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Induction of Ca^{2+}-Activated K^+ Current and Transient Outward Currents in Human Capillary Endothelial Cells

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Abstract. Human capillary endothelial cells (HCEC) in normal media contain noninactivating outwardly rectifying chloride currents, TEA-sensitive delayed rectifier K^+ currents and an inward rectifier K^+ current. Two additional ionic currents are induced in HCEC when the media are allowed to become conditioned: A Ca^{2+}-activated K^+ current (BKCA) that is sensitive to iberiotoxin is induced in 23.5% of the cells, a transient 4-AP-sensitive K^+ current (A current) is induced in 24.7% of the cells, and in 22.3% of the cells both the transient and BKCA currents are coinduced. The EC_{50} for Ca^{2+} activation of the BKCA current in HCEC from conditioned media is 213 nM. RNA message for BKCA (hSlo clone) is undetecable after PCR amplification in control cells but is seen in those from conditioned cells. The induction of BKCA current is not blocked by conditioning with inhibitors of nitric oxide synthase, cyclo-oxgenase or lypooxygenase pathways. Apparently the characteristics of human endothelial cells are highly malleable and can be easily modified by their local environment.

Keywords: Endothelial cells – Transient outward current — A current — BKCA — hSlo — Ca-activated K current — Conditioned media

Introduction

Endothelial cells contain ion channels that are thought to play an important role in the regulation of intracellular Ca^{2+} and the subsequent release of endothelial cell relaxing factors (Adams et al., 1989). To date, ionic currents in endothelial cells have been characterized from both large and small vessels of bovine, porcine and human. The ionic currents present and their magnitudes vary greatly from these different species and even from seemingly identical preparations studied by different investigators. For example some studies on endothelial cells have reported a prominent transient outward rectifier current, a calcium-activated potassium current and even the appearance of calcium currents; while many others see no evidence for these channel types (Nilius, Viana & Droogmans, 1997; Revest & Abbott, 1992). Cells in culture release many autocrine and metabolic factors that change the overall composition of the media in which they are growing. We have investigated the effects of conditioning of media by the endothelial cells on their ionic currents. The dramatic differences we see between conditioned and nonconditioned media may explain the conflicting observations reported from endothelial cell studies, and more importantly suggest a high degree of plasticity in these cells that would be expected to play an important role in their physiology.

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Materials and Methods

Cell Culture

Human capillary endothelial cells (HCEC) were obtained from Cell Systems Corporation (Kirkland, WA). Cells were cultured in growth medium for human microvascular endothelial cells (4M1–500, Cell Systems Corporation). The composition of this medium was: modified DME/F-12 (1:1), 15 mM HEPES buffer, 10% FBS, 50 mg/ml heparin, 50 mg/ml αFGF (bovine pituitary origin). For control cells, culture media were replaced every two days and cells passaged before day seven. In conditioned media cultures, the cells were conditioned in two different ways: (i) Cells were maintained at low density by passaging on day 5–6 but the culture media were allowed to become conditioned by only replacing it after passaging. (ii) Culture media were changed every 2 days but cultures were maintained at high density by passaging between days 10–20. Either procedure was effective in producing changes in ion channel composition and no difference could be seen between the type or magnitude of ionic currents induced.

Human capillary endothelial cells were cultured on 35 mm Corning tissue culture dishes in media and serum as described. They had a flattened (0.1–0.5 μM thick) cobblestone appearance that precluded reproducible whole-cell patch recording. Recordings from these flattened cells revealed a large degree of electrical coupling that manifested itself as an inability to clamp cells to a depolarizing step potential. To eliminate this problem and to produce a thicker cell profile, both conditioned and unconditioned cells were trypsinized and replated at low density 10–30 min prior to recording (Vaca & Kunze, 1993). Isolated endothelial cells responsed robustly to histamine, bradykinin and Vascular Endothelial Growth Factor (VEGF) suggesting that the trypsin had little deleterious effect on surface receptors.

