Expression and function of native potassium channel ($K_V\alpha1$) subunits in terminal arterioles of rabbit

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1. In this study we investigated the expression and function of the $K_V\alpha1$ subfamily of voltage-gated K^+ channels in terminal arterioles from rabbit cerebral circulation.

2. K^+ current was measured from smooth muscle cells within intact freshly isolated arteriolar fragments. Current activated on depolarisation positive of about −45 mV and a large fraction of this current was blocked by 3,4-diaminopyridine (3,4-DAP) or 4-aminopyridine (4-AP), inhibitors of K_V channels. Expression of cRNA encoding $K_V1.6$ in *Xenopus* oocytes also generated a 4-AP-sensitive K^+ current with a threshold for activation near −45 mV.

3. Immunofluorescence labelling revealed $K_V1.2$ to be specifically localised to endothelial cells, and $K_V1.5$ and $K_V1.6$ to plasma membranes of smooth muscle cells.

4. K_V channel current in arteriolar fragments was blocked by correolide (which is specific for the $K_V\alpha1$ family of K_V channels) but was resistant to recombinant agitoxin-2 (rAgTX2; which inhibits $K_V1.6$ but not $K_V1.5$). Heterologously expressed $K_V2.1$ was resistant to correolide, and $K_V1.6$ was blocked by rAgTX2.

5. Arterioles that were mildly preconstricted and depolarised by 0.1–0.3 nM endothelin-1 constricted further in response to 3,4-DAP, 4-AP or correolide, but not to rAgTX2.

6. We suggest that $K_V\alpha1$ channels are expressed in smooth muscle cells of terminal arterioles, underlie a major part of the voltage-dependent K^+ current, and have a physiological function to oppose vasoconstriction. $K_V\alpha1$ complexes without $K_V1.5$ appear to be uncommon.

That K^+ channel activation has a vasodilator function is well established by the fact that there is vasodilatation in response to ATP-sensitive K^+ (K_{ATP}) channel-opener drugs and vasoconstriction in response to block of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels by iberiotoxin or following knock-out of the BK_{Ca} channel β_1-subunit (Brayden & Nelson, 1992; Mayhan & Faraci, 1993; Beech, 1997; Prior *et al.* 1998; Brenner *et al.* 2000). The focus of this study was on another family of K^+ channels – the voltage-gated K^+ channels (K_V channels). We did this firstly because of the common observation that K^+ current through K_V channels in vascular smooth muscle cells is dominant in the physiological potential range −45 to 0 mV (Beech & Bolton, 1989; Volk *et al.* 1991; Robertson & Nelson, 1994; Ahn & Hume, 1997). Secondly, in smooth muscle cells of many blood vessels K_{ATP} channels are normally closed and BK_{Ca} channels activate only at more positive potentials (Faraci & Heistad, 1993; Wang & Mathers, 1993; Gebremedhin *et al.* 1994; Prior *et al.* 1998; Jackson & Blair, 1998). It is also notable that K_V channels are inhibited by $[Ca^{2+}]_i$ above the physiological level, whereas BK_{Ca} channels are activated (Gelband & Hume, 1995; Cox & Petrou, 1999), and in the spontaneously hypertensive rat, K_V channel current is suppressed and BK_{Ca} channel expression and current are enhanced (Martens & Gelband, 1996; Liu *et al.* 1997, 1998). Thus, it appears reasonable to speculate that K_V channels are physiologically important and that there is a switch over to dominance of BK_{Ca} channels only when $[Ca^{2+}]_i$ is high in response to high concentrations of vasoconstrictor agonist or in disease.

It might be surprising that K_V channels should be relevant in a cell type that often functions without firing action potentials. However, there is evidence from work on smooth muscle in major arteries that K_V channel currents provide tonic negative feedback against sustained small depolarisations evoked by stretch (e.g. Knot & Nelson, 1995; Quan & Sobey, 2000). It is plausible, therefore, that K_V channels have a major role in controlling physiological blood pressure. We sought evidence that K_V channels serve this function in smooth muscle cells of terminal arterioles, which have a primary role in controlling blood pressure and local tissue perfusion. Having found evidence for such a role we aimed to reveal mammalian K_V channel genes that are expressed and functionally important, focusing on the equivalents of the *Shaker* gene of *Drosophila*, i.e. the *KCNA* genes encoding $K_V\alpha1$ subunits (Coetzee *et al.* 1999).

METHODS

Male Dutch dwarf rabbits (1.0–1.5 kg) were killed by an intravenous overdose of 70 mg kg^{-1} sodium pentobarbitone, in accordance with the Code of Practice as set out by The UK Animals Scientific Procedures Act 1986. The brain was removed and placed in ice-cold Hanks' solution bubbled with 100% O_2.

Pieces of pial membrane were removed from the cerebral cortex and incubated with 0.032 mg ml^{-1} protease and 0.2 mg ml^{-1} collagenase in Hanks' solution at 37 °C for 10 min. The mixture was then kept at 4 °C for 15 min and subsequently agitated using a fire-polished Pasteur pipette before being washed with enzyme-free Hanks' solution followed by centrifugation at 1000 r.p.m. for 1 min. Superficial supernatant was discarded and the remaining suspension of arterioles was stored on glass coverslips at 4 °C in Hanks' solution and used within 10 h. Isolated arterioles used for experiments had an external diameter of about 30–40 μm and a thick wall of circularly arranged smooth muscle cells (Fig. 2A), in contrast to venules (Fig. 2B). This visual distinction was used to ensure that all recordings were from arterioles. Endothelial cells may appear to be present in these vessels (Guibert & Beech, 1999); in some cases they can be seen clearly under the light microscope (Fig. 2C) and can be detected by immunostaining (Fig. 4C). However, short arteriolar fragments can be visually identified as endothelium denuded (Fig. 2A). In confirmation that many vessel fragments are endothelium denuded, antibody specific for endothelial nitric oxide synthase detects endothelial cells in only about 25% of fragments, and arterioles dilate very rarely in response to 1 μM acetylcholine (C. Guibert, R. Flemming and D. J. Beech, unpublished observations).

