

# Characterization of a charybdotoxin-sensitive intermediate conductance $\text{Ca}^{2+}$ -activated $\text{K}^{+}$ channel in porcine coronary endothelium: relevance to EDHF

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**1** This study characterizes the  $\text{K}^{+}$  channel(s) underlying charybdotoxin-sensitive hyperpolarization of porcine coronary artery endothelium.

**2** Two forms of current-voltage (I/V) relationship were evident in whole-cell patch-clamp recordings of freshly-isolated endothelial cells. In both cell types, iberiotoxin (100 nM) inhibited a current active only at potentials over +50 mV. In the presence of iberiotoxin, charybdotoxin (100 nM) produced a large inhibition in 38% of cells and altered the form of the I/V relationship. In the remaining cells, charybdotoxin also inhibited a current but did not alter the form.

**3** Single-channel, outside-out patch recordings revealed a  $17.1 \pm 0.4$  pS conductance. Pipette solutions containing 100, 250 and 500 nM free  $\text{Ca}^{2+}$  demonstrated that the open probability was increased by  $\text{Ca}^{2+}$ . This channel was blocked by charybdotoxin but not by iberiotoxin or apamin.

**4** Hyperpolarizations of intact endothelium elicited by substance P (100 nM;  $26.1 \pm 0.7$  mV) were reduced by apamin (100 nM;  $17.0 \pm 1.8$  mV) whereas those to 1-ethyl-2-benzimidazolinone (1-EBIO, 600  $\mu\text{M}$ ,  $21.0 \pm 0.3$  mV) were unaffected ( $21.7 \pm 0.8$  mV). Substance P, bradykinin (100 nM) and 1-EBIO evoked charybdotoxin-sensitive, iberiotoxin-insensitive whole-cell perforated-patch currents.

**5** A porcine homologue of the intermediate-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channel (IK1) was identified in endothelial cells.

**6** In conclusion, porcine coronary artery endothelial cells express an intermediate-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channel and the IK1 gene product. This channel is opened by activation of the EDHF pathway and likely mediates the charybdotoxin-sensitive component of the EDHF response. *British Journal of Pharmacology* (2002) **137**, 1346–1354. doi:10.1038/sj.bjp.0705057

**Keywords:** Endothelium; EDHF; hyperpolarization; calcium-activated potassium channels; charybdotoxin; iberiotoxin; apamin; IK1 gene product

**Abbreviations:**  $\text{BK}_{\text{Ca}}$ , large conductance calcium-activated potassium channels; EDHF, endothelium-derived hyperpolarizing factor;  $\text{IK}_{\text{Ca}}$ , intermediate conductance calcium-activated potassium channels;  $\text{SK}_{\text{Ca}}$ , small conductance calcium-activated potassium channels; 1-EBIO, 1-ethyl-2-benzimidazolinone; RACE, rapid amplification of cDNA ends

## Introduction

The vascular endothelium controls vessel tone by releasing nitric oxide (Furchgott & Zawadzki, 1980) and prostacyclin (Moncada & Vane, 1979) as well as by a third pathway which involves hyperpolarization of the vascular smooth muscle (reviewed by Busse *et al.*, 2002). This 'endothelium-dependent hyperpolarizing factor (EDHF)' pathway is inhibited by a combination of the toxins apamin and charybdotoxin, 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). Given the specificities of these toxins (reviewed by Garcia *et al.*, 1991; Castle, 1999),

small- and intermediate-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels ( $\text{SK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$ , respectively) but not large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels ( $\text{BK}_{\text{Ca}}$ ) are implicated in the EDHF pathway.

In the vasculature,  $\text{SK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$  are expressed in endothelial cells (Sakai, 1990; Marchenko & Sage, 1996; Köhler *et al.*, 2000; Burnham *et al.*, 2002) but not in smooth muscle cells with the contractile phenotype while  $\text{BK}_{\text{Ca}}$  are mainly expressed in myocytes (Zygmunt *et al.*, 1997; Neylon *et al.*, 1999; Quignard *et al.*, 2000). Furthermore, the combination of apamin and charybdotoxin blocks EDHF-mediated vasodilatation if selectively applied to the endothelium and inhibits the hyperpolarization of the endothelial cells produced by acetylcholine or bradykinin (Edwards *et al.*, 1998; 2000; Doughty *et al.*, 1999; Ohashi *et al.*, 1999). Finally, the increase in endothelial intracellular calcium concentration, provoked by the agonist, is not inhibited by the two toxins (Ghisal &

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Morel, 2001). Altogether, these experimental results suggest that the hyperpolarization of the endothelial cells is the critical initiating step in the EDHF-mediated responses (Quignard *et al.*, 2000; Edwards *et al.*, 2000; Busse *et al.*, 2002) and that apamin plus charybdotoxin exert their effects at this site (Edwards *et al.*, 1998).