Electrophysiology

Human endothelial cells were recorded in whole-cell voltage clamp with the patch-clamp technique (Hamill et al., 1981). Patch electrodes were pulled from borosilicate glass (Sutter Inst. O.D. 1.0 mm, I.D. 0.5 mm) and had a resistance of 3–6 Mohms. The input resistance of cells at −60 mV measured in excess of 1 Gigaohm. Seal resistance was greater than 20 Gigaohms. All recordings were done at room temperature. An EPC9 amplifier with the acquisition program Pulse-PulseFit from HEKA (Lambrecht Germany) was used for recording, data acquisition, and Igor Pro (WaveMetrics, Oregon), Origin (Microcal Software, MA) and Excel (Microsoft, CA) were used for analysis. Series resistance compensation was used and currents were filtered at 3kHz. The standard pipete solution for whole-cell recordings was (in mmol/l): 120 K-aspartate, 20 KCl, 10 EGTA, 5 MgATP, 5 Phosphocreatine-Na_2, 1 $MgCl_2$, 5 HEPES (pH 7.2 with KOH). In the experiments where intracellular Ca^{2+} was measured, EGTA was omitted and 50 μM Fura 2 acid was added. The standard bath recording solution consisted of (in mmol/l) 140 NaCl, 5.4 KCl, 15 Dextrose, 1.7 $CaCl_2$, O.8 $MgSO_4$, 0.4 KH_2PO_4, 0.3 Na2HPO_4, 5 HEPES (pH 7.4 with NaOH). The internal solution for Chloride channel recordings contained (in mmol/l): 100 NMDG-glutamate, 40 NMDG-Cl, 2 EGTA, 0.5 Ca, 2 $MgCl_2$, 5 MgATP, 10 HEPES (pH 7.3 with tris). The external solution for Cl channel recording contained (in mmol/l): 140 NMDG-Cl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES (pH 7.4 with tris). Iberiotoxin was purchased from Peptide Institute, NS 1619 from RBI, Fura2 acid from Molecular Probe, 5 Nitro-2-(3-phenyl propylamine) benzoic acid (NPPB) from RBI, L-NIO from Calbiochem and ionomycin, meclomen, indomethacine and all salts from Sigma.

Photometry

Intracellular Ca^{2+} was monitored simultaneously with patch-clamp recordings in some of the Ca^{2+} activated K^+ channel (BKCA) experiments. A T.I.L.L. Photonics (Germany) photometer adapted for the EPC-9 (Applied Scientific Instrumentation) was used to monitor the fura ratio. Calcium concentration was calculated from Rmin/Rmax, and K_d values entered into the Pulse Fura program and verified against a calcium calibration curve generated with Molecular Probes calibration kit F-6774. Calcium concentrations reported with this technique represent the mean of four individual measurements taken during the voltage ramp.

Molecular Biology

For detection of hSlo messenger RNA, human endothelial cells were allowed to condition their media by passaging cells only when they were close to confluent. Control cells were maintained at low density (<60%) by more frequent passaging. Cells were analyzed for the absence or presence of BKCA current at the time of harvest (28 days) and only conditioned cells had BKCA currents. Total cellular RNA was extracted using RNAzol (Tel-test) quantified, and the integrity of the RNA verified by running a small amount of nondenaturing agarose gels.

First strand synthesis of cDNA was done according to the Gene Amp RT-PCR protocol (Perkin-Elmer, Amplitaq) using 500 ng total cellular RNA. PCR was carried out under standard conditions as described by the manufacturer (Perkin-Elmer, Amplitaq) using 1.5 mM $MgCl_2$. Primers used for hSlo amplification from the synthesized first strand products were 5′-ccatttgtggagaattcaggg-3′ (upstream primer) and 5′-cagagcttcaagctccagga-3′ (downstream primer), yielding a 411 bp product. β-actin was used as a positive internal standard using the same amplification protocol as for hSlo but with 25 rather than 35 cycles. Primers used were 5′-atcggcaccacaccttctacaatgagctgcg-3′ (upstream) and 5′ cgtcatactcctgcttgctgatccacatctgc-3′ (downstream), yielding a 838 bp fragment. The amplification conditions were: 94°C, one minute, 35 cycles of 94°C (30 sec), 58°C (30 sec) and 72°C (30 sec), 72°C 3 min. PCR products were analyzed on agarose gels stained with ethidium bromide.

