Functional expression of Kir2.x in human aortic endothelial cells: the dominant role of Kir2.2

Yun Fang,1 Gernot Schram,2 Victor G. Romanenko,1 Congzhu Shi,1 Lisa Conti,3 Carol A. Vandenbeng,3 Peter F. Davies,1 Stanley Nattel,2 and Irena Levitan1

1Institute for Medicine and Engineering and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; 2Department of Medicine, Montreal Heart Institute, and University of Montreal, Montreal, Quebec, Canada; 3Department of Molecular, Cellular and Developmental Biology and Neuroscience Research Institute, University of California, Santa Barbara, California

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VASCULAR ENDOTHELIAL CELLS constitute the inner lining of blood vessels and are actively involved in the regulation of vascular tone. K⁺ channels set the negative resting membrane potential of endothelial cells (ECs), providing the driving force for Ca²⁺ influx and regulating Ca²⁺-dependent intracellular signaling (29). Clamping the membrane potential at a depolarized value (12, 31) provides evidence suggesting that only Kir2.1 and Kir2.2 contribute significantly to native endothelial Iₑ (15). Furthermore, our studies suggest that Kir2.2 provides the dominant conductance in these cells. potassium channels; inward rectifier potassium channel

MATERIALS AND METHODS

Cell culture. HAECs were purchased at passage 2 from BioWhittaker Cambrex (Rutherford, NJ) and maintained between passages 3 and 5 in 2% fetal bovine serum endothelium growth medium 2 (EGM-2; Cambrex). Porcine aortic endothelial cells (PAECs) were freshly harvested from adult pig aortas (juvenile Yorkshire females) as previously described (32). Briefly, ECs were gently scraped from a 2-cm² region located at the inner wall of the descending thoracic aorta and transferred directly to EC culture medium EGM-2. Cell purity staining was observed, indicating the absence of contaminating smooth muscle cells. Institutional approval was obtained for this study, and animals were treated according to US Department of Agriculture and institutional guidelines.

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Electrophysiology. Ionic currents were measured using the whole cell and cell-attached configurations of the standard patch-clamp technique. Pipettes were pulled (SG10 glass; Richard-Link, Plainfield, NJ) to produce a final resistance of 3–5 MΩ and generated high-resistance seals without fire polishing. A saturated salt agar bridge was used as a reference electrode. Currents were recorded with an EPC9 amplifier (HEKA Electronic, Lambrecht, Germany) and accompanying acquisition and analysis software (Pulse and PulseFit; HEKA Electronic) running on a PowerCenter 150 (MacOS) computer. Pipette and whole cell capacitance was automatically compensated. Whole cell capacitance and series resistance were compensated and monitored throughout each recording. Whole cell current was recorded during 500-ms linear voltage ramps or a series of voltage steps from −160 to −110 mV to +60 mV at an interpulse interval of 5 s. The standard external solution contained (in mM) 150 NaCl, 6 KCl, 10 HEPES, 1.5 CaCl2, 1 MgCl2, and 1 EGTA, pH 7.3. In some experiments, 156, 96, or 60 mM extracellular KCl was used with equimolar substitution of KCl for NaCl to maintain osmolarity. The pipette contained (in mM) 145 KCl, 10 HEPES, 1 MgCl2, 1 EGTA, and 4 ATP, pH 7.3. For the cell-attached configuration, single-channel recordings were obtained in 1.6-s sweeps with a 0.1-ms sampling interval and were filtered at 500 Hz. Bath and pipette solutions for single-channel recordings contained (in mM) 156 KCl, 10 HEPES, 1.5 CaCl2, 1 MgCl2, and 1 EGTA, pH 7.3. All experiments were performed at room temperature (22–25°C).

RNA isolation and RT-PCR. Total RNA was extracted using the Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA). Highly purified total RNA was treated with DNase I to remove traces of genomic DNA. The integrity and quantity of RNA were evaluated using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano Chips assay kit (Agilent Technologies, Waldbronn, Germany). cDNA was generated using SuperScript II reverse transcription reagents (Invitrogen, Carlsbad, CA) with oligo(dT) primers. PCR primers (Table 1) were designed on the basis of known human Kir2.1 (GenBank accession no. U12507), Kir2.2 (GenBank accession no. AB074970), Kir2.3 (GenBank accession nos. U07364 and U24056), and Kir2.4 (GenBank accession no. AF081466) sequences with oligo primer analysis software (Molecular Biology Insights, Cascade, CO). Primer specificity was confirmed by performing a BLAST search. PCR was performed for 35 cycles consisting of denaturation (98°C), annealing (60°C), and extension (72°C).

