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Characterization of an apamin-sensitive small-conductance Ca^{2+}-activated K^+ channel in porcine coronary artery endothelium: relevance to EDHF

*,[1]M.P. Burnham, [2]R. Bychkov, [2]M. Félétou, [1]G.R. Richards, [3]P.M. Vanhoutte, [1]A.H. Weston & [1]G. Edwards

[1]School of Biological Sciences, University of Manchester, Manchester M13 9PT; [2]Département de Diabétologie, Institut de Recherche Servier, 92150 Suresnes, France and [3]Institut de Recherches Internationales Servier, 92410 Courbevoie, France

1 The apamin-sensitive small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) was characterized in porcine coronary arteries.

2 In intact arteries, 100 nM substance P and 600 μM 1-ethyl-2-benzimidazolinone (1-EBIO) produced endothelial cell hyperpolarizations (27.8 ± 0.8 mV and 24.1 ± 1.0 mV, respectively). Charybdotoxin (100 nM) abolished the 1-EBIO response but substance P continued to induce a hyperpolarization (25.8 ± 0.3 mV).

3 In freshly-isolated endothelial cells, outside-out patch recordings revealed a unitary K^+ conductance of 6.8 ± 0.04 pS. The open-probability was increased by Ca^{2+} and reduced by apamin (100 nM). Substance P activated an outward current under whole-cell perforated-patch conditions and a component of this current (38%) was inhibited by apamin. A second conductance of 2.7 ± 0.03 pS inhibited by d-tubocurarine was observed infrequently.

4 Messenger RNA encoding the SK2 and SK3, but not the SK1, subunits of SK_{Ca} was detected by RT–PCR in samples of endothelium. Western blotting indicated that SK3 protein was abundant in samples of endothelium compared to whole arteries. SK2 protein was present in whole artery nuclear fractions.

5 Immunofluorescent labelling confirmed that SK3 was highly expressed at the plasmalemma of endothelial cells and was not expressed in smooth muscle. SK2 was restricted to the peri-nuclear regions of both endothelial and smooth muscle cells.

6 In conclusion, the porcine coronary artery endothelium expresses an apamin-sensitive SK_{Ca} containing the SK3 subunit. These channels are likely to confer all or part of the apamin-sensitive component of the endothelium-derived hyperpolarizing factor (EDHF) response.
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Keywords: EDHF; porcine coronary artery; endothelium; apamin; 1-EBIO; SK1; SK2; SK3

Abbreviations: BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channel; BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; 1-EBIO, 1-ethyl-2-benzimidazolinone; EDHF, endothelium-derived hyperpolarizing factor; FITC, fluorescein isothiocyanate; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channel; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel

Introduction

In the combined presence of inhibitors of nitric oxide and prostacyclin synthesis, ligands such as acetylcholine, substance P and bradykinin or increases in luminal flow still evoke vasodilation (Nagao & Vanhoutte, 1991; Zhang *et al*., 1994; Petersson *et al*., 1997; Izzard & Heagerty, 1999). Under these conditions, vessel relaxation is endothelium-dependent and is believed to be generated through the action of an endothelium-derived hyperpolarizing factor (EDHF; Garland *et al*., 1995; Félétou & Vanhoutte, 2000; Edwards & Weston, 2001a).

Microelectrode experiments have shown that the critical initiating step in the EDHF pathway is endothelial cell hyperpolarization and that this component is sensitive to a combination of the toxins apamin and charybdotoxin (Edwards *et al*., 1998; 2000) but not apamin and iberiotoxin (Corriu *et al*., 1996; Zygmunt & Högestätt, 1996; Petersson *et al*., 1997; Chataigneau *et al*., 1998; Edwards *et al*., 1998; 2000). Iberiotoxin and charybdotoxin are two scorpion venom-derived peptides but while the former is selective for large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), the latter also inhibits the intermediate-conductance Ca^{2+}-activated K^+ channel (IK_{Ca}) and some voltage-dependent potassium channels (Garcia *et al*., 1991). Apamin is a selective inhibitor of the small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}; Castle, 1999). Together with other lines of evidence, these observations have led to the hypothesis that the inhibition of EDHF by apamin plus charybdotoxin is due to their inhibition of SK_{Ca} and IK_{Ca} located on the vascular endothelium (Edwards *et al*., 1998). Three SK_{Ca}

*Author for correspondence; E-mail: matt.b@man.ac.uk

subunits (SK1, SK2 and SK3) have been cloned which all form apamin-sensitive channels when expressed in *Xenopus* oocytes or mammalian cells (Köhler *et al*., 1996; Hosseini *et al*., 1999; Shah & Haylett, 2000; Strøbæk *et al*., 2000; Grunnet *et al*., 2001a).

The objective of the present study was to characterize further the apamin-sensitive component of the EDHF pathway in the porcine left anterior descending coronary artery. Patch-clamp and sharp microelectrode techniques were used to measure K^+ currents and membrane potential changes, respectively, in freshly-isolated endothelial cells and whole vessels. In addition, RT–PCR and Western blotting techniques coupled with immunofluorescence histochemistry were employed to identify and localize K^+ channel proteins in the cells of this artery. A preliminary account of some of these observations has been presented (Burnham *et al*., 2001). Data obtained concerning the endothelial IK_{Ca} will be reported in a separate study.

Methods

Tissue dissection

Pig hearts were taken from Large-White pigs (male or female, 25 kg) anaesthetized with a mixture of tiletamine plus zolazepam (i/m., each 10 mg kg^{-1}) or obtained from the local abattoir and transported to the laboratory in ice-cold Krebs solution (mM: NaCl 118, KCl 3.4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11). Left anterior descending coronary arteries were dissected free of surrounding tissue while maintained in ice-cold Krebs solution.

Patch-clamp experiments

Endothelial cells were mechanically isolated by placing the endothelial surface of an opened artery in contact with a Matrigel® pre-treated cover-slip and applying gentle pressure to the back of the artery with a brush. K^+ currents were measured at room temperature (20–24°C) using outside-out or perforated-patch configurations of the patch-clamp technique. The external solution contained (mM): NaCl 140, $CaCl_2$ 1.8, $MgCl_2$ 1, KCl 5.4, Na-HEPES (pH 7.4) 10. Recording pipettes (3–8 MΩ resistance) were filled with a solution containing (mM): K-aspartate 80, KCl 40, NaCl 20, $MgCl_2$ 1, Mg-ATP 3, EGTA 10, K-HEPES (pH 7.4) 5. EGTA was titrated with Ca^{2+} to provide solutions containing 100, 200 and 500 nM free Ca^{2+} using the methods of Fabiato (1988). For perforated-patch experiments, whole-cell access was achieved within 2–5 min by the inclusion of nystatin (50–100 μg ml^{-1}) in the pipette solution. Drugs were added cumulatively to the recording chamber in bolus doses.

