Characterization of ion channels in human coronary artery endothelial cells

Katesirin Ruamyod, Wattana B. Watanapa*, Chairat Shayakul***

Abstract

Endothelial ion channels play important roles in regulating coronary vascular tone by modulating endothelial intracellular Ca^{2+} concentration, which in turn controls the production and release of vasoactive substances. These transport molecules either provide direct Ca^{2+} influx pathway through Ca^{2+}-permeable non-selective cation channels, or influence Ca^{2+} electrochemical driving force via alterations in the K^+ and Cl^- conductance. We aimed to characterize the type and contribution of ionic currents in human coronary artery endothelial cells (HCAECs), using whole-cell patch clamp technique. Average peak whole-cell current amplitude of HCAECs at +60 mV was 8.07 ± 0.31 pA/pF (n = 336). The percentages of total currents blocked in 10 μM La^{3+}, 250 μM DIDS, 1 nM apamin, and 10 μM clotrimazole, were 36.11 ± 1.42% (n = 8), 20.34 ± 2.81% (n = 8), 15.51 ± 1.92% (n = 6), and 19.82 ± 2.02% (n = 10) at +60 mV, suggesting the fractions contributed by non-selective cation, Cl^-, small-conductance Ca^{2+}-sensitive K^+ (SK_{Ca}), and intermediate-conductance Ca^{2+}-sensitive K^+ (IK_{Ca}) channels, respectively ($P < 0.05$). In addition, 1 mM TEA and 100 nM iberiotoxin could suppress 16.51 ± 5.35% (n = 6) and 16.02 ± 3.99% (n = 5) of the control currents at +60 mV, indicating that the fraction responsible by large-conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channel was about 16%. Thus, HCAEC currents at +60 mV were mostly (≥ 50%) made up of K^+ currents, which included, at least, BK_{Ca}, IK_{Ca}, and SK_{Ca} currents. The rest of the currents passed through NSC and Cl^- channels. Finally, 100 μM Ba^{2+}, a specific blocker of inward rectifier potassium (K_{ir}) channel, inhibited 37.06 ± 4.59% (n = 6) at -100 mV. These observations could be a basis for further investigation on the role of endothelial ion channels in coronary vascular physiology in human.

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Introduction

Human coronary artery endothelial cells (HCAECs) are pivotal in the maintenance of coronary vascular tone. They exert significant autocrine and paracrine actions on the underlying coronary vascular smooth muscle cells, which in turn control coronary blood flow. Dysfunctional or injured coronary endothelial cells are associated with atherosclerotic changes and coronary artery disease.[1-3]

Studies in other endothelial cells, most notably those of human umbilical veins, have found various types of ion channels. Non-selective cation (NSC) channels, which mostly consist of transient receptor potential (TRP) channels,[4] are the principal influx routes for Ca^{2+} and other cations.[5] K^+ channels help determine resting membrane potential, control nitric oxide release, and mediate actions of vasoactive substances, such as endothelium-derived hyper-polarizing factors.[4,6] K^+ channels in endothelial cells are subdivided according to the structure and function into three main groups: Ca^{2+}-activated (K_{Ca}), inward rectifier (K_{ir}), and ATP-sensitive (K_{ATP}) K^+ channels.[4] Finally, Cl^- channels are important for the regulation of cell volume, intracellular pH and membrane potential in endothelial cells.[4]

Only a few reports have studied HCAEC ion channels electrophysiologically, despite their involvement in coronary vascular regulation and potential therapeutic significance. In one study, K_{ir} and NSC currents were reported;[7] in another, outwardly rectifying Cl^- current was described.[8] Two more recent studies focused on K_{Ca} currents in this cell type.[9,10] Data from a quantitative RT-PCR study showed that HCAEC expressed K^+, Cl^- and transient receptor potential (TRP) channel subunits.[11] Therefore, to assess the functional types and relative contributions of ion channels in HCAECs, we studied their ionic currents by employing whole-cell patch clamp technique and various ion channel blockers.