Arteriolar diameter measurements were made by placing arterioles in a modified culture dish on the stage of an inverted trinocular microscope (Nikon TMS, Japan), which had an attached video camera (Sony, Japan). The external diameter of arteriolar segments was measured using a video-dimension analyser (Living Systems Instrumentation, VT, USA) and calibrated using a stage micrometer. The signal was captured on-line via an A/D converter (Picolog software, Pico Technology, Cambridge, UK) and stored on a computer. All diameter measurements were made in artificial cerebrospinal fluid (aCSF) gassed with 5% CO_2–95% O_2 at 37 °C.

Current- and voltage-clamp experiments were performed using conventional whole-cell recording (Quinn & Beech, 1998). Gigaseals were formed directly on a smooth muscle cell embedded in an intact arteriolar segment (Fig. 2A). The patch-clamp amplifier used was an Axopatch-1D (Axon Instruments, USA), and command- and data-sampling protocols were controlled by Patch and Vclamp 6 software (Cambridge Electronic Design, UK). Except for the capacity current recordings in Fig. 2, current signals were filtered at 1 kHz and sampled at 2 kHz. Capacity currents were filtered at 10 kHz and sampled at 20 kHz. Patch pipettes were made from borosilicate glass capillary tubing with an outside diameter of 1 mm and inside diameter of 0.58 mm (Clark Electromedical Instruments, UK). Patch pipettes had resistances, after fire-polishing and when filled with solution, of 2–3 MΩ. Voltage clamp was applied to arteriolar segments < 150 μm in length. All patch-clamp experiments were performed at 23 °C. Substances were applied to arterioles by exchanging the solution in the recording chamber, which took < 30 s.

For immunofluorescence experiments, enzymatically isolated arterioles were allowed to adhere to polylysine-coated glass slides prior to fixation in 2% paraformaldehyde for 30 min. This was followed by a 60 min incubation at room temperature in 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in phosphate-buffered saline (PBS). Rabbit polyclonal antibodies were used to detect $K_V\alpha1$ subunits. Antibodies were targeted to rat (R) or mouse (M) sequences: anti-$K_V1.2R_{K(461–480)}$, anti-$K_V1.5R_{(578–598)}$ and anti-$K_V1.6R_{K(509–526)}$, kindly provided by Dr H. G. Knaus (Koch *et al.* 1997), and anti-$K_V1.2R_{A(417–498)}$ and anti-$K_V1.5M_{(513–602)}$, obtained from Alomone Labs (Israel). The locations of the peptide sequences in the channels are shown subscripted in parentheses. All antisera were used at 1 : 250 dilution. Goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC; Sigma) was used as the secondary antibody. Use of enzymatically cleaned arterioles minimised background staining associated with using anti-rabbit secondary antibody on rabbit tissue. For double labelling, anti-smooth muscle α-actin antibody pre-conjugated to indocarbocyanine 3 (α-SMA-Cy3; Sigma) was used at 1 : 200 dilution. The specificity of all antibodies was investigated by parallel experiments in the absence of primary antibody, and in the case of anti-$K_V1.5R_{(578–598)}$ the specificity was also confirmed by preadsorbing the antibody with 10 μM antigenic peptide. Stained cells were viewed using a fluorescence microscope with a ×40 1.3-NA Nplan objective (Nikon, Japan). Images were captured with a Hamamatsu 4880-82 cooled CCD camera at five focal planes separated by 0.5 μm, background subtracted, and haze removed by a deconvolution algorithm (Improvision, UK).

Xenopus laevis were killed by an overdose of tricaine anaesthetic followed by destruction of the brain and spinal cord. Oocytes were surgically removed from the abdomen and transferred into Ca^{2+}- free

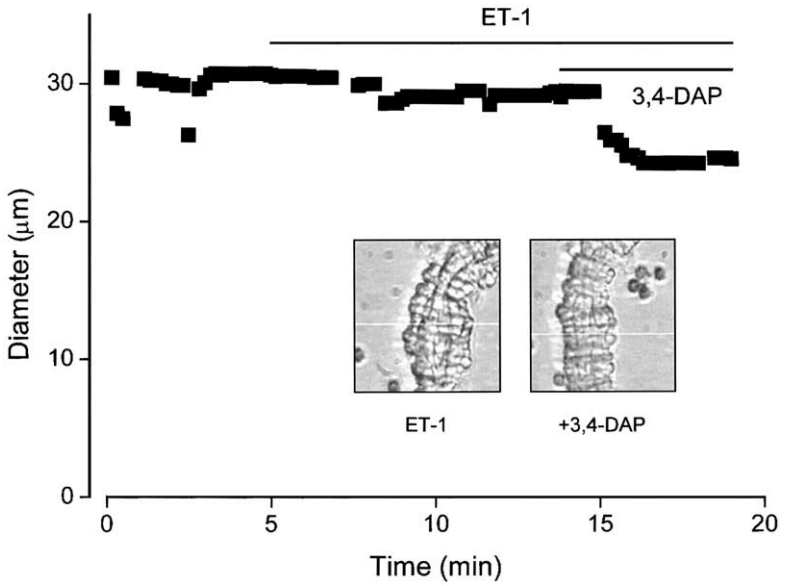

Figure 1. Contraction in an arteriole exposed to 3,4-diaminopyridine, a blocker of K_V channels

The filled squares in the plot indicate the external diameter of the arteriole. The bath solution was aCSF and a small level of preconstriction was induced with 0.3 nM endothelin-1 (ET-1) prior to application of 1 mM 3,4-diaminopyridine (3,4-DAP). Actual video images of the arteriole are shown inset for the two conditions. The white horizontal line in each image is the scan line used to detect the outer edge of the arteriole. It was moved slightly during the recording to ensure that edge-detection was maintained.