Patch-clamp data were recorded at 5–10 kHz using an Axopatch 200B amplifier (Axon Instruments) with compensation for series resistance and cell capacitance. Data were filtered at 1 kHz, digitized using an Axon interface and data analysis was performed using pClamp-6 software (Axon Instruments). Variance analysis and histogram distribution were used to determine the unitary conductance of single K^+ channels. Both methods were compared and provided similar results. Mean current (I) and variance (σ^2) were calculated for the variance analysis (Bychkov *et al*., 1997). Least-squared parabolic fits to the resulting values were used to derive estimates of single channel properties according to the equation $\sigma^2 = Ii - (I^2/N)$, where N is the number of channels in the patch and i the unitary current amplitude.

Micro-electrode studies

Segments of intact vessel were opened longitudinally, pinned to the Sylgard base of a heated 10 ml bath and superfused with Krebs solution (10 ml min^{-1}, 37°C, gassed with 5% CO_2 in O_2) containing 300 μM N^G-nitro-L-arginine and 10 μM indomethacin. Endothelial cells were impaled using microelectrodes filled with 3 M KCl (resistance 40–80 MΩ). Successful impalements were signalled by a sudden change in membrane potential which remained stable for at least 2 min before the experiment was commenced. Substance P and 1-EBIO were each added as bolus injections directly into the bath in quantities calculated to give (transiently) the final concentrations indicated. Charybdotoxin and apamin were added to the reservoir of Krebs superfusing the bath. Only recordings from impalements that were maintained successfully for the entire protocol were analysed.

Recordings were obtained using a conventional high impedance amplifier (Intra 767, WPI Instruments) and digitized for analysis using a MacLab system (AD Instruments). 50 Hz interference at the amplifier output was selectively removed using an active processing circuit (Humbug, Digitimer).

Gene-specific RT–PCR

Samples of endothelium were obtained as luminal scrapings. Pig brain tissue was taken from the hippocampal area. Total RNA was isolated using RNeasy Mini Prep kits (Qiagen) and, following DNase treatment (Gibco Life Technologies), RNA was reverse-transcribed using Sensiscript (Qiagen), all according to manufacturers' instructions. Forward and reverse primers for RT–PCR (35 cycles, 64°C annealing temperature and 1.5 mM Mg^{2+}) were as follows: GAPDH, 5′-GACCACTTCGTCAAGCTCATTTCC and 5′-GATGGTACATGACGAGGCAGGTC; SK1, 5′-CCCACATAGTCATGAACAGCCACAG and 5′-GGATCTCCCGGGCATGGTAGAGG; SK2, 5′-CCCCGAGATCGTGGTGTCTAAGC and 5′-CACACACCAGTATTTCCAAGCAGATG; SK3, 5′-TCCATGTTTTCGTTGGCCCTG and 5′-AGCATGACTCGGGCGATCAGGTA. Products were resolved by electrophoresis on 1.5% (w v^{-1}) agarose gels and the identity of each was confirmed by cloning and sequencing (Big Dye Chemistry).

The SK3 cDNA was sequenced using the additional primers 5′-GACACTTCTGGGCACTTCCATGA and 5′-CAACTGCTTGAACTTGTGTATGG to amplify overlapping segments of the coding region. 5′- and 3′- RACE (Rapid Amplification of cDNA Ends) was performed (GeneRacer kit, Qiagen) to obtain the complete cDNA sequence. Each product was sequenced in triplicate.

Western blotting

Whole arteries were homogenized on ice using ground-glass homogenisers in (mM): Tris pH 7.5 20, sucrose 250,

EDTA 5, EGTA 10, protease inhibitor (Sigma P2714). Nuclear and post-nuclear fractions were produced by centrifuging for 5 min at 12,000 r.p.m. in a bench-top centrifuge. Samples of endothelium were obtained by scraping the luminal surface with a clean scalpel blade. Protein estimation was performed with the method of Bradford (1976).

Sample preparation for sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) on 10% (w v^{-1}) acrylamide separating gels and electrophoretic transfer to polyvinylidene difluoride membrane was performed as previously described (Laemmli, 1970; Towbin *et al*., 1979). Membranes were blocked overnight in 50 mg ml^{-1} non-fat dried milk in Tween-Tris-buffered saline (Tween-TBS; 1 μl ml^{-1} Tween-20, 20 mM Tris pH 8.0, 150 mM NaCl). Anti-SK2 and SK3 primary antibodies were used with and without control antigen pre-incubation (incubated for 1 h with 3 fold excess by weight of control peptide) and diluted to 1 μg ml^{-1} in Tween-TBS containing 10 mg ml^{-1} bovine serum albumin (BSA). Membranes were incubated with primary antibody for 1 h. Detection was achieved using horseradish peroxidase-conjugated secondary antibodies and chemiluminescent reagents.

Immunofluorescence histochemistry

Arteries were fixed (McLean & Nakane, 1974) for 20 min, cryoprotected overnight in 0.3 g ml^{-1} sucrose in phosphate-buffered saline (PBS), and embedded in OCT® compound. Cryostat sections (thickness 8 μm) were collected on silanated microscope slides. To label intact endothelium viewed *en-face*, small fragments of artery were first pinned flat, fixed and washed in PBS. Both preparations were treated with 1 mg ml^{-1} SDS in PBS for 30 min, washed in PBS and blocked for 1 h or overnight with blocking buffer (50 μl ml^{-1} normal goat serum, 10 mg ml^{-1} BSA in PBS). Primary antibodies were prepared with and without control peptide pre-incubation, diluted to 6 μg ml^{-1} in blocking buffer and applied for 1 h. In some cases, negative controls were performed by omission of primary antibody. Secondary antibodies conjugated with Cy3 or fluorescein isothiocyanate (FITC) were applied for 30 min, with either 4,6-diamidino-2-phenylindole (DAPI; 6 μg ml^{-1} final) or propidium iodide (1 μg ml^{-1} final) included as nuclear labels. Sections were viewed on a Zeiss Axioplan 2 microscope equipped with Hamanatsu CCD camera and Zeiss KS300 software. Identical microscope, camera and software settings were used when imaging labelled sections and negative controls.

Materials

Anti-SK2 and anti-SK3 (Alomone Labs), supplied with control peptides, anti-von Willebrand's factor (Novocastra) and secondary antibody conjugates (Jackson Immunoresearch) were used. Apamin, synthetic charybdotoxin and synthetic iberiotoxin were obtained from Latoxan, France and Matrigel® was obtained from Becton Dickinson (MA, USA). OCT® compound was purchased from R.A. Lamb (UK). All other substances were supplied by Sigma, except 1-ethyl-2-benzimidazolinone (1-EBIO; Aldrich), levcromakalim (SmithKline Beecham) and substance P (RBI).

Data analysis

All values are given as mean ± standard error (s.e.m.). The number of tested cells or arteries from individual animals is given by n. Statistical analysis was carried out using the paired or unpaired Student's t-test and a value of $P<0.05$ was considered statistically significant.