Materials and Methods

Cell culture

Commercially available HCAECs (tested positive for von Willebrand factor, factor VIII, and acetylated LDL uptake, and negative for smooth muscle α-actin; Lonza, Walkersville, MD, USA) were cultured in

*Department of Physiology, Faculty of Medicine Siriraj Hospital;
**Department of Medicine, Faculty of Medicine Siriraj Hospital, Bangkok 10700, Thailand.

Corresponding author: Wattana B. Watanapa, MD, PhD
E-mail: wattana.wat@mahidol.ac.th

conventional culture medium (Lonza) consisting of endothelial cell basal medium, 5% fetal bovine serum, 0.4% bovine brain extract, 0.1% recombinant human epidermal growth factor, 0.1% hydrocortisone, 30 μg/ml gentamycin sulfate, and 15 ng/ml amphotericin-B. When cells were 70-80% confluent, they were subcultured and seeded in culture dishes containing poly-L-lysine-coated cover slips.

Electrophysiology

Whole-cell currents were recorded in response to a ramp protocol, consisting of a 200-ms voltage ramp from -100 to +80 mV (holding potential, -40 mV), using an Axopatch 200B patch-clamp amplifier and a Digidata 1440A analog-to-digital converter (both from Axon Instruments, Foster City, CA, USA). Low resistance glass pipettes used for whole-cell recording, were pulled from borosilicate glass capillaries (1.2 mm outer diameter, 0.69 mm inner diameter) using commercially available pullers (PP-830, Narishige, Tokyo, Japan), followed by fire-polishing with a microforge (MP-830, Narishige). The filled pipette resistance was 2-5 MΩ in the bath solution. The signal was filtered at 2 kHz (low-pass Bessel filter). Pipette and whole-cell capacitance were compensated.

Solutions

The external solution (bath) contained (in mM) 140 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, and 10 HEPES (pH 7.3, adjusted with NaOH; 320-325 mOsmol/kg). The standard pipette (internal) solution contained (in mM): 40 KCl, 100 K-aspartate, 1 $MgCl_2$, 4 $CaCl_2$, 5 HEPES, 7 EGTA, and 3 MgATP (pH 7.2, adjusted with KOH; 290-300 mOsmol/kg). The free Ca^{2+} concentration of the internal solution was calculated to be 245 nM (Patton C. CaMgATPEGTA Program version 1.0, using constants from NIST database. http://maxchelator.stanford.edu).

Chemicals and reagents

Tetraethylammonium (TEA), 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid, (DIDS), Ba^{2+}, iberotoxin and La^{3+} were dissolved in deionized water. Clotrimazole was dissolved in DMSO; final DMSO concentrations did not exceed 0.1%. Apamin was dissolved in l% acetic acid and the final solution was pH-adjusted before using in an experiment. All chemicals were obtained from Sigma Chemicals (St Louis, MO, USA) except apamin (from Calbiochem, San Diego, CA, USA).

Experimental procedure

Ion channel types were identified by using specific ion channel blockers and, if possible, the characteristic current-voltage relation. Membrane currents were first recorded in a control external solution, then after exposure to a channel blocker (dissolved in identical external solution), and finally on returning to the control external solution for washout currents and for testing repeatability. Blockers used were 100 μM Ba^{2+} (a K_{ir} channel blocker),[12] 1 mM TEA or 100 nM iberotoxin (large-conductance K_{Ca} or BK_{Ca} channel blockers),[13] 10 μM clotrimazole (an intermediate-conductance K_{Ca} or IK_{Ca} channel blocker),[14] 100 nM apamin (a small-conductance K_{Ca} or SK_{Ca} blocker),[15] 250 μM DIDS (a Cl^- channel blocker),[16] and 10 μM La^{3+} (a NSC channel blocker).[17]

Data Analysis

All data were initially analyzed with pClamp 10.0 program. Subsequent simple calculations were done in Microsoft® Excel. Membrane potentials were corrected off-line for liquid junction potentials. Plotting of current density-voltage (I-V) curves and bar graphs, as well as statistical analyses, were carried out with GraphPad PRISM 5 (GraphPad Software, San Diego, CA, USA). Data from a cell were discarded if the seal resistance was less than 1 GΩ, the maximum voltage error was more than 3 mV, or the cell current amplitude at +60 mV was less than 20 pA. Currents were normalized with the cell capacitance, yielding current density in pA/pF. Results were expressed as mean ± SEM. Data from blocker experiments were expressed as % inhibited and tested for normality using Kolmogorov-Smirnov test. Since all blocker data were normally distributed, one-sample *t* test was used for testing the statistical significance of the inhibition; $P < 0.05$ was considered significant.