Results

Human capillary endothelial cells (HCEC) had an average membrane potential of −57.6 ± 1.2 mV and input resistance of 5.0 ± 0.7 GΩ ($n = 12$). Figure 1 shows whole cell voltage clamp recordings from three different HCEC that demonstrate the kinds of ionic currents present. These include: a TEA (10 mM)-sensitive delayed rectifier type current with peak values of 192.8 ± 36.3 pA at +100 mV ($n = 5$) (Fig. 1*A*); a noninactivating outwardly rectifying Cl current blocked by Cl substitution with methyl sulfonate ($n = 3$) or the chloride channel blocker NPPB (100 μM) ($n = 12$), with a peak current value of 227.3 ± 37.1 pA at +100 mV ($n = 15$) (Fig. 1*B*) and a barium-sensitive inward rectifier current with peak current values of −360.8 ± 49.2 pA at −120 mV ($n = 38$) (Fig. 1*C*). The TEA-sensitive K^+ outward current was present in 60% of the cells ($n = 15$); the Cl current was seen in 52% of the cells ($n = 19$) and the inward rectifier K^+ current was found in 98% of the cells ($n = 120$). Similar ionic currents have been described for other endothelial cells maintained in culture (Adams et al., 1989; Revest et al., 1992; Nilius et al., 1997).

Human capillary endothelial cells maintained in media that they have conditioned had similar resting membrane propertites but contained outward currents not seen in cells cultured in control media. Figure 2 gives an overview of the ionic currents seen in human capillary endothelial cells maintained in conditioned media. Figure 2*A* is from an HCEC maintained in normal medium. Figure 2*B* shows an HCEC maintained for five days in conditioned medium. The cell responds to depolarizing voltage pulses with a large transient outward current. Figure 2*C* is from a different cell maintained for eight days in conditioned medium. This cell responds to depolarizations with large noisy outward currents. Figure