In an earlier study, in porcine coronary artery endothelial cells, an apamin-sensitive  $K^+$  channel which is likely to be involved in the EDHF response was proposed to be an  $SK_{Ca}$  containing the SK3 subunit (Burnham *et al.*, 2002). The purpose of the present study was, in the same cells, to characterize the  $K^+$  channels sensitive to charybdotoxin which are also involved in EDHF-mediated responses.

## 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, gassed with 5%  $CO_2$  in  $O_2$ ). Left anterior descending coronary arteries were dissected free of surrounding tissue while maintained in ice-cold Krebs solution.

### *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  $\mu M$   $N^G$ -nitro-L-arginine and 10  $\mu M$  indomethacin. Endothelial cells were impaled using micro-electrodes filled with 3 M KCl (resistance 40–80 M $\Omega$ ). 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).

### *Patch-clamp experiments*

Endothelial cells were mechanically isolated and placed on cover-slips pretreated with Matrigel<sup>®</sup> basement membrane matrix. This isolation procedure did not allow to determine the side of the endothelial cells which was studied (luminal or abluminal).  $K^+$  currents were measured at room temperature (20–24°C) using whole-cell, 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, and Na-HEPES 10 (pH 7.4). Recording pipettes (3–8 M $\Omega$  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 5 (pH 7.4). EGTA was titrated with  $Ca^{2+}$  to provide solutions containing 100, 200 and 500 nM free  $Ca^{2+}$ . For perforated-patch experiments, whole-cell access was achieved within 2–5 min by the inclusion of nystatin (50–100  $\mu g$   $ml^{-1}$ ) in the pipette solution.

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 unitary conductance. Both methods were compared and provided similar results. Mean current ( $I$ ) and variance ( $\sigma^2$ ) were calculated for the variance analysis. Least squared parabolic fits to the resulting values were used to derive estimates of single channels properties according to the equation  $\sigma^2 = Ii - (I^2/N)$ , where  $N$  is the number of channels in the patch and  $i$  the single channel current amplitude.

### *3' and 5' RACE*

Samples of porcine coronary artery endothelium were obtained by scraping the luminal surface of an artery with a clean scalpel blade. cDNA for 3' and 5' RACE (Rapid Amplification of cDNA Ends) was produced using a GeneRacer kit (Qiagen). Amplification was performed at 56°C with 1.5 mM  $Mg^{2+}$  using the gene-specific, nested primers (forward) 5'-CTGAGAGGC-AGGCTGTCAATGC and 5'-TCACGTTCTGACCATTGGCTA and (reverse, in presence of 10% dimethylsulphoxide) 5'-CGAGTGTGCTTGTAGTACATCCA and 5'-GACTCCTTCATCTCTTTGGCATA. Products were cloned using TOPO-TA cloning kits (Invitrogen) and sequenced in triplicate using Big Dye Terminator kit (PE Applied Biosystems) by the central sequencing facility of the School of Biological Sciences, University of Manchester.

### *Materials*

Apamin, synthetic charybdotoxin and iberiotoxin were obtained from Latoxan, France and Matrigel was obtained from Becton Dickinson (MA, U.S.A.). All other substances were supplied by Sigma, except 1-EBIO (1-ethyl-2-benzimidazolinone; Aldrich), and substance P (RBI).

### *Data analysis*

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

## Results

### *Whole-cell currents and effects of toxins*

Freshly-isolated endothelial cells were studied with the whole-cell configuration of the patch-clamp technique (500 nM free-

$Ca^{2+}$  pipette solution). The voltage steps were in increments of 10 mV (300–500 ms duration) between  $-150$  and  $+150$  mV from a holding potential of  $-60$  mV. The cells displayed two forms of current-voltage (I/V) relationship (Figure 1). One group of cells (62%) exhibited 'linear', voltage-independent whole-cell I/V relationships. The remaining cells exhibited 'non-linear' I/V relationships at positive potentials, with a peak in outward current at  $+50$  mV and another increase at potentials above  $+100$  mV (Figure 1). The reversal potential of the whole-cell current was similar in cells exhibiting linear ( $-68.8 \pm 1.7$  mV;  $n=16$ ) and non-linear ( $-73.4 \pm 2.4$  mV;  $n=10$ ) I/V relationships and both were close to the calculated  $K^+$  equilibrium potential under these conditions ( $-79$  mV for 120 mM  $K^+$  external and 5.4 mM  $K^+$  internal gradient). In the perforated patch configuration, after rupture of the cell membrane, dialysis of the cell with a concentration of 500 nM free calcium increased the net currents. Then, increasing the extracellular potassium concentration from 5 mM to 140 mM significantly increased the outward and inward potassium currents and shifted the reversal potential from  $-78$  mV to 0 mV (data not shown).