Ringer solution. Follicular cells were dissociated from oocytes by incubation in 1 mg ml^{-1} collagenase (Type 1A) at 23°C for 2 h. Oocytes were microinjected with 50 nl of water or cRNA (20–25 ng) encoding human $K_V1.6$ (GenBank accession number NP002226) or rat $K_V2.1$ (GenBank accession number P15387). Oocytes were then incubated in Barth's solution (mM: NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; Hepes, 10; $MgSO_4$, 0.82; $Ca(NO_3)_2$, 0.33; $CaCl_2$, 0.41; pH 7.4) at 19°C. Recordings were made 2 days later using a GeneClamp 500 amplifier (Axon Instruments) for two-electrode voltage clamp. Recording electrodes were pulled from borosilicate tubing (Clark Electromedical Instruments) and had resistances of 0.5–5 MΩ when filled with 3 M KCl solution. Current and voltage signals were digitised by a 1401 A/D converter (Cambridge Electronic Design) and stored on a computer. The extracellular bathing solution during recordings was Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 7.4). During recordings, oocytes were placed in a 0.2 ml recording chamber through which solution flowed at about 2 ml min^{-1}.

Results are expressed as means ± S.E.M. The n values indicate the number of arterioles in electrophysiological and diameter experiments or the number of staining protocols per animal in immunofluorescence experiments, which mostly involved observation of >1 arteriole and >1 microscope slide. Groups of data were compared using Student's unpaired t test and statistical significance was concluded if $P<0.05$. Mathematical functions were fitted to data points and data are presented using Origin 6 software (Microcal Inc., USA).

Hanks' solution contained (mM): NaCl, 137; KCl, 5.4; $CaCl_2$, 0.01; NaH_2PO_4, 0.34; K_2HPO_4, 0.44; D-glucose, 8; Hepes, 5. aCSF contained 0.5 μM tetrodotoxin and (mM): NaCl, 125; KCl, 1.72; $NaHCO_3$, 24; $MgSO_4$, 1.74; KH_2PO_4, 1.17; D-glucose, 5.35; $CaCl_2$, 2.47; EDTA, 0.023. Standard bath solution contained (mM): NaCl, 130; KCl, 5; D-glucose, 8; Hepes, 10; $MgCl_2$, 1.2; $CaCl_2$, 1.5. The pipette solution contained (mM): KCl, 130; EGTA, 0.2; $MgCl_2$, 2; Hepes, 10; Na_2ATP, 3; NaGTP, 0.5. The final pH of all solutions was titrated to 7.4 using NaOH. Solutions containing 3,4-diaminopyridine (3,4-DAP) or 4-aminopyridine (4-AP) were pH 7.4. BSA (0.01 %) was present in all solutions in experiments involving recombinant agitoxin-2.

EGTA, EDTA, Hepes, protease (Type E), collagenase (Type 1A), glibenclamide, apamin, penitrem A, barium chloride ($BaCl_2$), fatty acid-free bovine serum albumin (BSA), 18α-glycyrrhetinic acid, dimethylsulphoxide (DMSO) and 4-AP were from Sigma. 3,4-DAP was from Fluka Chemie AG. Recombinant agitoxin-2 (rAgTX2) was from Alomone Labs. Endothelin-1 (ET-1) was from Calbiochem. General salts were from BDH or Sigma/Aldrich. 18α-Glycyrrhetinic acid was prepared as a 10 mM stock in 100 % DMSO and freshly added to solutions and vortexed prior to experiments.

RESULTS

Enzymatically isolated arterioles appear functionally normal. They constrict in response to 20–60 mM extracellular K^+ (Quinn & Beech, 1998; Quinn *et al.* 2000) and 0.1–10 nM ET-1 (Guibert & Beech, 1999) or arginine vasopressin (data not shown). To study K_V channel function, arterioles were first exposed to 0.1–0.3 nM ET-1, which induces a small degree of tone and depolarisation to

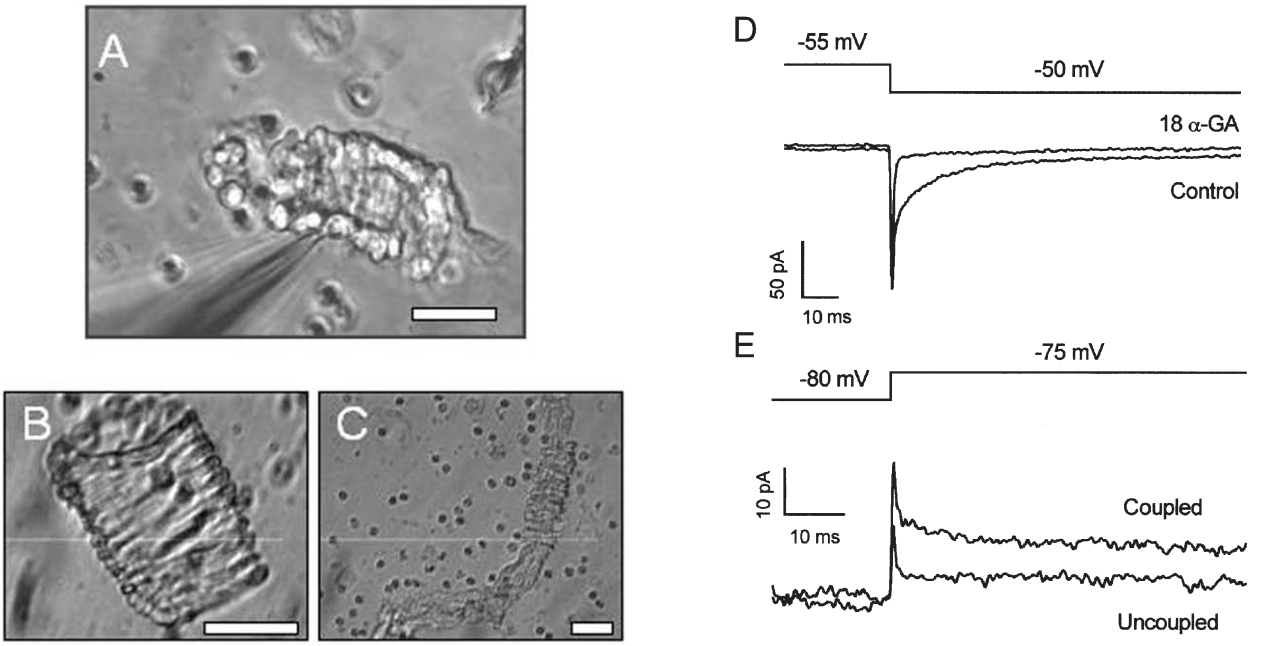

Figure 2. Conventional whole-cell patch-clamp recordings from smooth muscle cells within intact arterioles