Results

Sharp micro-electrode recordings from intact endothelium

Endothelial cells of porcine coronary arteries were impaled with sharp micro-electrodes and a membrane potential of -49.1 ± 0.4 mV ($n=4$) was observed under control conditions (Figure 1). Both 100 nM substance P (used to activate the EDHF pathway) and 600 μM 1-EBIO (an opener of Ca^{2+}-sensitive K^+ channels), induced robust hyperpolarizations (substance P, 27.8 ± 0.8 mV; 1-EBIO, 24.1 ± 1.0 mV; each $n=4$). Supplementing the bathing solution with 100 nM charybdotoxin abolished the response to 1-EBIO but did not modify the hyperpolarization to substance P (25.8 ± 0.3 mV, $n=4$). Further addition of 100 nM apamin to the superfusing

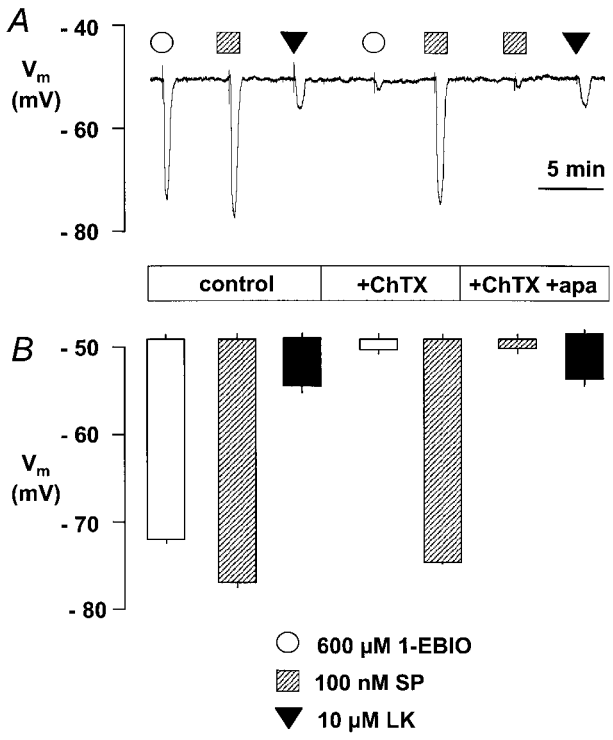


Figure 1 Determination using charybdotoxin and apamin of the Ca^{2+}-activated K^+ channels activated by substance P (SP) and 1-EBIO. Membrane potential responses in porcine coronary artery endothelium to transient exposure to 100 nM substance P and 600 μM 1-EBIO were recorded using sharp micro-electrodes. The addition of 100 nM charybdotoxin (ChTX) and 100 nM apamin (apa) to the superfusing bath solution is indicated. A representative trace (A) and the data derived from four such experiments (B: columns represent the mean membrane potential (V_m) and s.e.mean before and after exposure to each hyperpolarizing agent in the absence or presence of the inhibitors as indicated) is shown. Levcromakalim (LK, 10 μM) was included as an internal control.

solution abolished the response to substance P. Neither the hyperpolarization to the ATP-sensitive K^+ channel opener levcromakalim (10 μM; before 5.5 ± 0.4 mV; after 5.2 ± 0.6 mV; $n=4$) nor the resting membrane potential was affected by either of these toxins (Figure 1).

Patch-clamp analysis of K^+ currents in freshly-isolated endothelial cells

Outside-out patch configuration, single-channel recordings were performed with a 250 nM free Ca^{2+} pipette solution and asymmetric, quasi-physiological K^+ concentrations: internal solution containing 125 mM K^+ and external solution containing 5.4 mM K^+ (with 1.8 mM Ca^{2+} and 100 nM charybdotoxin). Under these conditions, two types of channel were observed (representative traces given in Figure 2A,B). Unitary conductances of 6.8 ± 0.04 and 2.7 ± 0.03 pS ($n=9$ and 7, respectively) were determined by plotting unitary current amplitude against membrane potential under asymmetric 5.4/125 mM K^+ concentrations (Figure 2C,D). The 6.8 pS channel showed essentially no voltage-dependency, with only a slight increase in open probability over the tested voltage range. Under symmetrical K^+ conditions, the unitary conductance of this channel was 10.1 ± 0.2 pS ($n=6$). The 2.7 pS conductance was observed infrequently (7 out of 38 patches) and was voltage-insensitive.

The effect of Ca^{2+} and toxins on the open probability of the 6.8 pS conductance was determined at a holding potential of 0 mV using pipette solutions containing 100, 250 and 500 nM free Ca^{2+} and in the bath presence of 100 nM charybdotoxin. In the absence of toxins, the open probability increased from 0.20 ± 0.03 ($n=8$) to 0.52 ± 0.05 ($n=8$) and to 0.77 ± 0.06 ($n=7$) for 100, 250 and 500 nM Ca^{2+}, respectively (Figure 3). With 250 nM Ca^{2+} in the pipette, the addition of 100 nM apamin significantly reduced the open probability of the channel from 0.53 ± 0.04 to 0.10 ± 0.06 ($n=7$, Figure 3). In the presence of 100 nM charybdotoxin (with 250 nM Ca^{2+} in the pipette), the open probability of the 6.8 pS channel (0.35 ± 0.05, $n=6$) was only marginally lower than that of controls recorded in this set of experiments (0.52 ± 0.05, $n=8$).

The 2.7 pS conductance was observed infrequently, making a detailed characterization difficult. However, in outside-out patches with 500 nM Ca^{2+} pipette solution, the open probability of the channel recorded at 0 mV (0.62 ± 0.07) was reduced to 0.34 ± 0.06 by 0.1 μM and to 0.12 ± 0.02 by 1 μM d-tubocurarine, an inhibitor of SK_{Ca} ($n=5$, Figure 4; Castle, 1999). The effect of d-tubocurarine on the 6.8 pS