The effects of cumulative addition to the bathing solution of the  $Ca^{2+}$ -activated  $K^+$  channel blockers iberiotoxin, charybdotoxin and apamin (100 nM each) were determined on the whole-cell currents described above. Iberiotoxin significantly inhibited currents only at potentials more positive than  $+50$  mV, in both cells with the linear and non-linear I/V relationships while the addition of charybdotoxin inhibited whole-cell currents at all tested potentials

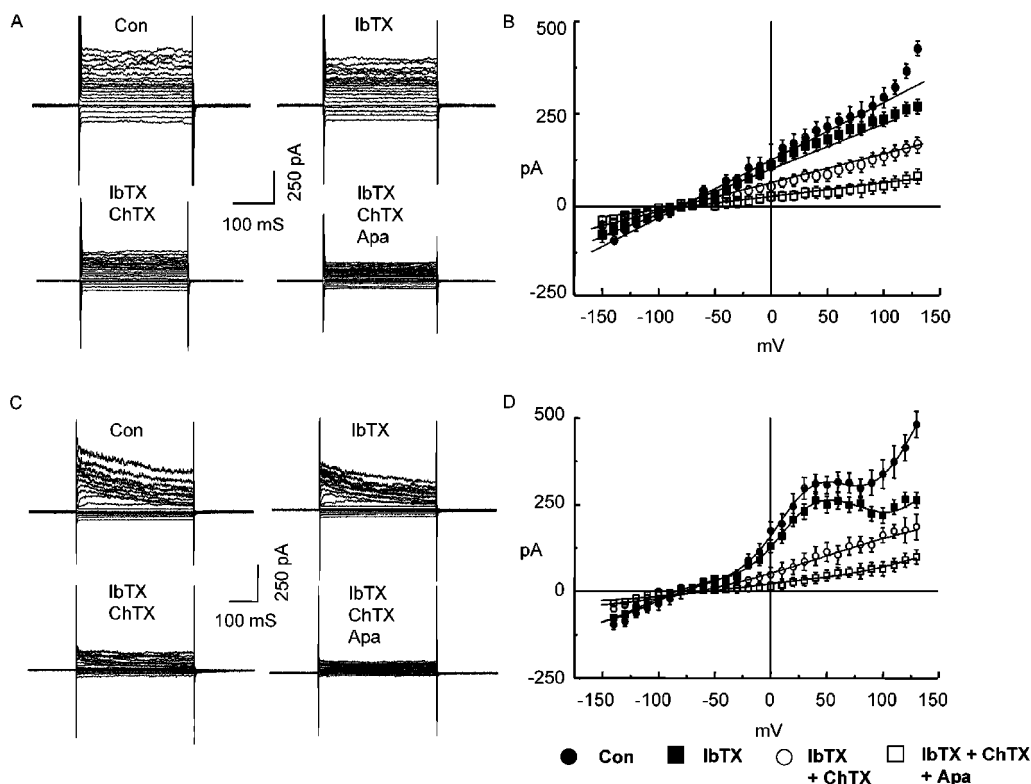
(Figure 1). Furthermore, charybdotoxin abolished the non-linear component of the I/V relationship observed between  $-20$  and  $+80$  mV in that group of cells. The addition of apamin produced a further decrease in whole-cell current in both types of cells (Figure 1).

As shown in Figure 1, the values of the reversal potentials of the currents (either the linear or the voltage-dependent current) is not affected by the presence of iberiotoxin or iberiotoxin+charybdotoxin. Furthermore, the subtracted charybdotoxin-sensitive current showed a similar reversal potential (data not shown).

### Single-channel recordings

Single-channel recordings were obtained from freshly-isolated endothelial cells using the outside-out patch configuration with 250 nM free  $Ca^{2+}$  pipette solution under asymmetric, quasi-physiological  $K^+$  concentrations (120 mM  $K^+$  internal and 5.4 mM  $K^+$  external solutions). In addition to the two  $SK_{Ca}$  that we have previously described (Burnham *et al.*, 2002), a third channel was detected with a unitary conductance of  $17.1 \pm 0.4$  pS ( $n=9$ ) in the range  $-20$  to  $+40$  mV as determined by plotting unitary current amplitude against membrane potential (Figure 2). This 17.1 pS conductance showed weak voltage-sensitivity, with open probability decreasing at potentials above  $+50$  mV ( $0.64 \pm 0.03$  at  $+50$  mV and  $0.40 \pm 0.03$  at  $+80$  mV,  $n=5$ ).

The gating effects of  $Ca^{2+}$  and toxins on the 17.1 pS conductance were determined in outside-out patches. With



**Figure 1** Effect of iberiotoxin, charybdotoxin and apamin on 'linear' and 'non-linear' whole-cell  $K^+$  currents in porcine coronary artery endothelial cells. Currents were elicited by 10 mV step pulses between  $-150$  and  $+150$  mV from a holding potential of  $-60$  mV using 500 nM free  $Ca^{2+}$  pipette solution. Cells displayed 'linear' (A and B) and 'non-linear' (C and D) I/V relationships. Representative current traces (A and C) and the mean data (B and D,  $n=9$ ) obtained under control conditions (Con) and after the cumulative application of iberiotoxin (100 nM; IbTX), charybdotoxin (100 nM; ChTX) and apamin (100 nM; Apa).