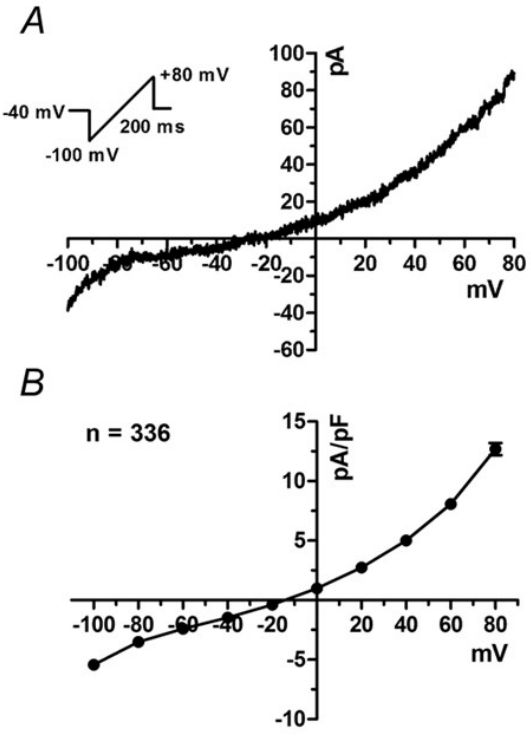

Figure 1 HCAEC membrane currents, elicited by 200 ms ramp protocol between -100 and +80 mV from a holding potential of -40 mV (inset). A) A current from a representative cell; the x axis shows corresponding voltages. B) Average I-V relationship from 336 cells. Error bars are SEM.