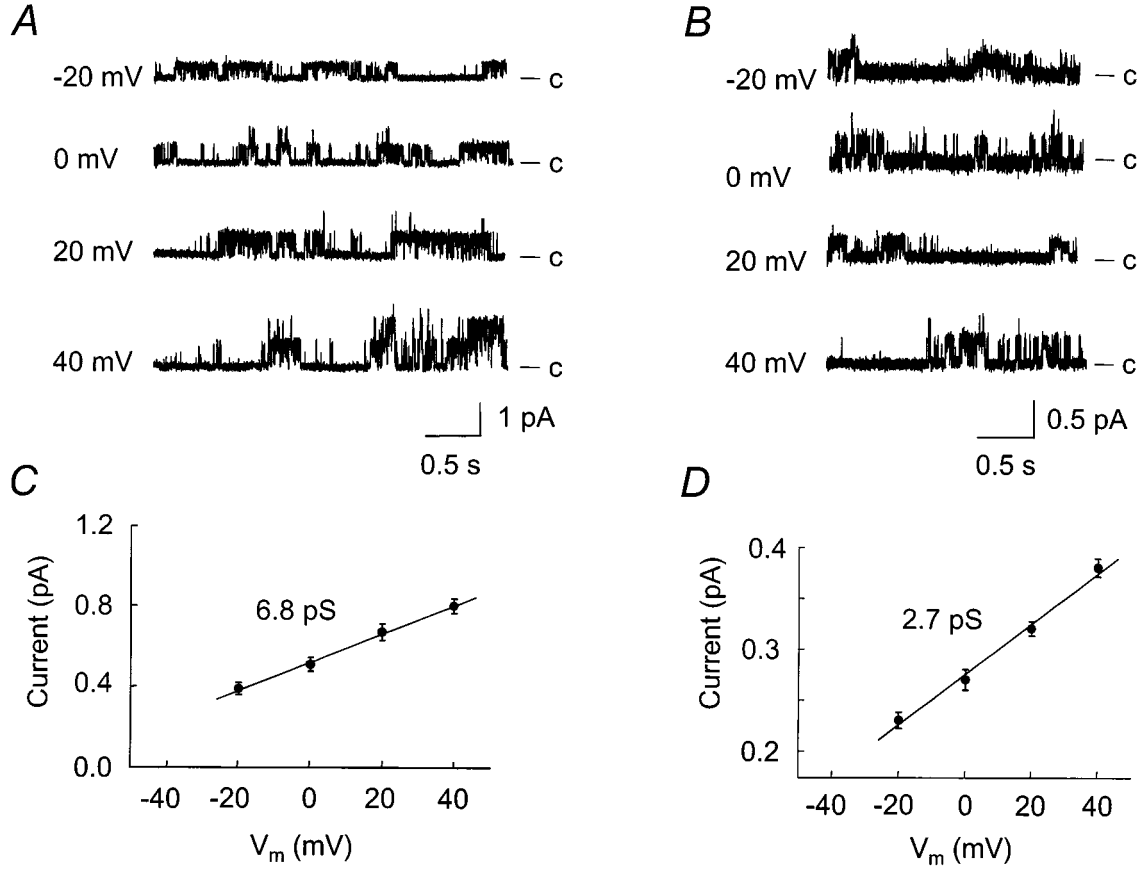


Figure 2 Outside-out patch recordings from freshly-dissociated endothelial cells. Two types of small conductance channel were observed in single-channel recordings obtained with 250 nM free Ca^{2+} pipette solution in the presence of 100 nM charybdotoxin (physiological K^+ gradient). Representative traces for each type of channel, recorded at the indicated membrane potentials are shown in (A) and (B) in which 'c' represents the closed state. Unitary conductances were determined by variance analysis (with the assumption of a single channel) of activity recorded at −20, 0, 20 and 40 mV. Data were fitted to give slope conductances of 6.8 and 2.7 pS for each channel (C and D).

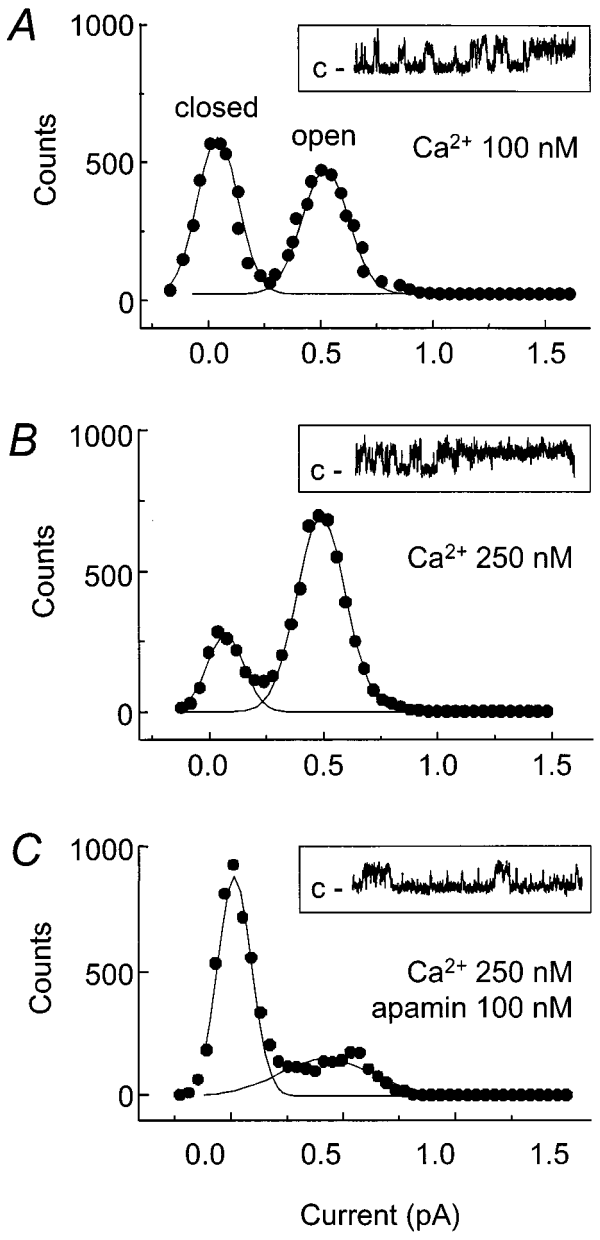


Figure 3 Effect of Ca^{2+} and apamin on the 6.8 pS channel activity. Data obtained in the outside-out patch configuration under a quasi-physiological K^+ gradient (in the absence of charybdotoxin). Distribution of unitary conductance amplitude recorded at 0 mV together with representative traces ('c' indicates the closed state): (A), with 100 nM Ca^{2+} pipette solution; (B), with 250 nM Ca^{2+} pipette solution; (C), with 250 nM Ca^{2+} pipette solution after the application of 100 nM apamin.

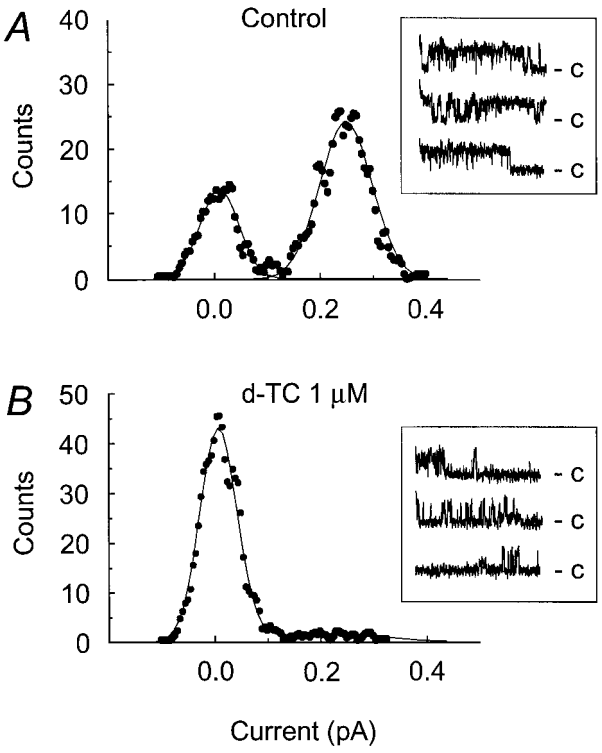


Figure 4 Effect of d-tubocurarine on the 2.7 pS channel activity. Data obtained at 0 mV from outside-out patches under a quasi-physiological K^+ gradient with 500 nM Ca^{2+} pipette solution. Unitary conductance amplitude distributions together with representative traces ('c' indicates the closed state): (A), control; (B), with 1 μM d-tubocurarine (d-TC) in the bathing solution.

channel was not investigated. The 2.7 pS channel was observed in presence of ChTX but not apamin ($n=7$).