A, digital image of an arteriolar fragment with an attached patch pipette (out of focus object entering the image from the left corner). B, an isolated venule. C, a long isolated arteriole with exposed endothelial cells. Small circular cells are erythrocytes. The scale bars in A–C represent 30 μm. D and E, capacity currents recorded from arteriolar fragments in response to 5 mV steps in voltage. D, two currents from the same arteriole, one under control conditions, and the other in the presence of 50 μM 18α-glycyrrhetinic acid (18α-GA). E, two current traces from different arterioles, one showing electrical coupling between cells (Coupled) and the other in which the patched cell was spontaneously uncoupled from other cells in the arteriole (Uncoupled).

about −40 mV (Guibert & Beech, 1999). Tetrodotoxin (0.5 μM) was included to block effects originating from nerve terminals not removed by the collagenase–protease treatment. In the continued presence of ET-1, arterioles constricted strongly (and lengthened and straightened) in response to 1 mM 3,4-DAP (Fig. 1) or 1 mM 4-AP (not shown, but see Fig. 7A), which are inhibitors of many K_V channels (Robertson & Nelson, 1994; Coetzee *et al.* 1999). The reduction in external diameter in response to 3,4-DAP was 32.0 ± 4.0% of the initial diameter ($n = 4$). The maximum possible reduction of external diameter, starting in the absence of ET-1, is about 40% (Quinn & Beech, 1998; Guibert & Beech, 1999). Thus, a significant role for K_V channels in controlling arteriolar diameter is indicated.

Smooth muscle cells within arterioles were subjected to patch-clamp recording (Fig. 2A) to determine the properties of the aminopyridine-sensitive current. Cells within these arteriolar fragments were often electrically coupled as shown by the presence of fast and slow components of capacity current elicited following a square step in voltage (Fig. 2D and E). The slow, but not the fast, capacity current was inhibited by 18α-glycyrrhetinic acid (Fig. 2D), which is a gap junction blocker (Davidson & Baumgarten, 1988; Taylor *et al.* 1998). Occasionally, the slow component was

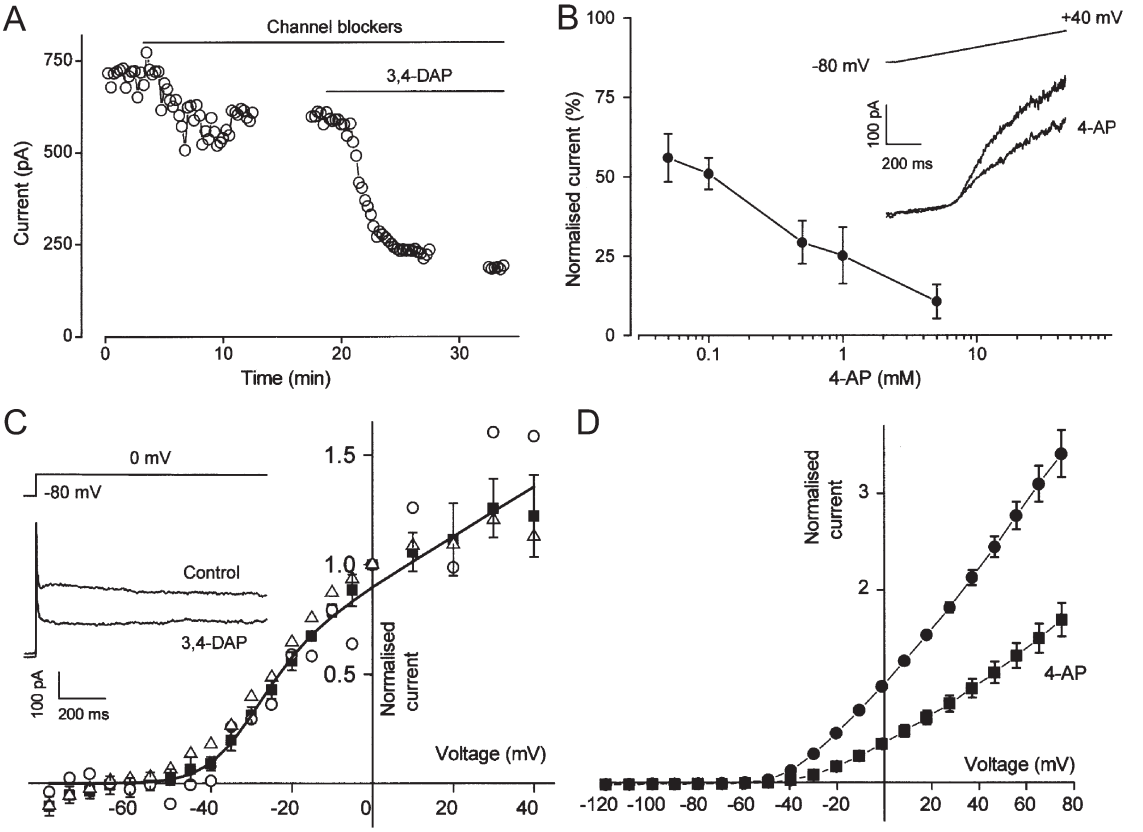

Figure 3. K_V channel currents in arterioles and through heterologously expressed $K_V1.6$