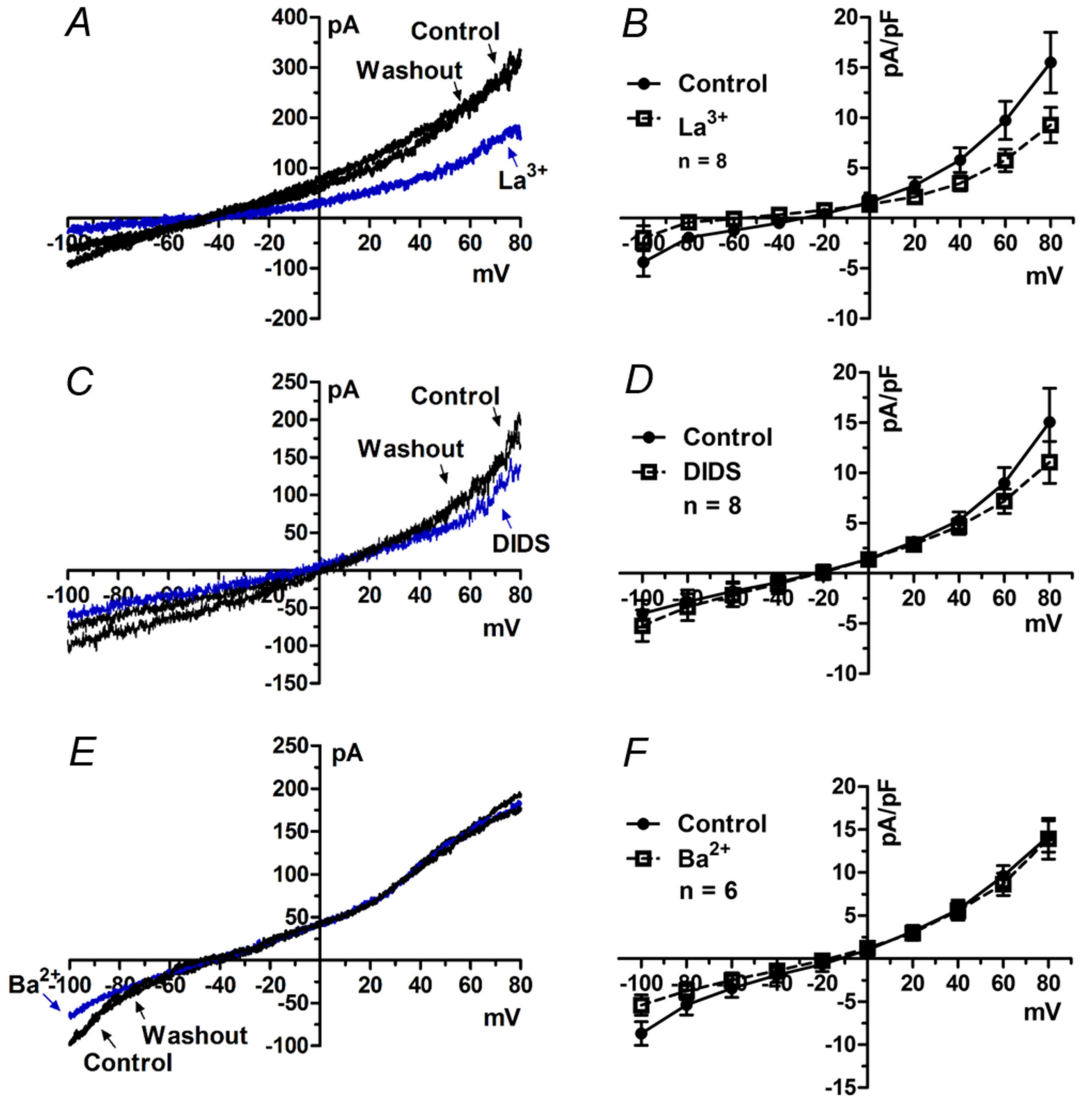

Figure 2 HCAEC currents in La^{3+}, DIDS or Ba^{2+}. Left panels show representative ramp currents in control external solution (Control), in 10 μM La^{3+} (A), 250 μM DIDS (C), or 100 μM Ba^{2+} (E), and on return to control external solution (Washout). Right panels (B, D or F) are corresponding average I-V curves; washout currents were not obtained for all cells and therefore the average were not shown. Error bars are SEM; n = number of cells.

Results

HCAEC whole-cell capacitance and currents

The average whole-cell capacitance of HCAECs was 12.38 ± 0.31 pF (n = 336). Typical membrane currents in response to the ramp protocol are shown in Figure 1. Currents from HCAECs displayed slightly non-linear current-voltage relationship at both negative and positive potentials. The average current magnitude at +60 mV and -100 mV were 87.59 ± 3.07 and -60.75 ± 2.55 pA, respectively, and average current density at +60 mV and -100 mV were 8.07 ± 0.31 and -5.43 ± 0.21 pA/pF, respectively. In our experiments, the average series resistance was 9.06 ± 0.14 MΩ, yielding an average maximum voltage error of 0.79 ± 0.03 mV.

Identification of ion channel types in HCAECs

In the presence of 10 μM La^{3+}, a non-specific blocker of NSC channel, in the external solution, the total currents at +60 mV were significantly and reversibly inhibited by 36.11 ± 1.42% (n = 8; P = < 0.0001). Typical current tracings from an experiment and average I-V curves from eight cells are shown in Figures 2A and 2B, respectively. When 250 μM DIDS, a non-specific blocker of Cl^- channel, was included externally to test the contribution of Cl^- currents, the whole-cell currents at +60 mV were also significantly and reversibly inhibited by 20.34 ± 2.81% (n = 8; P = 0.0002). Representative current tracings and average I-V curves are shown in Figures 2C and 2D, respectively. Figures 2E and 2F show typical currents from a cell and average I-V curves in experiments with 100 μM Ba^{2+}, a specific blocker of K_{ir} channel. As expected, 100 μM Ba^{2+} mainly suppressed the inward currents. The Ba^{2+}-sensitive component at -100 mV was 37.06 ± 4.59% (n = 6; P = 0.0005).