Quantitative real-time PCR (QRT-PCR). Quantitative real-time PCR (QRT-PCR) was performed using the FastStart DNA Master SYBR Green I kit and the LightCycler system (Roche Applied Science, Indianapolis, IN). Mg2+ concentration, annealing temperature, and primer concentration were optimized for each gene according to the manufacturer's instructions. To distinguish specific amplifications from nonspecific amplifications, a melting (dissociation) curve for amplicons was generated. Melting curve analysis of Kir2.x amplicons resulted in a single peak, indicating the formation of a single amplicon for each targeted gene and the lack of primer-dimer formation. Standard curves were generated with serial dilutions of known cDNA copy number for each gene to determine the copy number in the experimental sample. The standard curves were log linear for at least four orders of magnitude (10^2–10^6 copies). To measure the relative abundance of Kir2.x mRNA in HAECS, cDNAs were synthesized from 1.5 μg of total RNA in a 20-μl reaction. The cDNA reaction (0.1 μl) was used in 20-μl QRT-PCR reactions under optimized conditions. RNA extraction and cDNA synthesis, followed by QRT-PCR, were performed for all targeted Kir2.x genes in each of four biological samples. Each QRT-PCR measurement was performed in triplicate.

Immunoblotting. For preparation of total membrane (TM) samples, cells were scraped into buffer A, composed of (in mM) 150 NaCl, 20 HEPES, 5 EDTA, pH 7.4, protease inhibitor cocktail (PIC), and 1 μg/ml pepstatin; homogenized in a Dounce tissue grinder; and centrifuged for 10 min at 1,000 g. The pellet was resuspended in buffer A, dounced, and recentrifuged for 10 min at 1,000 g. The combined supernatant was centrifuged for 1 h at 200,000 g (SW40Ti rotor; Beckman). The pellet was resuspended in Laemml buffer and sonicated. Sample protein was measured using a bicinchoninic acid protein assay kit (Bio-Rad). Proteins were resolved with 12% SDS-PAGE at reducing conditions, followed by transfer to polyvinylidene difluoride membranes (Amersham). Channel-specific rabbit anti-peptide antibodies to Kir2.1 (rat amino acids 390–411), Kir2.2 (rat amino acids 390–410), and Kir2.3 (human amino acids 2–19) were prepared and purified by performing affinity chromatography or protein A chromatography as previously described for Kir2.2 (36). The membranes were probed with anti-Kir2.1 (1:1,000 dilution), anti-Kir2.2 (1:250 dilution), and anti-Kir2.3 (1:1,000 dilution), with dilutions optimized by probing Chinese hamster ovary (CHO) cells overexpressing Kir2.x subunits. Kir2.x-specific bands were detected using horseradish peroxidase-conjugated secondary antibodies (The Jackson Laboratory, Bar Harbor, ME). Finally, immunoreactivity was visualized using ECL Plus reagent (Amersham). The specificity of the antibodies was tested by transfecting COS-1 cells with Kir2.x constructs and harvesting the cells 2 days after transfection as previously described (19). Samples were run on 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with affinity- or protein A-purified antibodies (1:250 dilution) to Kir2.1, Kir2.2, Kir2.3, followed by probing with secondary antibodies conjugated to horseradish peroxidase and visualized using SuperSignal West Dura (Pierce).

Table 1. Molecular sequence and expected length of RT-PCR products for the different human Kir2.1, Kir2.2, Kir2.3, and Kir2.4 primers