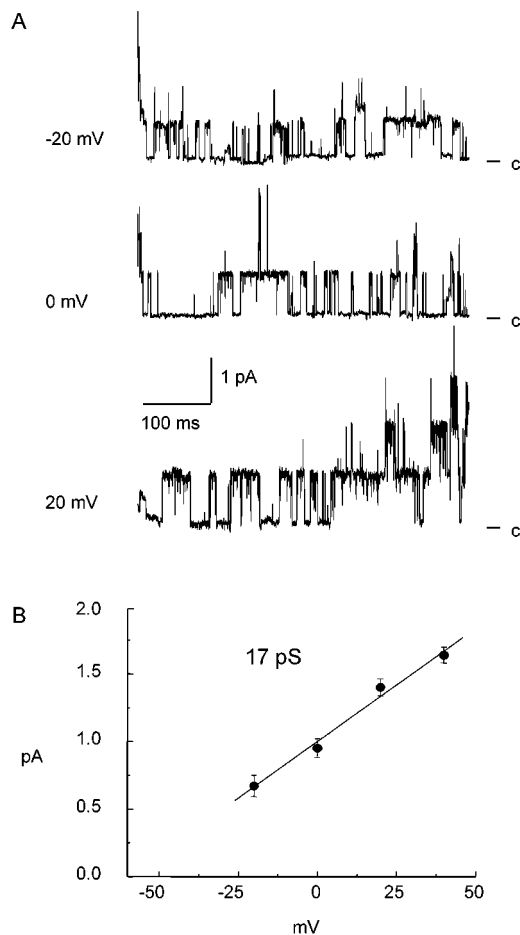
100, 250 or 500 nM free  $Ca^{2+}$  in the pipette solution and at a holding potential of 0 mV, the open probability increased from  $0.17 \pm 0.02$  ( $n=8$ ) to  $0.38 \pm 0.04$  ( $n=8$ ) and to  $0.65 \pm 0.03$  ( $n=7$ ), respectively (Figure 3). With 250 nM free  $Ca^{2+}$  in the pipette solution, addition of iberiotoxin (100 nM) to the bathing solution did not affect the channel open probability ( $0.47 \pm 0.11$  and  $0.44 \pm 0.08$  in its absence and presence, respectively;  $n=7$ ; Figure 3). However, supplementing the bathing solution with charybdotoxin (100 nM) decreased the channel open probability to  $0.14 \pm 0.04$  ( $P < 0.05$ ;  $n=7$ ; Figure 3). The open probability of the channel was not significantly affected by apamin (100 nM) alone ( $0.42 \pm 0.03$  and  $0.44 \pm 0.02$  in its absence and presence, respectively;  $n=6$ ).

In some experiments, either in the whole cell or the outside-out configuration the concentration of  $1 \mu\text{M}$  free  $Ca^{2+}$  was studied. However, under these experimental conditions, within 2 min of the cell membrane rupture,  $Ca^{2+}$  elicited activation of other currents. In the whole cell configuration, these calcium-induced currents had a reversal

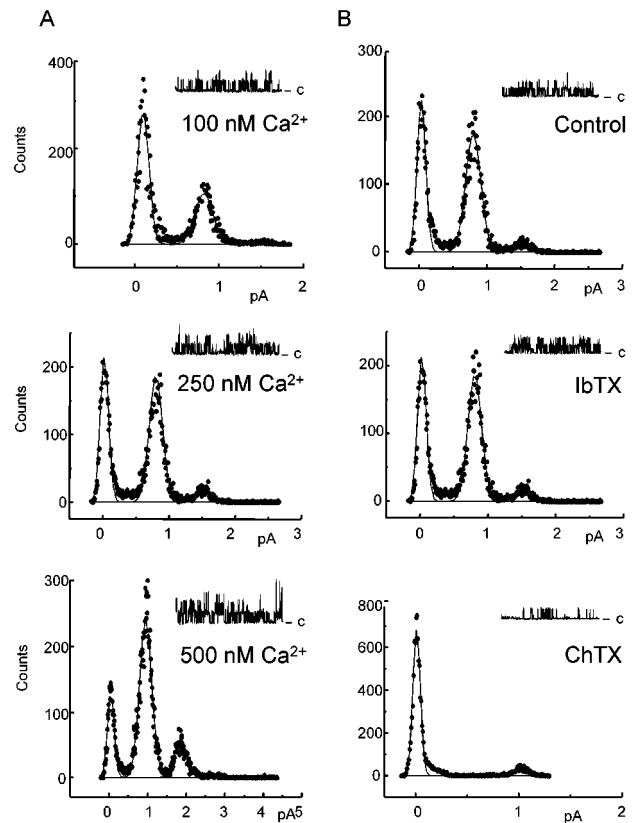
potential which varied between  $-35$  and  $-17$  mV. In outside-out patches, noisy currents with no clear opened and closed states were activated by this elevated calcium concentration.

