars 10 μ m. C. Mean data showing the external diameter of arterioles exposed to Et-1 alone or in combination with DIDS expressed as a fraction of the initial resting diameter prior to drug exposure.