<table>
<thead>
<tr>
<th>Kir Gene</th>
<th>Primer Sequence</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir2.1</td>
<td>S′-GATCGGTCACCGAATAGCAG-3′</td>
<td>199</td>
</tr>
<tr>
<td>Kir2.1F</td>
<td>S′-GCGTGGTCACCGAATTAGCAG-3′</td>
<td>199</td>
</tr>
<tr>
<td>Kir2.1R</td>
<td>S′-GCGTGGTCACCGAATTAGCAG-3′</td>
<td>199</td>
</tr>
<tr>
<td>Kir2.2</td>
<td>S′-GAGAACCTGCGAACTCATACG-3′</td>
<td>410</td>
</tr>
<tr>
<td>Kir2.2F</td>
<td>S′-GAGAACCTGCGAACTCATACG-3′</td>
<td>410</td>
</tr>
<tr>
<td>Kir2.2R</td>
<td>S′-GAGAACCTGCGAACTCATACG-3′</td>
<td>410</td>
</tr>
<tr>
<td>Kir2.3</td>
<td>S′-GACGCTGACCTTCCTTCGCC-3′</td>
<td>361</td>
</tr>
<tr>
<td>Kir2.3F</td>
<td>S′-GACGCTGACCTTCCTTCGCC-3′</td>
<td>361</td>
</tr>
<tr>
<td>Kir2.3R</td>
<td>S′-GACGCTGACCTTCCTTCGCC-3′</td>
<td>361</td>
</tr>
<tr>
<td>Kir2.4</td>
<td>S′-GCGCTGGACCTTCCTTCGCC-3′</td>
<td>362</td>
</tr>
<tr>
<td>Kir2.4F</td>
<td>S′-GCGCTGGACCTTCCTTCGCC-3′</td>
<td>362</td>
</tr>
<tr>
<td>Kir2.4R</td>
<td>S′-GCGCTGGACCTTCCTTCGCC-3′</td>
<td>362</td>
</tr>
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Kir, inwardly rectifying K+ channel.
(WT) Kir2.x-WT with the respective dnKir2.x-WT in Xenopus oocytes as previously described in detail (40).

**Transfection.** CHO cells and HAECs were transfected with Kir2.x-WT or dnKir2.x constructs using LipofectAMINE (GIBCO-BRL) according to the manufacturer’s instructions. Electrophysiological recording and Western blot analysis were performed 24 h after transfection.

**Data analysis.** Statistical analyses of the data were performed using a standard two-sample Student’s t-test assuming unequal variances of the two data sets. Statistical significance was determined using a two-tailed distribution assumption and was set at 5% (P < 0.05). The time constants of voltage-dependent inactivation were measured by fitting a single exponential function, $V_i = A e^{-\frac{t}{\tau}}$, where $A$ is the current amplitude and $\tau$ is the time constant. The fits were obtained using the Levenberg-Marquardt algorithm with PulseFit software (HEKA Electronik, Lambrecht, Germany). Single-channel events were analyzed using TAC software (Braxton, Seattle, WA). The frequency distribution of single-channel conductance was fitted using a weighted sum of two Gaussians (bimodal distribution), assuming unequal means and equal variance, with Origin 6.0 software (Microcal Software, Northampton, MA).

**RESULTS**

Membrane conductance in HAECs is dominated by strong $I_K$. Under resting conditions, 85% of HAECs showed a pronounced $I_K$ with significant inward currents at voltages below the reversal potential and small outward currents at voltages above the reversal potential (Fig. 1A). The reversal potential averaged $-79 \pm 2$ mV, close to the theoretical reversal potential of a K$^+$-specific current under these recording conditions ($-80$ mV). No Ca$^{2+}$ was added to the pipette solution to suppress Ca$^{2+}$-activated K$^+$ channels. The strong rectification of the endogenous current in HAECs suggests that it is carried by members of the Kir2 family (28). Indeed, the current-voltage ($I$-$V$) relationship of the endogenous HAEC current appears to be identical to the $I$-$V$ relationship of currents carried by Kir2.x subunits expressed in CHO cells, a cell line that has no endogenous $I_K$. Typical current recordings of Kir2.1, Kir2.2, Kir2.3, and Kir2.4 are shown in Fig. 1A, inset. Each subunit was expressed separately in CHO cells. Endogenous HAEC current shows two typical features of the Kir2 channels: sensitivity to the level of extracellular K$^+$ concentration ([K$^+$]), (Fig. 1B) and voltage-dependent inactivation at hyperpolarizing voltages (Fig. 1C) (28, 29).

To verify that similar $I_K$ are observed in freshly isolated native cells, the currents from freshly isolated PAECs were also recorded within 1–4 h after their isolation. Pronounced $I_K$ were recorded in $\sim 76\%$ of PAECs examined, similar to our findings in HAECs. Both $I$-$V$ relationships and current densities of $I_K$ in freshly isolated PAECs were virtually identical to those in HAECs (Fig. 2). Maintaining the cells in culture for five passages had no significant effect on $I_K$ densities or rectification properties (Fig. 2B), supporting the physiological relevance of the HEAC system.