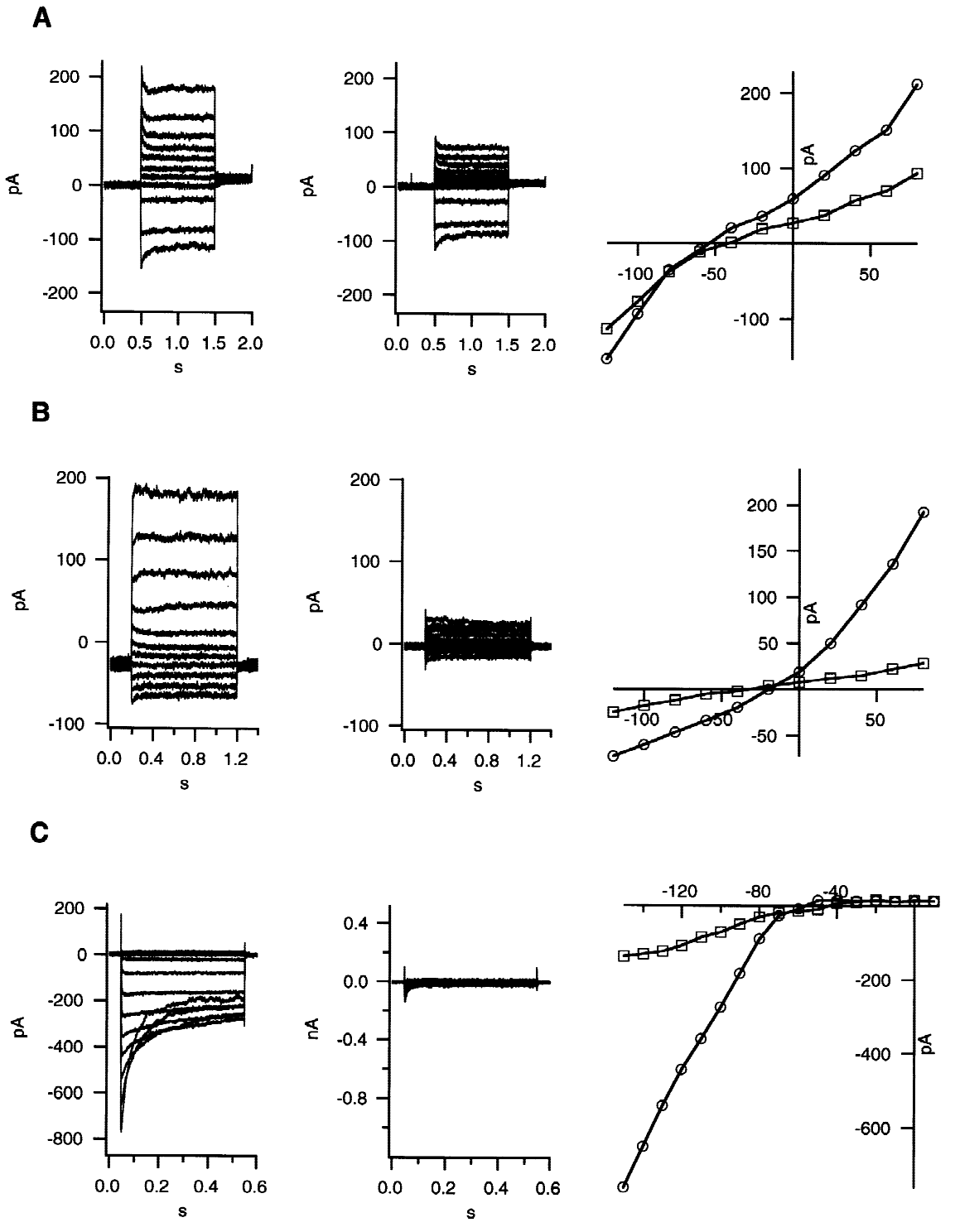

Fig. 1. TEA-sensitive K current, NPPB-sensitive Cl current and Ba-sensitive inward rectifier K current in human capillary endothelial cells from normal cultures. (*A*) Current traces of a HCEC held at −60 mV and pulsed from −120 to +80 mV with 20 mV steps before and after application of 10 mM TEA and corresponding current-voltage plot. (*B*) Current traces and current-voltage relation from a HCEC before and after application of 100 μM NPPB recorded with solutions designed to isolate Cl currents (Materials and Methods) (same voltage protocol as in *A*). (*C*) Current traces and current-voltage relation (*IV*) from another HCEC for control and after application of 50 μM Ba. Holding potential of −60 mV and 10 mV voltage steps from −150 mV to +10 mV, x axis: (*s* = time in seconds).