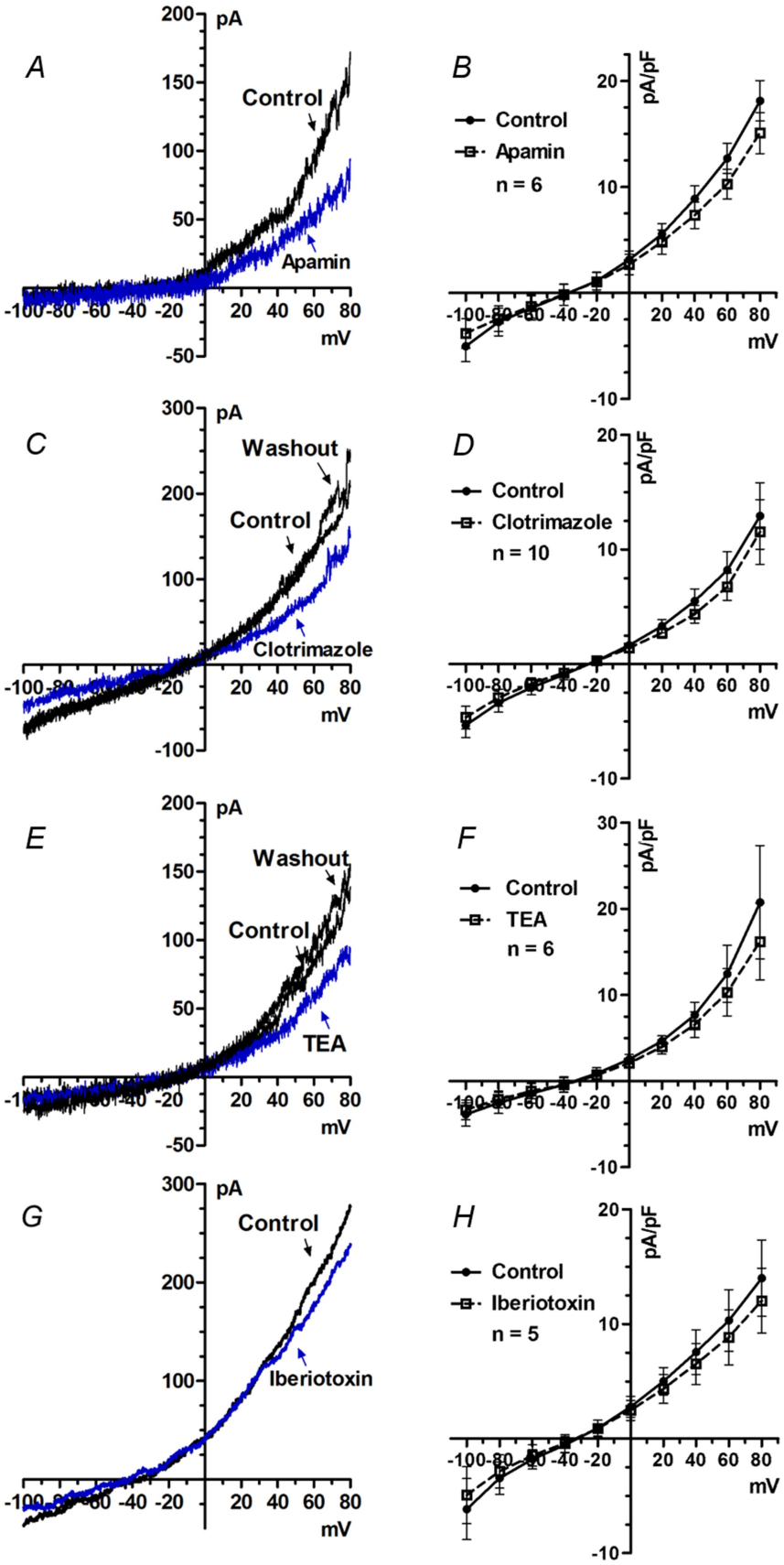

Figure 3 HCAEC currents in apamin, clotrimazole, TEA or iberiotoxin. Left panels show representative ramp currents in control external solution (Control), compared with that in 100 nM apamin (A), 10 μM clotrimazole (C), 1 mM TEA (E), or 100 nM iberiotoxin (G). Washout currents could be obtained only after clotrimazole and TEA. Right panels (B, D, F or H) are corresponding average I-V curves; in F, washout currents were not obtained for all cells and therefore the average were not shown. Error bars are SEM; n = number of cells.