**HAEC Kir2.x mRNA expression.** To determine which Kir2.x transcripts were expressed in HAECs, RT-PCR was performed. For all experiments, the RNA profile revealed two strong 28S and 18S ribosomal peaks, present at an $\sim 2:1$ ratio, with little tailing and a flat baseline as expected for high-integrity RNA (Fig. 3A). Table 1 lists the primer pairs used for RT-PCR of each of the four known Kir2 subunits. Figure 3Ba shows clear bands of the predicted size for all four Kir2.x PCR products (all primers were designed within the single exon that contained the reading frame of each subunit). No bands were observed when PCR was performed directly on RNA samples (reverse transcriptase-negative controls), indicating that the PCR products were free of contaminating genomic DNA. Note that the band for Kir2.3 is much weaker than the bands for the other three subunits, suggesting weaker Kir2.3 mRNA expression.
To exclude further the possibility of genomic contamination, expression of Kir2.1 and Kir2.4 was tested with intron-spanning primers for Kir2.1 and Kir2.4 (Fig. 3Bb). Kir2.2 has no intron; therefore, only nonspanning primers can be designed for this channel. The specific expression of all four Kir2.x subunits in HAECs was confirmed by sequencing the PCR products. All four PCR products were 99–100% identical to the reported sequences of human Kir2.x.

Relative abundance of HAEC Kir2.x mRNA using QRT-PCR. QRT-PCR was used to determine the relative expression levels of Kir2.x transcripts in HAECs. The melting curves of all PCR products showed single prominent peaks, indicating the specificity of the primers (Fig. 4A) and a lack of primer-primer dimerization (48). Figure 4B shows the PCR amplification standard curves generated with known amounts of Kir2.x cDNA. In Fig. 4B, threshold PCR cycle number was plotted as a function of the Kir2.x cDNA amount, in which the threshold cycle is defined as the onset of the log increase in PCR amplification. The relationship between the threshold cycle number and log concentration of the Kir2.x cDNA was linear. The standard curves were used to calculate the relative abundance of Kir2.x subunits in HAECs (Fig. 4C). Consistent with the RT-PCR results presented above, the expression of Kir2.3 was significantly lower than that of each of the other subunits. The mRNA level of Kir2.2 was higher than that of Kir2.1 in each of the four independent cell-derived samples. These observations suggest that not only Kir2.1 but also other Kir2.x subunits, Kir2.2 in particular, are candidates for making a substantial contribution to endogenous $I_K$ in HAECs.

Kir2.x protein expression in HAECs. The presence of Kir2.x protein in HAECs was assessed using Western blot analysis. Kir2.1, Kir2.2, and Kir2.3 proteins were probed with polyclonal antibodies raised against rat (for Kir2.1 and Kir2.2) or human (Kir2.3) Kir2.x subunits (36) (no antibody against Kir2.4 subunit was available). Kir2.x antibodies provided clear bands upon probing COS-1 cells transfected with mouse Kir2.1, rat Kir2.2, or human Kir2.3 subunits (Fig. 5A). Signal was detected only in the lane corresponding to the specific Kir2.x subunit for which the antibody was designed, and no signal was present in lanes containing other Kir2.x subunits or with nontransfected COS-1 cells, demonstrating the specificity of these antibodies for individual Kir2.x subunits. Similar bands were also observed upon probing CHO cells transfected with mouse Kir2.1 or Kir2.2 subunits or human Kir2.3 subunits (Fig. 5B), but no signal was observed in association with nontransfected CHO cells pointing to antibody specificity. CHO cells transfected with Kir2.x subunits presented prominent bands at ~50 kDa (molecular weights for Kir2.1, Kir2.2, and Kir2.3 subunits, predicted from their primary structures, were 48, 49, and 49.5 kDa, respectively). Probing of HAECs showed corresponding bands for Kir2.1 and Kir2.2 subunits, but not for the Kir2.3 subunit (Fig. 5B). The molecular weights

Fig. 2. Comparison of $I_K$ in HAECs and in freshly isolated or low-passage cultured porcine aortic endothelial cells (PAECs). A: superimposed currents recorded from freshly isolated PAECs and from HAECs at 156 mM extracellular [K+] solutions. B: average current densities recorded in HAECs (passages 3–5; n = 17), freshly isolated PAECs (n = 23), and low-passage cultured PAECs (passage 3, n = 5; passage 4, n = 3; passage 5, n = 6). Each data set is expressed as the mean ± SE.