Using the perforated-patch configuration with 500 nM free Ca^{2+} pipette solution, 100 nM substance P produced a transient and rapid increase in the whole-cell current followed by a steady-state activation (time-course data not shown). Substance P increased the total current in this steady-state phase by $55\pm9\%$ ($n=6$) at 90 mV and apamin (100 nM) reduced this increase to $34\pm6\%$ at 90 mV, ($n=6$, $P<0.05$; Figure 5). Charybdotoxin was absent throughout these experiments. Whole-cell currents from endothelial cells dialyzed with 10 mM EGTA were unaffected by application of 100 nM substance P ($n=5$, data not shown).

Analysis of SK_{Ca} subunit mRNA expression

RT–PCR primers for detection of SK1, SK2 and SK3 subunits were verified using template cDNA prepared from pig brain. Products of the appropriate size were amplified in each case (Figure 6), and product identity was confirmed by cloning and sequencing. Samples of cDNA prepared from the porcine coronary artery endothelium ($n=5$) contained SK3 (detected in 5 out of 5 samples) and SK2 (3 out of 5 samples) transcripts (Figure 6). No SK1 transcripts were detected. GAPDH housekeeping gene, included as a positive control, was detected in all cases. Using a combination of conventional RT–PCR and 5′ and 3′ RACE, the entire SK3 cDNA from porcine coronary artery endothelium was sequenced (Figure 6, GenBank accession number AY038049). This transcript displayed 93% identity with the human form in the coding sequence and included deletions in the two CAG repeat regions.

Immunofluorescence histochemistry

The endothelium was positively identified in cryostat sections by anti-von Willebrand's factor reactivity (Figure 7A). Autofluorescence intrinsic to the internal elastic lamina could

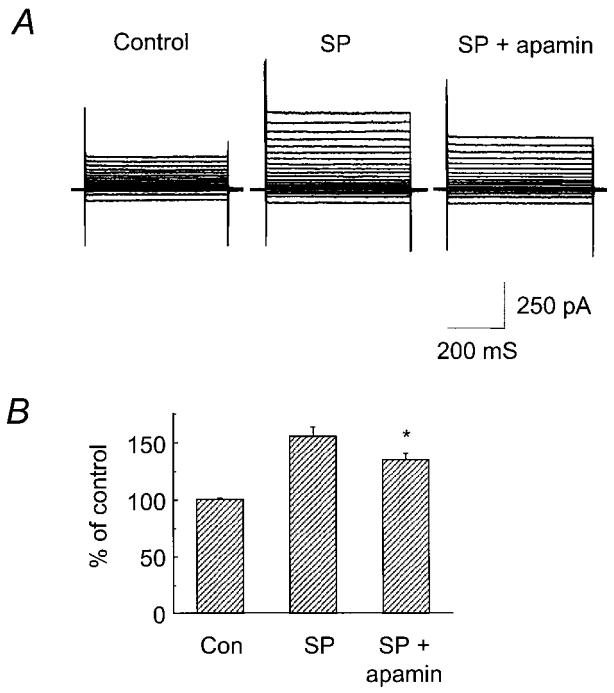


Figure 5 Effect of substance P on K^+ currents recorded using the perforated-patch configuration. K^+ currents were recorded under control conditions and during the steady state phase of current activation by 100 nM substance P in the presence and absence of 100 nM apamin. (A), superimposed traces represent K^+ currents elicited by 10 mV step pulses ranging from −100 mV to +90 mV from a holding potential of −60 mV with 500 nM free Ca^{2+} pipette solution. (B), bar graph representing steady-state activation of K^+ currents by substance P (SP) and a partial but significant block of substance P-activated current by 100 nM apamin at 90 mV ($n=6$).

be detected through the FITC filter-set (green fluorescence) and was purposefully included in images to divide visibly the endothelial and smooth muscle layers. This green autofluorescence was not visible when imaging the red-fluorescent Cy3-conjugated secondary antibody used for SK2 and SK3 detection. Nuclei were stained using the blue-fluorescent DAPI.

In artery sections, intense SK3 labelling was observed in the endothelium (Figure 7B, $n=4$), with reactivity in the smooth muscle limited to the occasional cell (not visible in Figure 7B). Labelling was completely absent when the primary antibody was pre-incubated with control peptide (Figure 7B, inset).

The endothelium was also viewed *en-face*, with nuclei labelled red using propidium iodide and SK3 reactivity detected with green-fluorescent (FITC) secondary antibodies. When observed in this orientation, endothelial cells were clearly identified because their nuclei appeared rounded while those of smooth muscle cells were elongated. In agreement with the observations from cryostat sections, intense SK3 labelling was visible throughout the endothelium (Figure 7C, $n=2$). SK3 appeared localized to the periphery of each endothelial cell, near or within the plasmalemma. No labelling was observed when the primary antibody was omitted (Figure 7C, inset).

SK2 immunoreactivity in artery sections was present in both endothelial and smooth muscle cells (Figure 7D). When viewed *en-face* (Figure 7E) or in sections, SK2 labelling was restricted to the area immediately surrounding the nuclear staining ($n=6$). No immunoreactivity was observed when the primary antibody was pre-incubated with control peptide (Figure 7D, inset) or omitted (Figure 7E, inset).

Identification of SK2 and SK3 protein by Western blotting

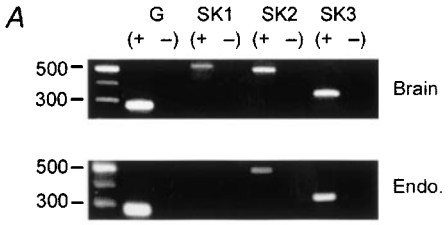
The SK_{Ca} protein content of endothelium only versus whole arteries was examined by Western blotting ($n=2$). Samples of whole artery were prepared as nuclear and post-nuclear fractions. Anti-SK3 immunoreactivity was absent from whole artery fractions but present in endothelium samples (Figure 7F, 10 μg protein each). The ~74 kDa SK3 band migrated close to the predicted molecular weight of hSK3 of 81 kDa, while a related epitope was present at ~134 kDa. Strong anti-SK2 immunoreactivity was observed at ~69 kDa, close to the predicted molecular weight of hSK2 of 64 kDa (Figure 7F). This reactivity was present in the nuclear, but absent from the post-nuclear, fractions of whole artery. The reactivity was also observed in endothelium samples. Detection of SK2 and SK3 bands was prevented in each case by pre-incubation with the appropriate control peptide (Figure 7F).