#### Substance P, bradykinin and 1-EBIO evoked currents

**Microelectrode studies** In a previous study (Burnham *et al.*, 2002), we tested for 1-EBIO-evoked activation of  $SK_{Ca}$  in porcine coronary artery endothelium as a charybdotoxin-resistant hyperpolarization. However, this activation would have been masked if the  $SK_{Ca}$  was partially charybdotoxin-sensitive. Consequently, we examined the apamin-sensitivity of 1-EBIO-evoked hyperpolarization in the present study. Under control conditions, intact porcine coronary artery endothelium impaled with 3 M KCl-filled microelectrodes displayed a membrane potential of  $-49.2 \pm 0.3$  mV ( $n=4$  for all; Figure 4). Under these conditions, substance P (100 nM) and the  $Ca^{2+}$ -sensitive  $K^+$  channel opener 1-EBIO ( $600 \mu\text{M}$ ) produced hyperpolarizations of  $26.1 \pm 0.7$  and  $21.0 \pm 0.3$  mV,



**Figure 2** Single-channel recordings obtained from outside-out patches taken from porcine coronary artery endothelial cells. Activity was recorded at the indicated holding potentials under asymmetrical  $K^+$  gradients with  $Ca^{2+}$  fixed at 250 nM in the pipette solution. Representative traces are shown, with 'c' indicating the closed state (A). Mean unitary currents were plotted against holding potential and fitted with a linear function (B). The slope conductance obtained was  $17.1 \pm 0.4$  pS.



**Figure 3** Effect of  $Ca^{2+}$  and toxins on 17.1 pS channel activity recorded from outside-out patches taken from porcine coronary artery endothelial cells. Distributions of unitary conductance amplitude recorded at 0 mV are shown, together with representative traces (closed state is indicated by 'c'). A Gaussian fit was performed for every single channel experiment, and the amplitude of single channels was determined with the best fit. Then, the mean of the obtained amplitudes was calculated. The effect of  $Ca^{2+}$  was determined using pipette solutions with free  $Ca^{2+}$  fixed at 100, 250 and 500 nM (panel A). The sensitivity of the channel to the cumulative application of iberiotoxin (IbTX, 100 nM) and charybdotoxin (ChTX, 100 nM) was recorded using 250 nM free  $Ca^{2+}$  in the pipette solution (panel B).

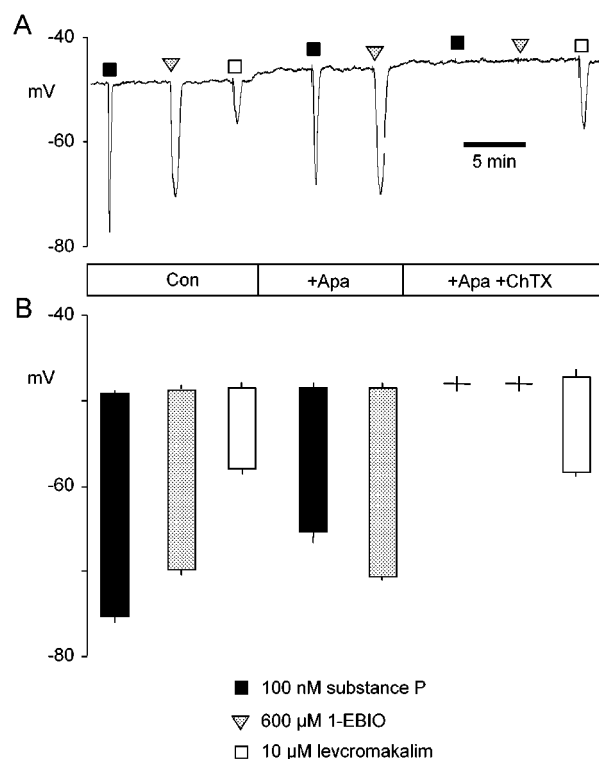
respectively. With apamin (100 nM) present in the bathing solution, the response to substance P was reduced ( $17.0 \pm 1.8$  mV) whereas the 1-EBIO response was unaffected ( $21.7 \pm 0.8$ ). The combination of apamin and charybdotoxin (100 nM each) abolished both responses. The small magnitude of the levcromakalim-evoked control hyperpolarizations ( $\sim 10$  mV, Figure 4) confirmed that the microelectrodes were impaled into endothelial cells, as myocytes produce much larger hyperpolarizations to this agent ( $\sim 30$  mV; Edwards *et al.*, 2001).

**Perforated-patch recordings** Substance P (100 nM) did not affect  $K^+$  currents in endothelial cells dialysed with 10 mM EGTA ( $n=5$ , data not shown). Using the perforated-patch whole-cell configuration at a holding potential of  $-40$  mV (500 nM free  $Ca^{2+}$  in the pipette solution), substance P produced a transient and rapid peak in the net outward current followed by a steady-state activation (Figure 5). This peak current ( $57.9 \pm 8.2$  pA;  $n=5$ ) was insensitive to iberiotoxin (100 nM) but inhibited by charybdotoxin (100 nM;  $15.3 \pm 4.1$  pA;  $n=5$ ). In the steady-state phase,  $K^+$  currents were elicited by 10 mV voltage steps between  $-120$  mV and

$+80$  mV from a holding potential of  $-80$  mV (Figure 5). Substance P increased the outward current at  $+50$  mV by  $152 \pm 8\%$  and this was significantly reduced to  $119 \pm 3\%$  by the presence of charybdotoxin (100 nM;  $n=9$ ).