Fig. 3. RT-PCR analysis of mRNA encoding different types of Kir2 channel subunits in HAECs. A: typical profile for total RNA of HAECs. B: PCR products for Kir2.x channels amplified with exon-based primers (a) or intron-spanning primers (b). Lanes marked with minus signs correspond to negative controls in which amplification was performed without adding RT during cDNA synthesis.
of Kir2.1 and Kir2.2 in HAECs were similar to mouse Kir2.1 and Kir2.2 expressed in CHO cells. The specificity of the Kir2.2 band was further confirmed by blocking the antibody with the antigen (36). In addition, a larger (65–70 kDa) band was observed in Kir2.2 subunits in some experiments. The latter observation is consistent with an earlier study showing that Kir2.2 isolated from rat brain presented a band at 64 kDa (36). The lack of a Kir2.3 band in HAECs suggests either that the channel is not expressed at the protein level or that its expression is below the detection limit of the antibodies. Indeed, this is consistent with the low level of Kir2.3 mRNA expression.

**Sensitivity of endothelial $I_K$ to $Ba^{2+}$ block and to pH.** Differences in the sensitivity of Kir2.x currents to extracellular $Ba^{2+}$ and pH provided a valuable assay to further distinguish Kir2.x subunit contributions to endogenous HAEC currents. All Kir2.x currents are blocked by extracellular $Ba^{2+}$, but while the sensitivities of Kir2.1, Kir2.2, and Kir2.3 are known to overlap (22, 34, 41), currents carried by Kir2.4 subunits have $Ba^{2+}$ sensitivity significantly lower than that of other Kir2.x subunits: the $IC_{50}$ values of Kir2.1–Kir2.3 range from 3 to 16 μM for Kir2.1, from 0.5 to 2.3 μM for Kir2.2, and from 10 to 18.5 μM for Kir2.3, whereas the $IC_{50}$ of Kir2.4 is >200 μM at −100 to −120 mV (22, 40, 41). It is also important to note that the $Ba^{2+}$ sensitivity of Kir2.x channels may depend on the extracellular [K+] (22, 40, 41). In the present study, $Ba^{2+}$ sensitivity was determined at 60 mM extracellular [K+], con-

Fig. 4. Quantification of Kir2.x mRNA level in HAECs using quantitative real-time PCR (QRT-PCR). A: melting curves of Kir2.x amplicons representing plots of fluorescence (−dF/dt) vs. temperature (°C). B: standard curves for the real-time PCR amplification of human Kir2.x cDNA representing threshold cycle number vs. the copies of purified human Kir2.x cDNA. C: mRNA expression levels for Kir2.x channels estimated from cDNA copy numbers normalized to Kir2.1 in the same biological sample. Four independent experiments (RNA isolation and cDNA synthesis, followed by QRT-PCR) were performed, and the values shown are means ± SE. *$P < 0.05$.

Fig. 5. Protein expression of Kir2.x in HAECs. Typical examples of Kir2.x immunoblots of total membrane fractions of COS-1 or CHO cells transfected with cDNA encoding mouse or rat Kir2.1 and Kir2.2 and human Kir2.3, as well as HAECs. A: characterization of antibody specificity. The same amount of cell lysate (5–10 μg/lane) was loaded for COS-1 cells transfected with mouse Kir2.1, rat Kir2.2, human Kir2.3, and nontransfected cells. Blots were probed with antibodies to Kir2.1 (top), Kir2.2 (middle), and Kir2.3 (bottom). B: same amount of protein (30 μg/lane) was loaded for transfected and nontransfected CHO cells. For HAECs, the gels were loaded with the maximal sample volume (80 μg total proteins) for all three Kir subunits. The Kir2.2 band was completely abolished by preabsorbing the antibody with the specific antigen. All of the experiments were repeated in at least three independent biological samples.
being inhibited at low extracellular pH and enhanced at high pH (3, 11), a lack of pH sensitivity of the endogenous currents in HAECs suggests that neither Kir2.4 nor Kir2.3 contributes significantly to the whole cell $I_{K}$ in HAECs as homotetramers. Therefore, on the basis of low transcript and undetectable protein expression (for Kir2.3), a lack of pH sensitivity, and the range of Ba$^{2+}$ sensitivity of the current, we conclude that the contribution of Kir2.3 and Kir2.4 homotetrameric channels to the endogenous current in HAECs is negligible. These observations, of course, do not exclude the possibility that Kir2.3 and/or Kir2.4 subunits may form functional heterotetramers with Kir2.1 and/or Kir2.2.