Discussion

It has long been assumed that endothelium-dependent smooth muscle hyperpolarization results from the release of a hyperpolarizing factor (i.e. EDHF) which opens smooth muscle K^+ channels. Such a view was apparently strengthened by the observation that charybdotoxin plus apamin (toxin inhibitors of K^+ channels) blocked the EDHF response. However, the smooth muscle location of the K^+ channel targets for charybdotoxin and aparmin has been challenged and an endothelial site proposed (Edwards *et al*., 1998). Furthermore, the ability of endothelial K^+ channels to stimulate smooth muscle hyperpolarization has been demonstrated in principle using 1-EBIO, an opener of these channels (Edwards *et al*., 1999). Substance P-induced smooth muscle hyperpolarization elicited by the EDHF pathway in porcine coronary artery is inhibited by blockade of SK_{Ca} and IK_{Ca} (Edwards *et al*., 2000). In this study, we have demonstrated that native porcine coronary artery endothelial cells express an apamin-sensitive SK_{Ca} that contains the SK3 subunit. The SK1 and SK2 subunits make a minor, if any, contribution to these channels. The SK3 channels are restricted to endothelial cells and are not expressed in myocytes, in agreement with the proposed endothelial site of action of apamin. Furthermore, we have shown that these apamin-sensitive channels are activated by substance P.

Microelectrode studies

Endothelial cell responses to modulators of IK_{Ca} and SK_{Ca} were determined using sharp micro-electrodes to record membrane potential. Impalement of endothelial cells rather than myocytes was verified by the responses to levcromakalim, which were small (~5 mV) compared to those recorded in the smooth muscle of this artery (~30 mV, Edwards *et al*.,