The effects of bradykinin were qualitatively similar to those of substance P. Voltage steps to  $+50$  mV from a holding potential of  $-80$  mV elicited outward currents that were potentiated by bradykinin. These currents ( $147.5 \pm 8.1\%$  of control;  $n=7$ ) were reduced by the addition of apamin ( $129.1 \pm 5.2\%$ ) and almost abolished by the further addition of charybdotoxin ( $108.3 \pm 3.4\%$ ; Figure 6). Similar protocols were used to assess the effects of 1-EBIO (300  $\mu$ M). Application of 1-EBIO increased outward current by  $164 \pm 8\%$  of control ( $n=7$ ; Figure 6) and this increase was unaffected by iberiotoxin (100 nM;  $157 \pm 6\%$ ). Cumulative addition of apamin (100 nM) produced a small but statistically significant inhibition of this current ( $142 \pm 10\%$ ) while addition of charybdotoxin (100 nM) completely abolished the current ( $81 \pm 10\%$ ).

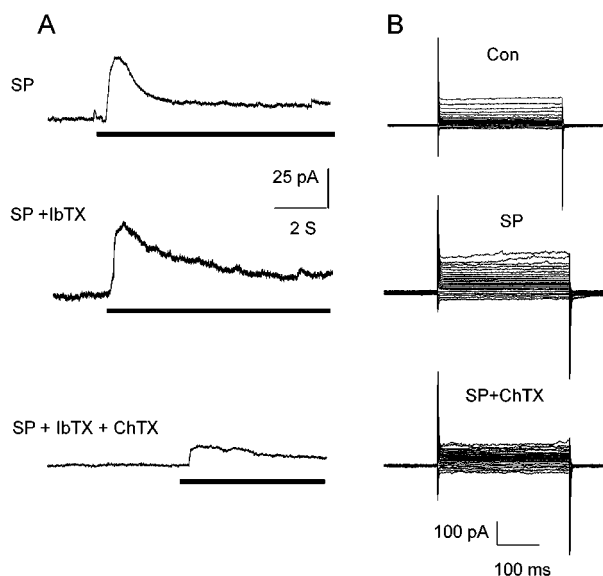
The reversal potentials of the charybdotoxin-sensitive currents activated by 1-EBIO, substance P or bradykinin were included between  $-68$  and  $-75$  mV and therefore very similar to the equilibrium potential for  $K^+$  ions under these experimental conditions.



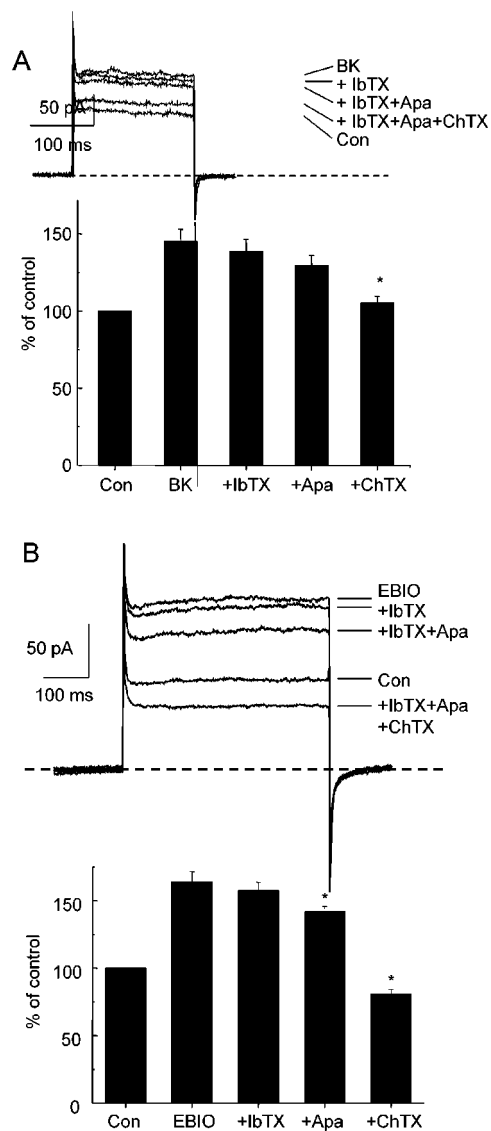
**Figure 4** Effect of apamin and the combination of apamin plus charybdotoxin on substance P- and 1-EBIO-evoked hyperpolarizations of porcine coronary artery endothelium. Substance P and 1-EBIO were added as bolus injections, calculated to give transiently the required final concentrations (100 nM and 600  $\mu$ M, respectively), to a recording chamber perfused with Krebs' solution containing apamin (Apa, 100 nM) and charybdotoxin (ChTX, 100 nM) as indicated. Membrane potential responses were recorded using microelectrodes filled with 3 M KCl. A representative trace (A) and mean data derived from four such experiments (B: columns represent the mean  $\pm$  s.e. mean resting membrane potential immediately before exposure (top of column) and mean  $\pm$  s.e. mean peak hyperpolarization (bottom of column) during exposure to each agent) are shown. Levcromakalim (10  $\mu$ M) was included as an internal control.