A, open circles show current amplitude at the end of 1 s ramp changes in voltage from −80 to +40 mV. 'Channel blockers' means replacement of extracellular Ca^{2+} by Mg^{2+}, and application of 100 nM penitrem A, 1 μM glibenclamide and 50 μM niflumic acid. 3,4-DAP (1 mM) was applied as indicated. Gaps in the trace indicate the periods during which current–voltage relationships were constructed. B, plot of mean ± S.E.M. ($n = 3$–5) concentration-dependent block by 4-aminopyridine (4-AP) of outward current at 0 mV. Currents were normalised to the pre-4-AP amplitude. Inset, voltage protocol and example currents for control and in the presence of 50 μM 4-AP. C, voltage dependence of 1 mM 3,4-DAP-sensitive current measured at the end of 1 s square voltage steps and normalised to the amplitude at 0 mV. △, coupled cells under control conditions (S.E.M. bars not shown, $n = 9$); ■, cells in the presence of 50 μM 18α-glycyrrhetinic acid ($n = 4$); ○, a spontaneously uncoupled cell. The curve is fitted to the 18α-glycyrrhetinic acid data and is the equation: $g_{max}(V - V_{rev})/\{1 + \exp((V - V_{1/2})/dV)\}$, in which $V_{1/2}$ is the half-activation potential (−31.4 mV), V_{rev} is the reversal potential (−85 mV), dV is the slope (6.5 mV) and g_{max} is the maximum normalised conductance. Inset, example currents for one arteriole. The bath solution contained 'channel blockers'. D, voltage dependence of current (means ± S.E.M.) through $K_V1.6$ expressed as a homomultimer in *Xenopus* oocytes under control conditions (●, $n = 7$) and in 1 mM 4-AP (■, $n = 7$). Currents were normalised to the amplitude at 0 mV.

spontaneously absent even though the patched cell remained in the vessel (Fig. 2E).

Whether or not there was electrical coupling between cells, an outward current component was activated by depolarisation. Furthermore, replacement of extracellular Ca^{2+} by Mg^{2+} and application of penitrem A, glibenclamide and niflumic acid (inhibiting voltage-dependent Ca^{2+} entry and blocking BK_{Ca}, K_{ATP} and Cl_{Ca} (Ca^{2+}-activated chloride) channels, respectively; Quinn *et al.* 2000) had only a small inhibitory effect, whereas 1 mM 3,4-DAP inhibited a major component of the current (Fig. 3A). A concentration–response curve was constructed for 4-AP, rather than 3,4-DAP, to facilitate comparison with other published studies. About 40 % inhibition occurred with a low concentration (0.05 mM), there was little further effect at 0.1 mM, but then further inhibition occurred at concentrations up to 5 mM (Fig. 3B). Thus, there appeared to be two components of voltage-dependent K^+ current, one very sensitive and one less sensitive to 4-AP.

The voltage dependence of 3,4-DAP-sensitive current was determined by measuring current amplitude at the end of long square steps in voltage, well after the settling time of the clamp (Fig. 3C). The current–voltage relationships were compared for arterioles with electrically coupled cells, arterioles in the presence of 18α-glycyrrhetinic acid and an arteriole in which the patched cell was spontaneously uncoupled. In each case the voltage dependence of current was similar. The apparent threshold of activation (when

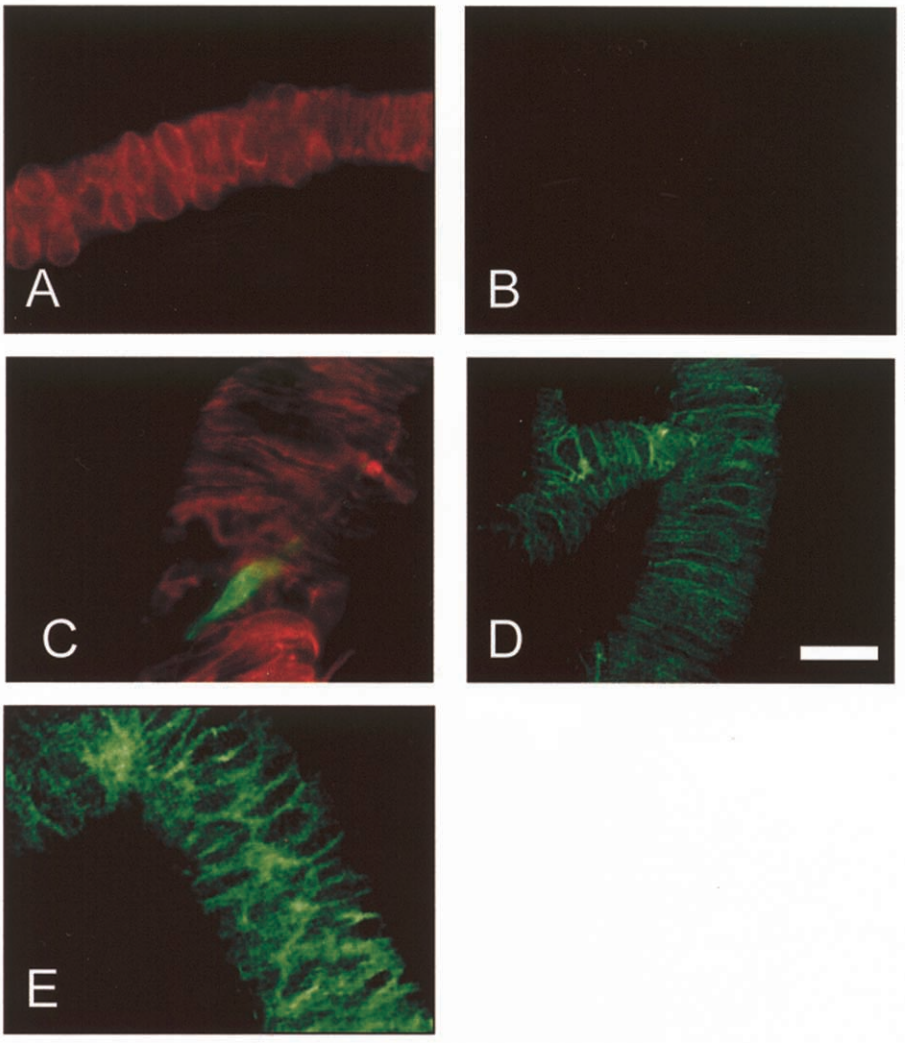

Figure 4. Immunofluorescence staining of native $K_V1.2$, $K_V1.5$ and $K_V1.6$ in enzymatically isolated arterioles

A and B, control experiments showing an arteriole stained with anti-α-SMA-Cy3 (A) and secondary antibody conjugated to FITC but without primary (anti-K_V) antibody (B). C, double staining with anti-α-SMA-Cy3 (red) and anti-$K_V1.2R_{K(461–480)}$ (green). A fracture in the arteriole is evident, through which an endothelial cell (green) is exposed. D, staining with anti-$K_V1.5R_{(578–598)}$. E, staining with anti-$K_V1.6R_{K(509–526)}$. Scale bar, 30 μm.