B

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TTGAGCCAGC GAGGAGTGAA NCTGANCCTG GCCTCACACG CTCCTGGAGG ACCACCTCCT  - 60
GAGACAGAGT TCTTTTTCCC CCTTCTTCTT CTTTCTCCAA GCGCCCCTCC TGCTCTCCCT  - 120
CCGTGCCCAG TACAATGCAT TCTTGAGTGG CAGCGTCTGG ACTCCAGGCA GCCCCAGAGA  - 180
ACCGAAGCAA GCCAAAGAGA GAGGGCTGGA GCCAAGACAC TCTGGTGGGG GAGCTTGGAT  - 240
GCCTGGCTTT CTTTGAGGAC ATCTTTGGAG CAAGGGTGGC TTTGGGGTGG GGGGCTGTGC  - 300
TGCAGGAAAT CCAGCCAGGC CCCAAGATGG ACACTTCTGG GCACTTCCAT GACTCCGGGG  - 360
TGGGAGACCT GGATGAAGAC CCCAAGTGCC CCTGTCCATC CTCCGGGGAT GAGCAGCAAC  - 420
AACAGCAGCC GCCGCCGCCG CCACCGCCGC CGGCACCACC AGCAGCCCCC CAGCAGCCCC  - 480
CGGGACCCCC GTTGCAGCCT CAGCCTCTGC AGCTTCAGCA GCAGCAGCAG CAACAGCAGC  - 540
AGCAGCCACC GCATCCCCTG TCTCAGCTCG CCCAACTCCA GAGCCAGCCC GTTCATCCCG  - 600
GCCTGCTACA CTCCTCTCCC ACTGCTTTCA GGGCACCCCC TTCGTCCAAC TCCACCGCCA  - 660
TCCTCCACCC TTCCTCCAGG CAAGGCAGCC AGCTCAATCT CAATGACCAC TTGCTTGGCC  - 720
ACTCTCCAAG TTCCACAGCC ACAAGTGGGC CTGGTGGAGG CGGCCGGCAC CGGCAGGCCA  - 780
GCCCCCTGGT GCACCGGCGG GACAGCAACC CCTTCACGGA GATCGCCATG AGCTCCTGCA  - 840
AGTACAGCGG TGGGGTCATG AAGCCCCTCA GCCGCCTCAG CGCCTCCCGG AGGAACCTCA  - 900
TTGAGGCTGA GCCTGAGGGC CAACCCCTCC AGCTCTTCAG CCCCAGCAAC CCCCCCGAGA  - 960
TCATCATCTC CTCCCGGGAG GACAACCATG CCCATCAGAC CCTGCTCCAC CACCCCAACG  - 1020
CCACCCACAA CCACCAGCAT GCCGGCACCA CTGCCAGCAG CACCACCTTC CCCAAAGCCA  - 1080
ACAAGCGGAA AAACCAAAAC ATTGGCTATA AGCTGGGACA CAGGAGGGCC CTGTTTGAAA  - 1140
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CACGTGAAGT CCAGCTTTTC GTGATCGACA ATGGTGCGGA CGACTGGCGG ATAGCCATGA  - 1380
CCTACGAGCG CATCCTCTAC ATCAGCCTGG AGATGCTGGT GTGTGCCATC CACCCCATTC  - 1440
CCGGCGAGTA CAAGTTCTTC TGGACGGCGC GCCTGGCCTT CTCCTACACA CCCTCGCGGG  - 1500
CCGAGGCCGA CGTGGACATC ATCCTGTCCA TCCCGATGTT CCTGCGCCTG TACCTGATCG  - 1560
CCCGCGTCAT GCTGCTGCAC AGCAAGCTCT TCACTGACGC CTCGTCCCGC AGCATCGGGG  - 1620
CCCTCAACAA GATCAACTTC AACACGCGCT TCGTCATGAA GACCCTCATG ACCATTTGCC  - 1680
CCGGCACCGT GCTGCTCGTG TTCAGCATCT CACTGTGGAT CATCGCTGCC TGGACCGTCC  - 1740
GTGTCTGCGA AAGGTACCAT GACCAGCAGG ACGTAACTAG TAACTTTCTG GGTGCCATGT  - 1800
GGCTCATCTC TATCACGTTC CTTTCCATTG GTTATGGGGA TATGGTGCCC CACACATACT  - 1860
GTGGGAAAGG TGTCTGTCTT CTCACGGGCA TCATGGGTGC AGGCTGCACT GCCCTGGTGG  - 1920
TGGCCGTGGT GGCCCGAAAG CTGGAACTCA CCAAAGCAGA GAAACATGTT CACAACTTCA  - 1980
TGATGGACAC TCAGCTGACC AAACGGATCA AGAATGCTGC AGCCAACGTC CTCCGAGAAA  - 2040
CATGGCTAAT CTATAAACAC ACAAAGCTGC TAAAGAAGAT TGACCACGCC AAAGTCCGGA  - 2100
AGCACCAGAG GAAGTTCCTG CAAGCTATCC ACCAACTGAG GAGCGTCAAG ATGGAGCAGA  - 2160
GGAAGCTGAG CGACCAAGCC AACACGCTGG TGGACCTTTC CAAGATGCAG AACGTCATGT  - 2220
ATGATTTAAT CACAGAACTC AATGATCGGA GTGAAGATCT GGAGAAACAA ATCGGCAGCC  - 2280
TGGAGTCTAA GCTGGAGCAT CTCACGGCCA GCTTCAATTC CCTGCCCCTG CTCATCGCGG  - 2340
ACACCCTGCG CCAGCAGCAG CAGCAGCTCC TGTCTGCCCT GATGGAGGCC CGGGGCGTCA  - 2400
GTGTGGCAGT GGGCACCACC CACACCCCAC TCTCCGACAG CCCGATCGGG GTCAGCTCCA  - 2460
CCTCCTTCCC GACCCCGTAC ACAAGTTCAA GCAGTTGCTA AATAAAACTC CCCACTCCAG  - 2520
AAGCNTTAAA AAAAAAAAAA AAAAAA
```

Figure 6 Detection of SK_{Ca} subunit mRNA in porcine coronary artery endothelium. (A), RT–PCR analysis of GAPDH (G), SK1, SK2 and SK3 expression in samples of endothelium (Endo.) and positive control pig brain prepared with (+) and without (−) reverse transcription. Size markers in base pairs are indicated. In endothelium cDNA, GAPDH ($n=5/5$), SK3 ($n=5/5$) and SK2 ($n=3/5$) were detected but not SK1 ($n=5$). (B), the complete SK3 sequence from porcine coronary artery endothelium was determined by RT–PCR and 5′ and 3′ RACE. Start and stop codons are underlined.

2001b). The effects of substance P were compared with those of the benzimidazolinone, 1-EBIO, an agent which is without effect on vascular smooth muscle but which opens charybdotoxin-sensitive IK_{Ca} channels in vascular endothelial cells (Edwards *et al*., 1999) and also activates apamin-sensitive channels formed by hSK1, rSK2 or rSK3 subunits expressed in *Xenopus* oocytes and mammalian HEK293 cells (Syme *et al*., 2000; Cao *et al*., 2001; Grunnet *et al*., 2001b; Pedarzani *et al*., 2001). Both agents generated robust hyperpolarizations of endothelial cells under control conditions and, as expected, substance P-induced hyperpolarization was fully blocked by charybdotoxin only in combination with apamin. Consistent with the findings of Coleman *et al*. (2001) in guinea-pig submucosal arterioles, the hyperpolarizing effect

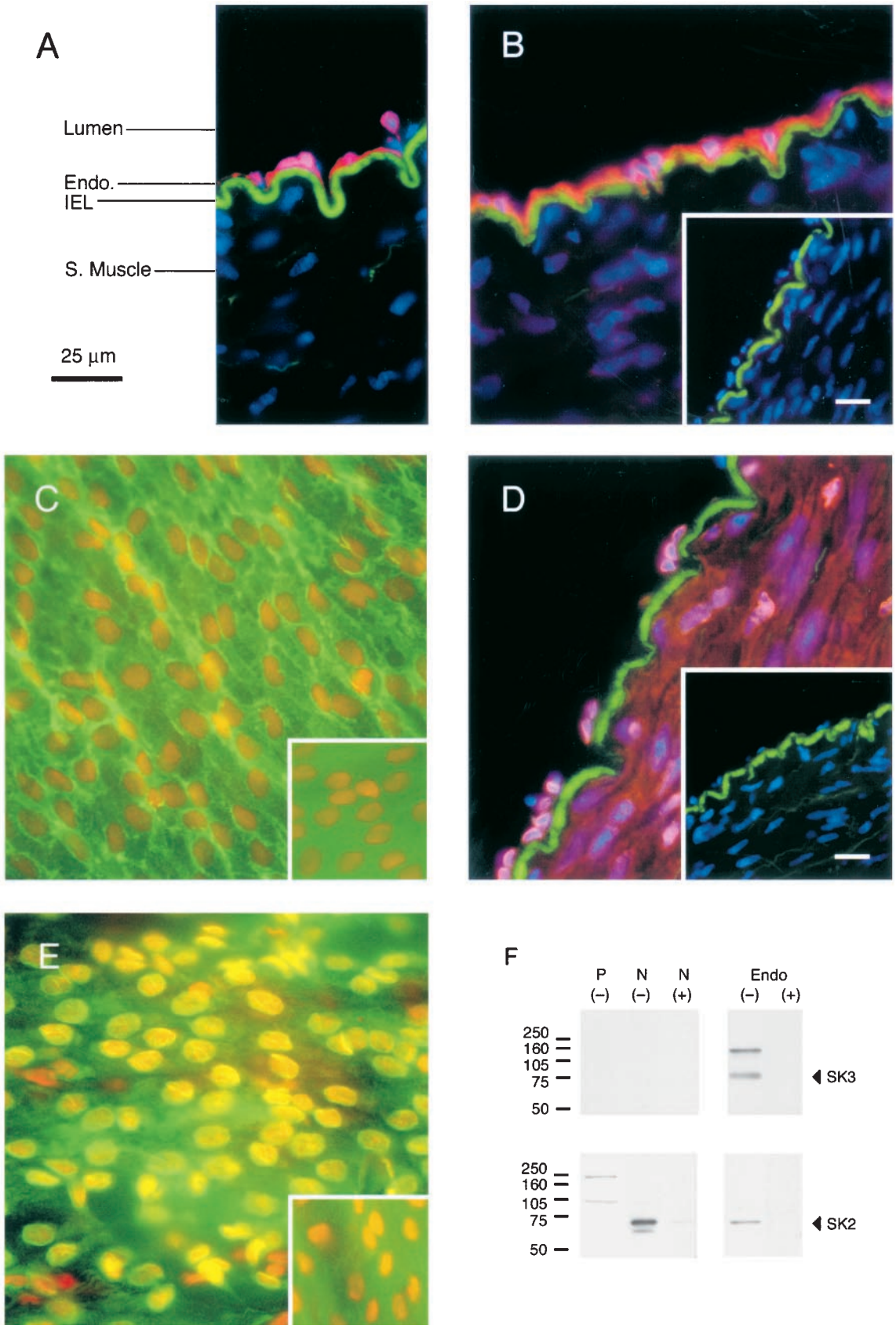

Figure 7 Localization of SK3 and SK2 protein in porcine coronary arteries by immunofluorescence labelling and Western blotting. (A), positive identification by von Willebrand's factor immunoreactivity (red) of the endothelium (Endo.) in a cross-section. Positions of the lumen, internal elastic lamina (IEL, green), smooth muscle (S. Muscle) and nuclei (blue) are shown. (B), cross-section labelled for SK3 (red) and nuclei (blue), with IEL (green) visible (inset, primary antibody pre-incubated with control

of the benzimidazolinone was completely blocked by charybdotoxin alone, indicating that the underlying current was carried by IK_{Ca} only (1-EBIO does not activate BK_{Ca}; Edwards *et al*., 1999) and without activation of SK_{Ca}. The absence of an apamin-sensitive current was unexpected, given that the 600 μM 1-EBIO used in the present study should have been sufficient to activate all SK_{Ca} subunit types, including SK3, when expressed in transfected cells. We suggest that the native SK_{Ca} are subjected to additional modulation and/or subunits that may be absent from heterologous expression systems, as previously proposed (Vergara *et al*., 1998; Clément-Chomienne *et al*., 1999).

Single-channel studies

Single-channel recordings from endothelial cell outside-out patches revealed a K^+ channel with a unitary conductance of 6.8 pS (under physiological, asymmetrical K^+ conditions). The associated current was virtually voltage-insensitive, activated by Ca^{2+}, blocked by apamin and resistant to charybdotoxin: collectively the hallmarks of an SK_{Ca} (Castle, 1999). A 2.7 pS channel, observed in only a small number of membrane patches, was difficult to study because of its small conductance and also because of contamination by currents from other channels in the same patch. This current was inhibited by d-tubocurarine and was observed in the presence of charybdotoxin but not in the presence of apamin. Therefore, this channel most likely represents an additional SK_{Ca}.

The few studies that have examined SK_{Ca} in native endothelial cells have produced results similar to our own. An SK_{Ca} with a unitary conductance of 9 pS was identified in rabbit aortic cells (Sakai, 1990) and channels with conductances of 6.7 and 2.8 pS identified in rat aortic cells (Marchenko & Sage, 1996). The unitary conductances of channels composed of SK1, SK2 or SK3 subunits are similar, approximately 9–10 pS, when the subunits are expressed in oocytes or mammalian cells (Köhler *et al*., 1996; Barfod *et al*., 2001), close to our observed conductance of 6.8 pS (10.1 pS in symmetrical K^+). While it is currently unknown whether native SK_{Ca} are homo- or heteromultimers, it appears that the molecular identity cannot be determined by conductance characteristics alone. Whether Ca^{2+}-activated K^+ channels of 2–3 pS are composed of SK subunits is unknown.