### 3' and 5' RACE

Gene-specific primers for 3' and 5' RACE were designed to homologous regions of rat and human  $IK_1$  sequences. Two RACE products were amplified from porcine coronary artery endothelium cDNA and constituted overlapping halves of the



**Figure 5** Characterization of substance P-evoked currents in porcine coronary artery endothelial cells determined using the perforated-patch configuration (holding potential of  $-40$  mV using 500 nM free  $Ca^{2+}$  in the pipette solution). Panel A: Outward currents resulting from the application of substance P (SP, 100 nM, indicated by horizontal bar) to the bathing solution in the presence of iberiotoxin (IbTX, 100 nM) and charybdotoxin (ChTX, 100 nM) are shown. Panel B: During the steady-state phase of the SP-evoked currents, 10 mV voltage steps between  $-120$  mV and  $+80$  mV were applied from a holding potential of  $-80$  mV. The effect of charybdotoxin is shown.



**Figure 6** Characterization of bradykinin and 1-EBIO evoked currents in porcine coronary artery endothelial cells recorded using the perforated-patch configuration.  $K^+$  currents were elicited by voltage steps to +50 mV from a holding potential of -80 mV (500 nM free  $Ca^{2+}$  in the pipette solution) in control conditions (Con), after application of bradykinin (BK, 100 nM) and after application of iberiotoxin (IbTX, 100 nM), apamin (Apa, 100 nM) and charybdotoxin (ChTX, 100 nM) ms shown (panel A, representative traces and mean data are shown,  $n=7$ ). Identical experiments were performed using 1-EBIO (300  $\mu$ M) in place of BK (panel B,  $n=7$ ).

1.97 kb full-length porcine homologue of IK1. This sequence was submitted to GenBank under accession number AY062036. The presence of an in-frame stop codon at position -84 in the 5' untranslated region suggested that the initiator codon was valid although the GenBank EST database contained no porcine sequences for comparison. The deduced amino acid sequence shared 90% identity with human IK1 (Figure 7), displaying 41 amino acid substitutions and a single deletion (alanine-133). Residues surrounding the  $K^+$  selectivity motif (GYG) were highly conserved, with sequence divergence largely restricted to the S3-S4 region and the C terminus.

## Discussion

This study demonstrates the presence of a charybdotoxin-insensitive, iberiotoxin-insensitive  $IK_{Ca}$  in freshly-isolated endothelial cells of the porcine coronary artery. This channel is activated by substance P, bradykinin and 1-EBIO and contributes to the endothelial cell hyperpolarization elicited by these compounds. Together with previously described  $SK_{Ca}$  (Burnham *et al.*, 2002), these channels generate the endothelial hyperpolarization thought to initiate the EDHF pathway in porcine coronary arteries and likely represent the site of action for the characteristic blockade of this pathway by charybdotoxin and apamin.

### *Porcine coronary artery endothelial cells express $IK_{Ca}/IK1$*

Whole-cell currents were dominated by  $K^+$  conductances, with reversal potentials close to the calculated  $K^+$  equilibrium potential. In the presence of the  $BK_{Ca}$ -specific inhibitor iberiotoxin, charybdotoxin blocked a component of the current activated by 500 nM free  $Ca^{2+}$  pipette solution, indicating that native porcine coronary artery endothelial cells express a charybdotoxin-sensitive  $K^+$  channel. Single-channel recordings from outside-out patches confirmed the existence of a channel with 17.1 pS unitary conductance under quasi-physiological  $K^+$  gradients. This conductance was stimulated by internal  $Ca^{2+}$  and was blocked by external charybdotoxin but not iberiotoxin or apamin. In addition, we detected and sequenced the mRNA for an  $IK_{Ca}$  in samples taken from intact porcine coronary artery endothelium.

Few studies of  $Ca^{2+}$ -activated  $K^+$  channels have been performed in native endothelial cells, although these channels are often reported in cultured endothelial cells. Sakai (1990) reported a 9 pS  $Ca^{2+}$ -activated  $K^+$  channel in native rabbit aortic endothelial cells. Sharma & Davis (1994) detected a  $Ca^{2+}$ -activated, voltage-insensitive, inwardly-rectifying  $K^+$  conductance of 11–25 pS in porcine coronary artery endothelial cells that had been cultured only overnight. Although the toxin-sensitivity was not determined in these studies, these properties are representative of an  $SK_{Ca}$  or  $IK_{Ca}$  (typically 5–20 pS and 20–60 pS, respectively; Castle, 1999). Marchenko & Sage (1996) observed a charybdotoxin-sensitive  $Ca^{2+}$ -activated  $K^+$  channel in rat aortic endothelium with a conductance of 6.7 pS under quasi-physiological and 18 pS in symmetric  $K^+$  solutions.