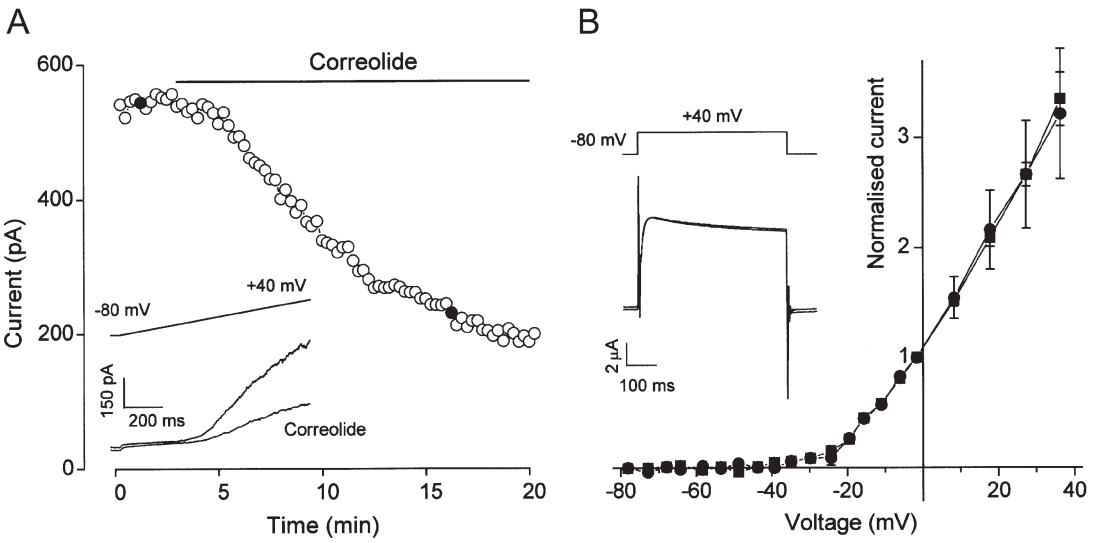

Figure 5. Effect of correolide on K^+ currents

A, plot of current amplitude at the end of 1 s ramp changes in voltage from −80 to +40 mV. Inset, two example current traces taken at the points marked by filled circles in the time-series plot. Correolide (1 μM) was bath applied. B, current–voltage relationships for $K_V2.1$ homomultimers expressed in *Xenopus* oocytes before (■) and after (●) application of 1 μM correolide (means ± S.E.M., $n = 3$). Currents were normalised to the amplitude at 0 mV. Inset, example current traces for control and correolide.

current was first detected) was near −45 mV, and the half-activation potential was not significantly different for the control ($n = 9$) and 18α-glycyrrhetinic acid ($n = 4$) data sets (−33.7 ± 2.5 mV compared with −30.4 ± 2.3 mV). For comparison, current–voltage relationships were constructed for $K_V1.6$ expressed as a homomultimer (Fig. 3D). Again the activation threshold was near −45 mV and the current was sensitive to 4-AP. These data indicate that $K_V\alpha1$ might underlie part of the native voltage-dependent K^+ current in arterioles.

Immunofluorescence staining with polyclonal antibodies was used to test for $K_V1.2$, $K_V1.5$ and $K_V1.6$ expression in arterioles. Arterioles were routinely double-stained with anti-α-SMA-Cy3 to confirm that structures were

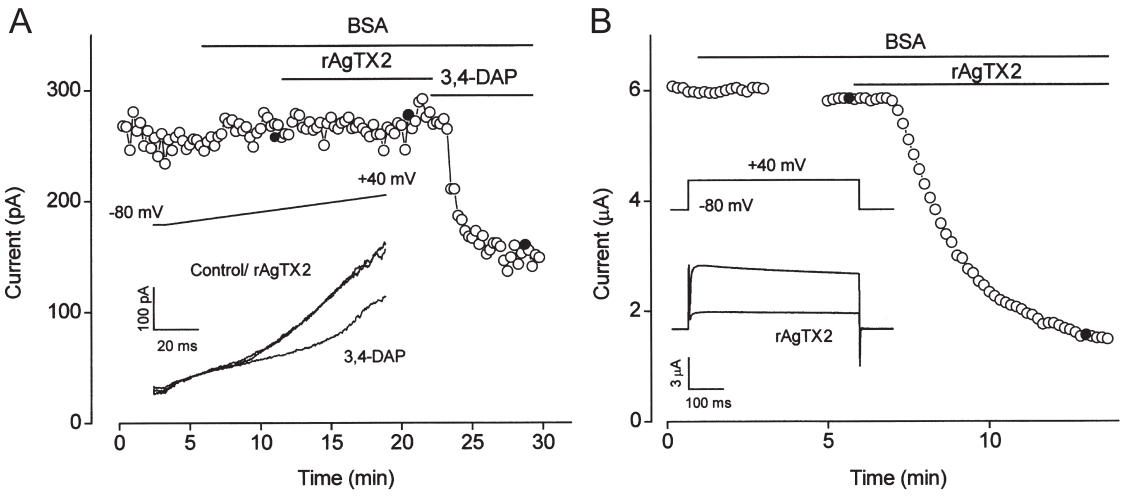

Figure 6. Effect of recombinant agitoxin-2 on K^+ currents

A, plot of arteriolar current amplitude at the end of 1 s ramp changes in voltage from −80 to +40 mV. Inset, three example current traces taken at the points marked by filled circles in the time-series plot. BSA (0.01 %), recombinant agitoxin-2 (rAgTX2; 1 nM) and 3,4-DAP (1 mM) were bath applied. B, plot of current amplitude at +40 mV for $K_V1.6$ homomultimers expressed in a *Xenopus* oocyte. BSA (0.01 %) and rAgTX2 (1 nM) were bath applied. The gap in the trace indicates the period during which a current–voltage relationship was constructed. Inset, example current traces taken at the points marked by filled circles in the time-series plot.