Molecular studies

The SK_{Ca} on native porcine coronary artery endothelial cells was apparently composed of SK3 subunits, with little contribution, if any, from SK1 or SK2 subunits. At the mRNA level, SK2 and SK3 but not SK1 transcripts were detected in samples of endothelium. The fact that SK3 transcripts were detected in all cases while SK1 and SK2 were not suggests that SK mRNA may be relatively unstable and that SK3 mRNA is the more abundant or resistant to degradation. The presence of short-lived SK1 transcripts cannot be totally excluded. In Western blot analysis, SK3 protein was easily detected in samples prepared from the endothelium as opposed to the whole artery. In addition, the anti-SK3 antibody detected a second protein of $\sim$134 kDa and this was also displaced by the control peptide. To aid in identification of this protein, the sequence of the SK3 cDNA detected by RT–PCR was determined in its entirety. This sequence, GenBank accession number AJ251016, was 93% identical in its coding region to the previously-identified human SK3 and therefore represents a porcine homologue with a predicted translation product of 80.4 kDa. Additionally, the predicted translation product contains amino acid sequence identical to the antigenic peptide used in production of the anti-SK3 antibody. Currently, no SK_{Ca} subunits of $\sim$134 kDa have been described, although it is possible that the higher weight band represents a tightly-associated subunit multimer.

In artery sections, the endothelium was positively identified as a single layer of cells which lined the lumen and which possessed immunoreactivity towards von Willebrand's factor. SK3 staining was abundant only in the endothelium and not in the smooth muscle cells. Furthermore, when viewed *en-face*, SK3 fluorescence was observed at the perimeter of each endothelial cell, suggesting a plasmalemmal location which would be consistent with a functional role in mediating endothelial hyperpolarizations. In contrast, SK2 reactivity was found in both endothelium and smooth muscle, and in both situations at an intracellular location surrounding the nuclear staining. To confirm this, whole artery samples for Western blot analysis were fractionated into nuclear and post-nuclear fractions. Strong SK2 immunoreactivity was present in the nuclear but not in the post-nuclear fraction, consistent with an intracellular localization.

Although the specificity of the anti-SK3 antibody has been confirmed in other studies, the specificity of the anti-SK2 antibody has been questioned (Khanna *et al*., 2001; Ro *et al*., 2001). The study of Khanna *et al*. (2001) determined that a high degree of non-specific cross-reactivity was present in Western blot analysis of cells transfected with rat and human SK subunits but not from native tissues. However, our Western blotting data indicate that the protein detected in porcine coronary artery was both the appropriate size for SK2 and detection was prevented by control peptide pre-incubation. We can only suggest that these differences may be due to the species and tissue examined or the expression of high levels of protein in transfected cells.

Substance P-evoked currents and relevance to EDHF

Substance P induced outward currents with a bi-phasic profile – a transient peak followed by steady state current

peptide). (C), arteries opened longitudinally allowing the endothelium to be viewed *en-face* were labelled for SK3 (green) and nuclei (red) (inset, primary antibody omitted). (D), cross-section labelled for SK2 (red) and nuclei (blue), with IEL (green) shown (inset, primary antibody pre-incubated with control peptide). (E), endothelium viewed *en-face* and labelled for SK2 (green) and nuclei (red) (inset, primary antibody omitted). Areas where green SK2 label and red nuclei overlap appear yellow or orange. All scale bars are 25 μm. (F), Western blot analysis of SK3 and SK2 protein expression. Whole artery samples were separated into post-nuclear (P) and nuclear (N) fractions. Additional samples were prepared from endothelium (Endo.) only. Primary antibodies were used with (+) and without (−) control peptide pre-incubation. Molecular weight markers are indicated (kDa). 10 μg protein loadings.

(data not shown, but see Sharma & Davis, 1994; Frieden *et al*., 1999). The steady-state current was partially sensitive to apamin, confirming the activation of an SK_{Ca} in endothelial cells by substance P. The apamin-insensitive residual steady-state current probably reflects activation of other Ca^{2+}-activated K^+ channels, as no substance P-evoked currents were detected in the presence of EGTA, and these will be investigated in future work. It has recently been suggested that substance P and bradykinin, both endothelium-dependent vasodilator agonists, activate separate K^+ conductances in *cultured* porcine coronary artery endothelial cells (Frieden *et al*., 1999). Under these conditions, apamin partially inhibited the substance P-evoked current without effect on the bradykinin-activated current. Iberiotoxin produced the opposite results. Thus, the present study is in agreement in that substance P activates an apamin-sensitive current in porcine coronary artery endothelium, which likely represents the apamin-sensitive component of the EDHF pathway. The inhibition of bradykinin-evoked currents in cultured endothelial cells by iberiotoxin (Frieden *et al*., 1999) may be less relevant to intact vessels, as EDHF responses induced by bradykinin are iberiotoxin-insensitive (Edwards *et al*., 2001b). One possibility is that, in intact arteries, bradykinin does not elevate intracellular Ca^{2+} sufficiently to activate BK_{Ca}, although there is also evidence that BK_{Ca} expression is up-regulated by the process of culturing endothelial cells (Kestler *et al*., 1998; Jow *et al*., 1999). Furthermore, the effects of bradykinin are difficult to interpret because it stimulates an additional hyperpolarizing effect which is independent of EDHF (Edwards *et al*., 2001b).

Conclusions

Endothelial cells of the porcine coronary artery express a plasmalemmal-located, apamin-sensitive SK_{Ca} channel which contains an SK3 subunit. The data do not support a prominent role for SK1 or SK2 subunits. The endothelial SK_{Ca} was activated by substance P and it is these channels which are most likely to mediate the apamin-sensitive component of the EDHF response in this artery.

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