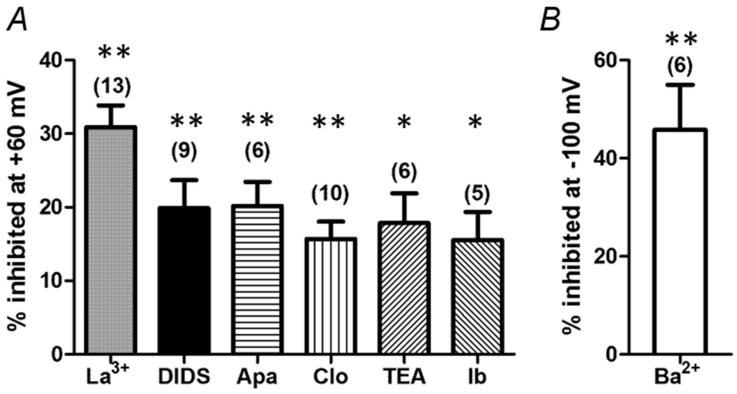

Figure 4 A) Bar graphs showing percentages of whole-cell HCAEC currents inhibited in the presence of 10 μM La^{3+}, 250 μM DIDS, 100 nM apamin, 10 μM clotrimazole, 1 mM TEA or 100 nM iberiotoxin externally (at +60 mV). B) Bar graphs showing the percentage of currents inhibited during exposure to 100 μM Ba^{2+} externally (at -100 mV). Error bars are SEM. **P* < 0.05; ***P* < 0.01; Apa, apamin; Clo, clotrimazole; Ib, iberiotoxin.

Figure 3 summarizes results from experiments to define the contribution of each K_{Ca} subtype. Experiments with external 100 nM apamin, a specific blocker of SK_{Ca} channel, demonstrated that the total currents at +60 mV were significantly reduced by 15.51 ± 1.92% (n = 6; *P* = 0.0005; washout current could not be obtained in these experiments; Figure 3A-B). With external 100 μM clotrimazole, a specific blocker of IK_{Ca} channel, clotrimazole-sensitive current at +60 mV was shown to be 19.82 ± 2.02% of control, on average (n = 10; *P* < 0.0001; reversible; Figure 3C-D). For BK_{Ca} channel, 1 mM TEA or 100 nM iberiotoxin (both specific for BK_{Ca}, ref. 15) applied externally could similarly block the HCAEC whole-cell currents by 16.51 ± 5.35% and 16.02 ± 3.99% (n = 6, *P* = 0.0273 and n = 5, *P* = 0.0160, respectively; Figure 3E-H). Finally, all results of blocker experiments were summarized as bar graphs in Figure 4.

Discussion

Our findings demonstrated the existence of K^+, NSC and Cl^-, in HCAECs. At +60 mV, K^+ currents appeared to collectively contribute around 50% of the total currents. The presence of four types of K^+ currents, i.e. K_{ir}, SK_{Ca}, IK_{Ca}, and BK_{Ca}, are consistent with those reported previously in coronary artery endothelium of porcine, guinea-pig and human.[7,15,18-20] However, a report did not find BK_{Ca} channel in bovine coronary endothelium.[21] It is therefore possible that BK_{Ca} channel expression could be species specific. As shown in this study, both 100 nM iberotixin and 1 mM TEA, a specific and a nonspecific BK_{Ca} channel inhibitors, respectively, could inhibit total currents to the same extent, thus confirming that BK_{Ca} channel is expressed in HCAECs, as in other human endothelial cells.[13] We used 100 μM Ba^{2+}, a high affinity blocker for K_{ir} channel,[20] to characterize K_{ir} currents in HCAECs. The finding that 100 μM Ba^{2+} could inhibit inward current more than outward current was consistent with the inward-rectifying property of K_{ir} channels. Other studies demonstrated the presence of K_{ir} current in guinea-pig or human coronary artery endothelial cells.[7,20]

Nonselective cation (NSC) currents in this study were identified by 10 μM La^{3+}, which is known to block most TRP channels.[22] We found that La^{3+}-sensitive currents comprised about 36% of total currents. This NSC fraction when added to those ascribable to Cl^- and K^+ channels (about 20% and 50%, respectively) equaled approximately 100%, suggesting that most NSC channels in HCAECs are La^{3+}-sensitive and could be attributed to TRP channels. This is in agreement with a recent quantitative RT-PCR study in cultured HCAECs, which demonstrated that several types of TRP channels were expressed in these cells.[11] Although most TRP channels are blocked by La^{3+}, it has been reported that TRPC4 and TRPM2 channels are not inhibited by this trivalent ion.[23,24] However TRPC4 and TRPM2 mRNAs were found to be expressed at relatively low levels in HCAECs,[11] and therefore may contribute little to HCAEC currents.