arterioles. Fluorescence was not observed in parallel control experiments in the absence of primary antibody (e.g. Fig. 4A and B). Inclusion of primary antibody revealed $K_V1.2$ to be localised to endothelial cells (Fig. 4C; $n = 5$ for anti-$K_V1.2R_{K(461–480)}$, and confirmed with anti-$K_V1.2R_{A(417–498)}$ (data not shown)). In contrast, $K_V1.5$ ($n = 5$ for anti-$K_V1.5R_{(578–598)}$) and $K_V1.6$ ($n = 6$ for anti-$K_V1.6R_{K(509–526)}$) were localised to the plasma membranes of arteriolar smooth muscle cells (Fig. 4D and E). Anti-$K_V1.5M_{(513–602)}$ also stained smooth muscle cells and staining with anti-$K_V1.5R_{(578–598)}$ was not observed in the presence of antigenic peptide (data not shown). Thus, $K_V\alpha1$ subunits are expressed in arteriolar smooth muscle.

Correolide is a triterpene from *Spachea correa* that selectively binds and blocks members of the $K_V\alpha1$ family of K_V channel proteins including $K_V1.5$ and $K_V1.6$ (Felix *et al.* 1999; Hanner *et al.* 1999; Koo *et al.* 1999; Kaczorowski & Garcia, 1999). Consistent with the involvement of $K_V\alpha1$ in the native arteriolar K^+ current, 1 μM correolide had a marked inhibitory effect (Fig. 5A), reducing currents at 0 and +40 mV by 43.5 ± 7.6 and 43.9 ± 4.6%, respectively ($n = 9$). Although correolide has no effect on an array of non-$K_V\alpha1$ proteins, it may have some effect on $K_V2.1$ at high concentrations (Felix *et al.* 1999). To check the significance of this effect in our experimental protocol, we expressed $K_V2.1$ cRNA in *Xenopus* oocytes. Correolide (1 μM) had no effect on $K_V2.1$ (Fig. 5B).

Agitoxin-2 is a peptide originally derived from *Leiurus quinquestriatus* venom. It is a potent blocker of $K_V\alpha1$ channels except $K_V1.5$, inhibiting $K_V1.6$ with a K_i of 37 pM (Garcia *et al.* 1994; Kaczorowski & Garcia, 1999; Kotecha & Schlichter, 1999; Tytgat *et al.* 1999; M. Garcia, personal communication). rAgTX2 (1 nM) had no effect on voltage-dependent K^+ current in arterioles at 0 or +40 mV (2.8 ± 2.8% effect at 0 mV, $n = 8$) even though the current was shown subsequently to be sensitive to 3,4-DAP (Fig. 6A). In contrast, 1 nM rAgTX2 blocked heterologously expressed $K_V1.6$ by 58.6 ± 8.1% ($n = 5$) using a square step voltage protocol (Fig. 6B) and by 72.4 ± 4.8% ($n = 5$) using the voltage ramp protocol described for Fig. 6A (data not shown).

Correolide (1 μM) was an effective vasoconstrictor of arterioles (Fig. 7B), reducing the diameter by 28.0 ± 3.5% ($n = 7$) relative to the effect of 3,4-DAP or 4-AP. In contrast, rAgTX2 (1 nM) had no effect (0.96 ± 0.96%, $n = 5$) on arterioles that were sensitive to 3,4-DAP or 4-AP (Fig. 7A).

DISCUSSION

We measured K_V channel current in smooth muscle cells within functional cerebral precapillary arterioles, blood vessels that have a primary physiological function in the control of blood pressure and local blood flow in the brain. The data show that current through K_V channels is a major current component and is active in the physiologically crucial voltage range of −45 to 0 mV. We also show expression of $K_V1.5$ and $K_V1.6$ proteins in the plasma membrane of arteriolar smooth muscle cells,

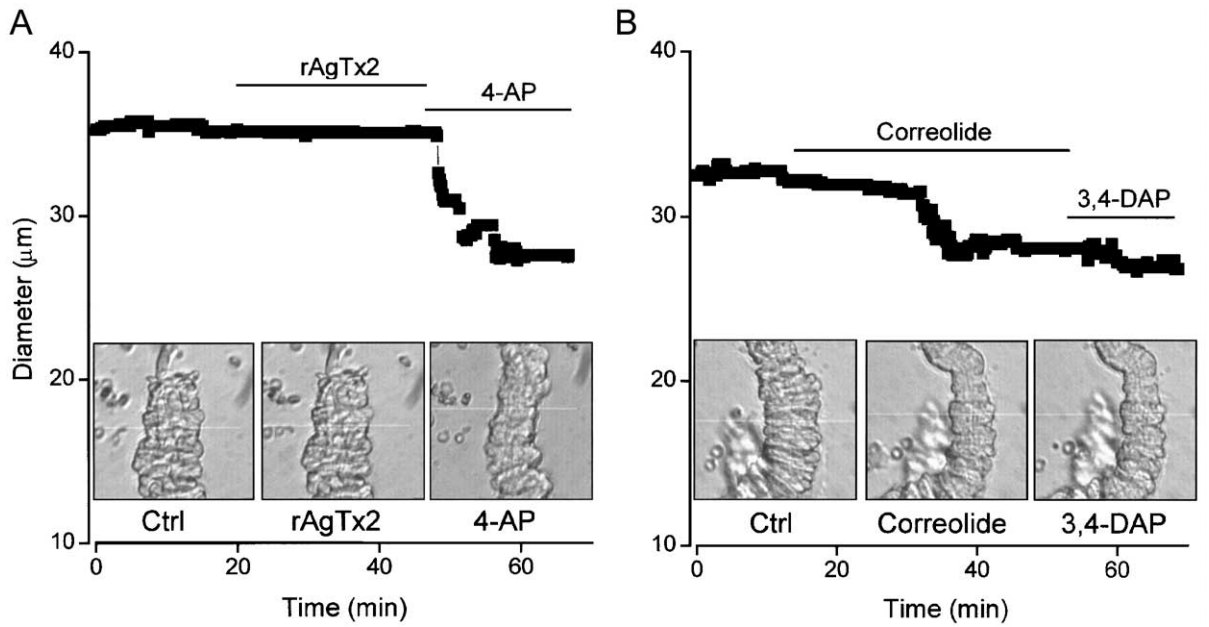

Figure 7. Effects of correolide and recombinant agitoxin-2 on arteriolar diameter