**Distribution of $I_{K}$ unitary conductance.** To further assess which Kir2.x channels constitute the endogenous $I_{K}$ conduc-

![Fig. 6. Ba$^{2+}$ block and pH sensitivity of the $I_{K}$ in HAECs. A: $I_{K}$ recorded in a single HAEC at 0.1, 5, 10, and 230 μM extracellular Ba$^{2+}$ (extracellular $[K^{+}]$ was 60 mM). B: concentration dependence of Ba$^{2+}$ block. Fractional block was determined at $-100$ mV and calculated as $(I_{control} - I_{Ba^{2+}})/I_{control}$, where $I_{control}$ is the current recorded before application of Ba$^{2+}$ and $I_{Ba^{2+}}$ is the current recorded at a specific Ba$^{2+}$ concentration. Each point is the mean ± SE ($n = 5$). The data were fitted with the function $I_{Ba^{2+}}/I_{control} = 1/(1 + [Ba^{2+}]/K_{d})$ where $K_{d}$ is the dissociation constant. C: $I_{K}$ amplitudes plotted as a function of extracellular pH normalized to the current measured with pH 7.5 solution in the same cell. The amplitudes were measured at $-100$ mV. The protocol for current recording and K$^{+}$ concentration ($[K^{+}]$) in bath and pipette solutions were identical to those in Fig. 5. Data are means ± SE ($n = 5$).](http://www.ajpcell.org/issue)

Consistent with an earlier study of $I_{K}$ in bovine endothelial cells (13), endogenous $I_{K}$ in HAECs is not sensitive to extracellular pH (Fig. 6C). Because both Kir2.3 and Kir2.4 currents show strong sigmoidal dependence on pH, with the currents...
tance in HAECs, unitary conductance was evaluated using single-channel analysis. The typical values of Kir2.x unitary conductance are 20–30 pS for Kir2.1, 35–40 pS for Kir2.2, 10–15 pS for Kir2.3, and 14–15 pS for Kir2.4 (16, 22, 26, 33, 42, 43). In HAECs, 90% of the channels had unitary conductance of between −20 and 45 pS (Fig. 7) as would be expected if only Kir2.1 and Kir2.2 channels contributed significantly to the whole cell endogenous $I_K$ in these cells. Furthermore, there appear to be two distinct peaks in the distribution of the unitary conductances, one with a mean at 25 pS and another at 35 pS (Fig. 7B), supporting the notion that both Kir2.1 and Kir2.2 contribute to the endogenous $I_K$ in HAECs. It is also noteworthy that the peak at 35 pS is more prominent, suggesting that Kir2.2 is the major Kir2.x channel in HAECs. The ratio between the integrals for the major and minor histogram peaks is 1.58, suggesting that Kir2.1 channels constitute ~40% and Kir2.2 constitute ~60% of the channel population. A double-peak histogram of unitary conductance, however, does not exclude the possibility that multiple channel populations contribute to the endogenous $I_K$ in HAECs. This possibility is addressed further using dnKir2.x constructs.

Inhibition of endogenous $I_K$ using dnKir2.x. Specific inhibition of currents in HAECs by dnKir2.x subunits was used to further discriminate channel subunits that underlie the endogenous currents. dnKir2.x constructs were generated by replacing the GYG region of the pore with AAA, a substitution known to prevent ion throughput (34, 40). The efficiency of the constructs was tested by coinjecting dnKir2.x RNA with the respective Kir2.x-WT into Xenopus oocytes. All four Kir2.x were efficiently inhibited by the appropriate dnKir2.x (Fig. 8).

To evaluate the relative contributions of Kir2.x channels to the endogenous $I_K$ in HAECs, cells were transfected one at a time with dnKir2.x constructs subcloned into a bicistronic vector containing an enhanced green fluorescent protein (EGFP) flu-
oesence marker. This system allowed fidelity for the identification of successfully transfected cells that was much higher than that of standard cotransfection procedures, and by subcloning the constructs into the bicistronic vector, complications inherent in tagging green fluorescent protein (GFP) to the molecule of interest were minimized. To avoid possible non-specific effects of overexpression, only cells with moderate fluorescence were used for current recordings. Transfection of HAECs with the EGFP marker alone had no effect on $I_K$. However, transfecting the cells with dnKir2.1 and dnKir2.2 significantly inhibited native $I_K$ current. It is also noteworthy that the effect of dnKir2.2 was significantly stronger than that of dnKir2.1 (Fig. 9, A and B). In contrast to dnKir2.1 and dnKir2.2, no significant effects were elicited by dnKir2.3 and dnKir2.4 (Fig. 9, A and B). These observations are consistent with assignment of the major $I_K$ conductances to Kir2.1 and Kir2.2 on the basis of the double-peak histogram of $I_K$ unitary conductances, as well as with the dominance of Kir2.2. The lack of dominant-negative effects of dnKir2.3 and dnKir2.4 suggests that Kir2.3 and Kir2.4 subunits do not contribute significantly to functional $I_K$ channels in HAECs.