Several authors have reported a molecular identity for  $IK_{Ca}$ , at least in pancreas, foetal brain and T lymphocytes (Ishii *et al.*, 1997; Joiner *et al.*, 1997; Logsdon *et al.*, 1997). Expression of the IK1 (also known as SK4; see Joiner *et al.*, 1997) sequence in *Xenopus* oocytes and mammalian cells resulted in charybdotoxin-sensitive, iberiotoxin- and apamin-insensitive 12–42 pS  $Ca^{2+}$ -activated  $K^+$  channels: the symmetry of the  $K^+$  gradient and inward-rectifier properties of the channel affecting conductance. To date, no other charybdotoxin-sensitive, iberiotoxin-insensitive  $Ca^{2+}$ -activated  $K^+$  channels have been cloned and therefore the porcine homologue of IK1 sequenced in the present study likely represents the molecular identity of the 17.1 pS channel. The porcine IK1 is 90% identical at the amino acid level to the human form, homology comparable to that shown by mouse and human sequences (Vandorpe *et al.*,



charybdotoxin-sensitive EDHF pathway (Edwards *et al.*, 2001).

1-EBIO is an activator of  $SK_{Ca}$  and  $IK_{Ca}$  (Devor *et al.*, 1996; Grunnet *et al.*, 2001) thought to modulate the interaction between the channel and its intrinsically-bound calmodulin  $Ca^{2+}$  sensor (Pedarzani *et al.*, 2001).  $BK_{Ca}$ , which is not gated by calmodulin, is insensitive to this compound and in the vasculature,  $K^{+}$  channels opened by 1-EBIO are limited to the endothelium (Edwards *et al.*, 1999; Walker *et al.*, 2001). In a previous study (Burnham *et al.*, 2002), we showed that the hyperpolarization of porcine coronary artery endothelium evoked by 1-EBIO was entirely blocked by charybdotoxin alone, despite recombinant channel studies suggesting that  $SK_{Ca}$  should also be opened. In the present study, it was hypothesized that an  $SK_{Ca}$  could have been activated by 1-EBIO but, if it displayed some sensitivity to charybdotoxin, this effect would have been masked. Consequently, we tested whether application of apamin would reveal an activation of  $SK_{Ca}$  by 1-EBIO. Although the hyperpolarization to substance P recorded using conventional microelectrodes was reduced by apamin alone, the response to 1-EBIO was unaffected. Furthermore, our single-channel analysis of the apamin-sensitive  $SK_{Ca}$  and charybdotoxin-sensitive  $IK_{Ca}$  of porcine coronary artery endothelium indicated a lack of 'cross-reactivity' to the other toxin (present study; Burnham *et al.*, 2002). Although 1-EBIO-evoked whole-cell currents were, in addition to being charybdotoxin-sensitive, slightly inhibited by apamin, this may represent the inhibition of a basal  $SK_{Ca}$  current due to the presence of  $Ca^{2+}$  in the pipette solution.

In cultured porcine coronary artery endothelial cells, a recent study by Sollini *et al.* (2002) reported the presence of

an apamin-insensitive  $Ca^{2+}$ -activated  $K^{+}$  channel. This channel was activated by both substance P and bradykinin, similar to the channel described in the present study. However, despite the fact that this channel in cultured endothelial cells was inhibited by charybdotoxin and resistant to apamin (both hallmark pharmacological features of  $IK_{Ca}$ ) and activated by 1-EBIO, Sollini *et al.* (2002) designated the channel as an  $SK_{Ca}$  ( $SK_{ap-ins}$ ), largely on the basis of its relatively low conductance (10.7 pS in asymmetrical  $K^{+}$  conditions; 30.9 pS in symmetrical  $K^{+}$ ). Thus,  $SK_{ap-ins}$  shares all the characteristics of the  $IK_{Ca}$  reported here, except that its conductance is low for an  $IK_{Ca}$  (typically 20–60 pS). The present investigation has clearly shown that freshly-isolated porcine coronary artery endothelial cells expresses the porcine homologue of IK1. We thus favour the view that an  $IK_{Ca}$  is present on these endothelial cells and that this is the correct designation of the charybdotoxin-sensitive, apamin-insensitive channel involved in the EDHF pathway in the porcine coronary artery.

In summary, the present study has shown that native porcine coronary artery endothelial cells express an  $IK_{Ca}$  and the IK1 gene product. This channel is opened by vasodilators such as substance P and bradykinin, which activate the EDHF pathway. Inhibition of the EDHF pathway by charybdotoxin and apamin is due to their blockade of  $IK_{Ca}$  and  $SK_{Ca}$  located on the endothelial rather than on smooth muscle cells. The presence of  $IK_{Ca}$  and  $SK_{Ca}$  on endothelial cells is associated with expression of IK1 and SK (particularly SK3) gene products (present study, Köhler *et al.*, 2001; Burnham *et al.*, 2002) and these are likely to be the channels which generate the endothelial hyperpolarization that underlies the EDHF response.

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