Among the three main components of HCAEC currents studied, Cl^- current contributed the least, only around 20%. In our study, 250 μM DIDS, a nonspecific Cl^- channel inhibitor, was used to identify Cl^- currents. Other electrophysiological studies in human umbilical vein and pulmonary artery endothelial cells found that Cl^- currents were completely inhibited by 250 μM DIDS (IC_{50} = 7-50 μM).[16,25,26] Cl^- channel subunits found in HCEACs were ClC-3, ClC-4 and ClC-7, which are voltage-gated and Ca^{2+}-activated Cl^- channels.[11] Studies in human umbilical vein endothelial cells similarly found only a small Cl^- current at normal pH (7.4) and osmolality (300-320 mOsmol/l).[27,28]

It is possible that using the magnitude of blocker inhibition to represent each current may over- or under estimate the fraction contributed by each channel. Specifically, some ion channel blockers used in our experiments could cross-inhibit other channels, albeit involving only small fractions. For example, TEA at 1 mM was used in this study for blocking BK_{Ca} channel (IC50 = 0.4 mM),[29] but this dose has been reported to slightly inhibit IK_{Ca} and SK_{Ca} (8-10%).[30,31] Apamin has a high affinity for SK_{Ca} (IC50 = 1-10 pM)[32] and does not cross-react with BK_{Ca}, but it could minimally inhibit IK_{Ca} (10%).[33] On the other hand, the antifungal clotrimazole is quite selective for

IK_{Ca} (IC_{50} = 70 nM) and has no effect on other K_{Ca} channels, although it could inhibit cytochrome P450 enzymes at submicromolar concentrations.[34]

Furthermore, differential pharmacology exists among SK_{Ca} channel subunits. Oocyte-expressed KCa2.2 and KCa2.3 subunits could be blocked by apamin with high sensitivity (IC_{50} = 60 pM and 2 nM, respectively), while KCa2.1 subunits are not blocked even by 100 nM apamin and have low sensitivity to d-tubocurarine (IC_{50} = 354.3 μM) and TEA (IC_{50} = 14.6 mM).[35,36] When co-expressed, KCa2.1 and KCa2.2 currents have intermediate apamin and d-tubocurarine sensitivity.[36] Among SK_{Ca} channel subunits, KCa2.3 was found to be expressed at higher levels relative to KCa2.1 and KCa2.2 in HCAECs.[11] However, it is currently unknown whether native SK_{Ca} in HCAEC are homo- or hetero-multimers. It is thus uncertain whether and how much apamin-sensitive currents underestimated the contribution of SK_{Ca} current.

The magnitude of K_{ATP} current was not determined in this study, though K_{ATP} channel subunits have also been found in HCAECs.[11,37] In our experiments, 3 mM MgATP was always present in the internal solution to prevent current rundown; this concentration has been shown to sufficiently block all K_{ATP} current in this cell type.[38] Therefore K_{ATP} may contribute little to whole-cell HCEAC currents. This is supported by the fact that the sum of inhibitory percentages for all channel types studied was approximately 100%.

Conclusion

This study demonstrated that HCAEC currents consisted of NSC, Cl^- and K^+ currents. K^+ currents were at least composed of large-, intermediate- and small-conductance Ca^{2+}-activated K^+ (BK_{Ca}, IK_{Ca} and SK_{Ca}) currents and inward rectifier K^+ (K_{ir}) current. In our conditions, K^+ currents collectively contributed the most to the total HCAEC current. These data provide a basis for further electrophysiological studies in human coronary artery endothelial cells.

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Conflict of Interest

None

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