Because dnKir2.1 only partially inhibited the macroscopic currents in HAECs, it was possible to extend the analysis to the single-channel level and investigate whether one or both peaks of unitary conductance were specifically affected by dnKir2.1. When dnKir2.1 constructs were expressed in HAECs, we observed a shift in the distribution of unitary conductance of endothelial $I_K$ channels, so that under these conditions, the histogram presented a single peak at 35 pS that corresponded to the unitary conductance of Kir2.2, whereas the minor peak at 25 pS, whose unitary conductance corresponded to Kir2.1 channels, was absent (Fig. 9C). These observations support the hypothesis that HAECs express two distinct populations of Kir2.x channels.

**DISCUSSION**

In the present study, we have performed a detailed analysis of the molecular identity of $I_K$ channels in HAECs. The general biophysical characteristics of whole cell $I_K$ in HAECs were similar to those reported earlier in other types of ECs (9, 13). Herein, however, we have shown that HAECs express not only Kir2.1 mRNA as previously described (7, 9, 13, 50) but also Kir2.2, Kir2.3, and Kir2.4 mRNA. Furthermore, analyses of the biochemical and pharmacological properties of whole cell HAEC $I_K$ current as well as the biophysical properties of single-channel events suggest that Kir2.2 contributes prominently and that Kir2.1 provides a significant but smaller contribution to endogenous HAEC current. Kir2.3 and Kir2.4, on the other hand, do not appear to be of physiological importance in HAECs. Consistent with these observations, a dnKir2.2 construct strongly suppressed endogenous $I_K$ in HAECs, whereas a dnKir2.1 construct had a smaller effect. Dominant-negative constructs of Kir2.3 and Kir2.4 had no significant effect on HAEC $I_K$. Together, these observations suggest that Kir2.2 channels provide the primary $K^+$ conductance in HAECs under resting conditions.

**Consideration of the system.** The low-passage HAECs used in the present study appear to be the best available cell model to investigate $I_K$ in human aortic endothelium. Although it was reported earlier that aortic endothelial cells freshly isolated from mouse or rabbit aortas lack Kir channels (39), we have shown in the present study that Kir are clearly active in freshly isolated PAECs. Furthermore, $I_K$ in low-passage PAECs were similar to those in freshly isolated cells. Thus the fraction (~80%) of cells that express Kir and the average current density of the current are similar in low-passage HAECs, low-passage PAECs, and freshly isolated PAECs, supporting the relevance of the low-passage HAEC system. The difference between the ECs isolated from mouse, rabbit, or pig aorta could be due to interspecies differences in Kir expression or to different isolation protocols. Interspecies differences in cardiac...
Kir subunit expression are well recognized (5, 24). It is not surprising that \( I_K \) in HAECS and in PAECs are more similar than \( I_K \) in mouse or rabbit endothelium, because it is generally accepted that the pig vasculature is more similar to the human vasculature than to that of mice or rabbits. Kir expression appears to vary in different types of ECs. Nilius and Schwarz (30) showed that only a fraction of ECs freshly isolated from the human umbilical vein expressed \( I_K \), and Himmel et al. (10) showed that microvascular ECs from human omentum lacked \( I_K \). In contrast, freshly isolated coronary ECs show pronounced \( I_K \) with a unitary conductance similar to that observed in aortic endothelium (45). It is also possible that the expression profiles of Kir channels are modified under different physiological or pathological conditions. Thus it is important to take into account both the differences among species and between ECs isolated from different vascular beds when comparing the properties of endothelial \( I_K \).