The filled squares in the plots indicate external diameter. The bath solution was aCSF and a small level of preconstriction was induced in both experiments with 0.3 nM ET-1. Actual video images of the arterioles are shown inset for the three conditions in each case. The white horizontal line in each image is the scan line used to detect the outer edge of the arteriole. It was moved slightly during recordings to maintain edge-detection. A, 1 nM rAgTX2 and 1 mM 4-AP. B, 1 μM correolide and 1 mM 3,4-DAP. Ctrl, control.

and reveal that there are electrophysiological and pharmacological similarities between the native K_V channel current and that carried by heterologously expressed KCNA6 cRNA. Importantly, non-specific K_V channel blockers and a selective $K_V\alpha1$ blocker are powerful arteriolar constrictor agents, suggesting that $K_V\alpha1$ channels play a major role in smooth muscle cells of these small blood vessels.

Inferences about the functional importance of $K_V\alpha1$ were made from *in vitro* studies of arteriolar diameter under quasi-physiological conditions, which included the presence of a low concentration of an endogenous vasoconstrictor (ET-1). The presence of ET-1 is important because it depolarises the arterioles to about −40 mV and causes a small amount of basal tone (Guibert & Beech, 1999). This is significant because, *in vivo*, arterioles are exposed to a small lumenal flow and pressure (30 mmHg), which causes an effect, at least by inference from studies of large arteries, similar to that of ET-1 (Knot & Nelson, 1995). *In vivo*, arterioles are also exposed to a range of endogenous vasoconstrictors, including ET-1 (Thorin *et al.* 1998). The induction of depolarisation or basal tone is probably important for the vasoconstrictor action of K_V channel inhibitors. The mean resting membrane potential of isolated cerebral arterioles in the absence of stimuli is −69 mV (negative of the threshold for activation of K_V channel current) and these arterioles are often resistant to a cocktail of K^+ channel inhibitors including 1 mM 3,4-DAP (Quinn *et al.* 2000).

The functional importance of K_V channels in arterioles is dependent on the existence of sustained channel activity, which may arise because of window-current, or because there is a non-inactivating channel state at physiological membrane potentials. There are three reasons to believe that sustained K_V channel activity exists. Firstly, the data in Fig. 3C show that 3,4-DAP-sensitive K_V channel current is non-inactivating over a time period of 1 s at 0 mV. Thus, if there is inactivation it must be very slow, and it would be even slower at, for example, −35 mV. Secondly, correolide and aminopyridine compounds caused arteriolar constriction, effects that could not occur in the absence of sustained non-inactivating K_V channel current. Thirdly, in rabbit basilar artery myocytes, there is a 3 pA sustained current component at −40 mV that is inhibited by 5 mM 4-AP (Robertson & Nelson, 1994).

We suggest that agitoxin-2 is ineffective on rabbit arterioles because Kv1.5 is expressed in the smooth muscle cells. Although $K_V1.6$ is also expressed, we presume that this subunit exists only within a hetero-multimer with $K_V1.5$, which prevents access of agitoxin-2 to the binding site on $K_V1.6$. Indeed, such an effect has been demonstrated for charybdotoxin, which also blocks $K_V1.6$ but not $K_V1.5$ (Russell *et al.* 1994; Coetzee *et al.* 1999). Strikingly, cerebral arterioles from mouse do not express $K_V1.5$ in the smooth muscle layer and are sensitive to agitoxin-2 (Cheong *et al.* 2001).

All previous studies suggesting a role for K_V channels in vascular smooth muscle have employed aminopyridine compounds. 4-AP causes contraction in pulmonary, coronary, basilar and middle cerebral arteries (Knot & Nelson, 1995; O'Rourke, 1996; Archer *et al.* 1998; Shimizu *et al.* 2000; Quan & Sobey, 2000). One problem with aminopyridines is their high pK_a (−log of the acid dissociation constant). Particularly at concentrations > 1 mM, they tend to alkalinise extracellular and intracellular solutions. Anti-proliferative effects of 4-AP have been attributed to intracellular alkalinisation (Pappas *et al.* 1994). Although it was shown that intracellular loading with anti-$K_V1.5$ antibody inhibited the effect of 4-AP in pulmonary artery, the antibody surprisingly did not cause contraction (Archer *et al.* 1998). Thus, evidence for the functional importance of $K_V\alpha1$ has been lacking. Following the development of correolide (Felix *et al.* 1999), we now show that $K_V\alpha1$ channels have a primary role in terminal cerebral arterioles. We also show that, as with large arteries, aminopyridine compounds are very effective vasoconstrictors, and demonstrate the existence of K_V channel current in the physiological voltage range and in smooth muscle cells that are still within the functional vessel, rather than in isolation.

Archer *et al.* (1998) and Hogg *et al.* (1999) previously observed a $K_V\alpha1$ channel ($K_V1.5$) in endothelial cells of pulmonary arteries. Although we did not detect $K_V1.5$ in cerebral endothelial cells we did detect $K_V1.2$. In endothelial cells, K_V channels do not have a well-recognised function (Nilius *et al.* 1997), although a 4-AP-sensitive K_V channel current exists and is regulated by stretch (Fan & Walsh, 1999). $K_V1.2$ may serve to inhibit depolarisation if it spreads electrotonically from smooth muscle cells.

In cerebral arteriolar smooth muscle cells we show that current through $K_V\alpha1$ contributes a critical balance against sustained excitation, preventing depolarisation at the steep rising phase of the voltage-gated Ca^{2+} channel activation curve. Too much $K_V\alpha1$ channel current will lead to quiescence, and too little to excess contraction and even regenerative spiking. Regulation of $K_V\alpha1$ function or gene expression presumably has major implications for the control of physiological blood pressure and local tissue perfusion.

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