**Molecular diversity of Kir2-based native currents.** In HAECS, the molecular diversity of Kir2 subunits at the transcript level is higher than the diversity of functional Kir. While for Kir2.3 this discrepancy could be explained by undetectable levels of protein expression due to very low transcription, the transcript level of Kir2.4 is similar to that of Kir2.1, suggesting that Kir2.4 functional expression is regulated at a posttranscriptional level. A discrepancy between the heterogeneity of \( K^+ \) channels at the transcript and functional levels was reported previously for Kir2.x channels in human myoblasts (8) and for voltage-gated \( K^+ \) channels in rat cardiomyocytes (2, 49), and it has been proposed that translational-postranslational steps may contribute a rate-limiting step to channel expression (38). Protein expression of Kir2.x subunits in HAECS is consistent with the functional expression of the channels.

The peak \( I_K \) unitary conductance levels in HAECS (25 and 35 pS) are similar to previously reported values in human umbilical vein endothelial cells (29 pS) and in bovine aortic endothelial cells and PAECs (30–42 pS) (13, 29, 30, 37). We observed a dual-peak distribution of \( I_K \) unitary conductances in HAECS. A similar distribution with an additional peak at a lower value (34 pS, 24 pS, and 11 pS) was reported in guinea pig cardiac myocytes (22). These peak values are consistent with the unitary conductances of Kir2.2, Kir2.1, and Kir2.3 expressed in *Xenopus* oocytes (34–36 pS, 21 pS, and 8–15 pS, respectively) (16, 33, 42). The exact values of Kir2.x unitary conductances can vary among cell types, but an ~10-pS difference between Kir2.1 and Kir2.2 is maintained. For example, Kir2.1 and Kir2.2 have conductances of 21 and 28 pS, respectively, when expressed in human myoblasts (8) and conductances of 31 and 42 pS, respectively, when expressed in human embryonic kidney HEK-293 cells (22). These variations have been attributed to the possible binding of an intracellular ligand (22). We propose that the observed bimodal distribution of unitary conductances suggests that both Kir2.1 and Kir2.2 contribute to the endogenous \( I_K \) in HAECS. Taking into account the difference in unitary conductances between both channels, Kir2.2 appears to be the dominant conductance, contributing ~70% of the whole cell \( K^+ \) current. These ideas are supported by the observation that dnKir2.1 suppressed only the lower-conductance portion of the single-channel current histogram.

Dissecting the contributions of different Kir2.x subunits to the endogenous \( I_K \) with dnKir2.x constructs further supports the hypothesis that both Kir2.1 and Kir2.2 are the main constituents of endothelial \( I_K \), with Kir2.2 having the dominant role. Earlier studies showed that coexpression of wild-type and dnKir2.x subunits resulted in the formation of Kir2.x heterotetramers (34, 40). Furthermore, Zobel et al. (52) demonstrated that both dnKir2.1 and dnKir2.2 inhibited endogenous \( I_K \) in myocytes by >50%, suggesting the formation of heterotetramers between dnKir2.x and endogenous Kir2.x subunits. Consistent with these findings, our data show that the sum of current inhibition by dnKir2.x in HAECS (dnKir2.2, ~85%; dnKir2.1, ~50%) produced a value >1, implying possible heterooligomerization of native Kir2.x subunits. It is important to note, however, that heterooligomerization between overexpressed dnKir and native Kir does not necessarily mean that native Kir form heterotetramers. As Zobel et al. (52) pointed out, if a significant proportion of endogenous Kir channels were Kir2.1 and Kir2.2 heterotetramers, then dnKir2.1 and Kir2.2 would be expected to inhibit endogenous \( I_K \) equally. That was indeed the case for rabbit cardiomyocytes, but in HAECS, dnKir2.1 had a weaker effect than dnKir2.2.

**Potential significance of our findings.** Multiple Kir2.x subunits are expressed in individual cells of several types, including cardiomyocytes (22, 46) and smooth muscle cells (14). It is noteworthy that because Kir2.x subunits have differential sensitivities to several modulatory systems, such as those related to protein kinase C (15), G protein-coupled receptors (6), and chaperone molecules (18, 19), the expression pattern of multiple Kir2.x subunits may underlie differences in tissue electrophysiological properties. Indeed, differential expression of multiple Kir2.x in atrial vs. ventricular myocytes was suggested to account for different resting potentials and excitability properties between the two heart tissues (15), and it was suggested that changes in Kir2.x expression profile may be important for plasticity of electrophysiological responses in arterial smooth muscles (14). In summary, this study is the first to demonstrate the expression of multiple Kir2.x subunits in ECs and to identify the relative roles of specific subunits of endogenous endothelial Kir.

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