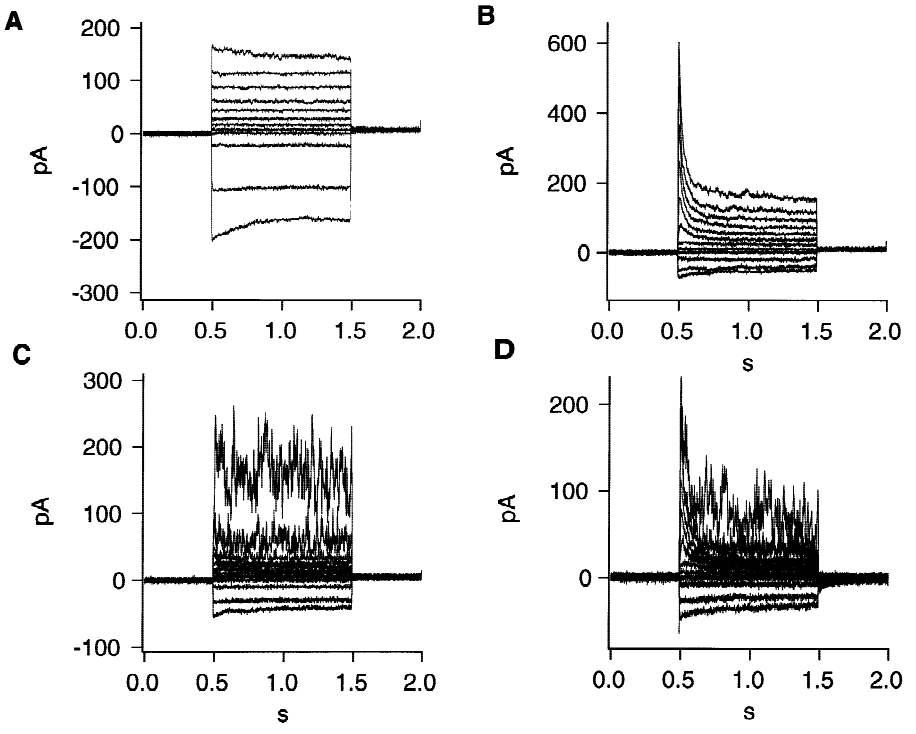

Fig. 2. Human capillary endothelial cells (HCEC) cultured in normal media have different outward currents from cells maintained in conditioned media. (*A*) Typical ($n = 120$) current traces in response to voltage steps (20 mV) for one second from −120 to +100 mV from a HCEC cultured in normal medium (holding potential = −60 mV), × axis: (s = time in seconds). (*B*) The current traces (note scaling) for the same voltage steps from a HCEC maintained for 5 days in conditioned medium. Note the large transient outward current at potentials above 0 mV (holding potential = −60 mV). (*C*) The current response to the same voltage steps (−120 to +100 mV) of a different HCEC maintained for 8 days in conditioned medium. Note the large noisy outward currents seen at the more depolarized potentials (⩾60 mV). (*D*) Another HCEC maintained in conditioned medium for 6 days. The current response to the voltage steps in this cell is a combination of the noisy outward current and the transient outward current seen in the previous cells.

2*D* is from a cell having both transient and noisy outward currents that was maintained in conditioned medium for six days. No correlation was seen between the number of days in conditioned media and the type or magnitude of ionic currents seen, although conditioning appeared to require a minimum of 4–5 days to produce an effect on ionic currents.

Cells maintained in normal media that do not show induction of the outward currents shown in Fig. 2 *B–D* can be induced by substituting their media with media from conditioned cultures. Four cells that had their control media substituted with five-day-old conditioned media from confluent cultures exhibited both noisy outward and transient outward currents two days later whereas their control counterparts did not.

The noisy outward current was seen by itself in 23.5% and with a transient outward current in 22.3% ($n = 85$) of the HCEC from conditioned media. The current appears similar to the large conductance calcium-activated potassium current described in rabbit aortic endothelial cells (Rusko et al., 1992) and in other preparations (Blatz & Magleby, 1987). Iberiotoxin is a specific inhibitor of this large conductance BKCA channel (Candia & Garcia, 1992). Figure 3 shows that application of 100 nM iberiotoxin greatly reduced the level and noise of the outward currents in this HCEC. Iberiotoxin blocked 61.1 ± 7.9% of the total outward current in 5 cells that had been maintained in conditioned media, but had no effect (< 5%) on HCEC maintained in nonconditioned media (n = 18). This result indicates that much of the outward current at very positive potentials in these conditioned cells is carried through iberiotoxin-sensitive BKCA channels.

RT-PCR was used to correlate the appearance of the iberiotoxin-sensitive BKCA channel with an increase in the mRNA encoding the hSlo α subunit, the human isoform of the BKCA channel. Primers were designed from 5′ region of the sequence. Cells were cultured under conditions shown to induce the appearance of BKCA and the transient outward currents in these cells, total cellular RNA was extracted, and RT-PCR used to identify the production of the hSlo α subunit. RT-PCR amplification of RNA from control unconditioned cells did not have any measurable hSlo α subunit message in the conditions used (35 cycles) (Fig. 4, lane 3) and no BKCA current was measured. Cells allowed to condition their media

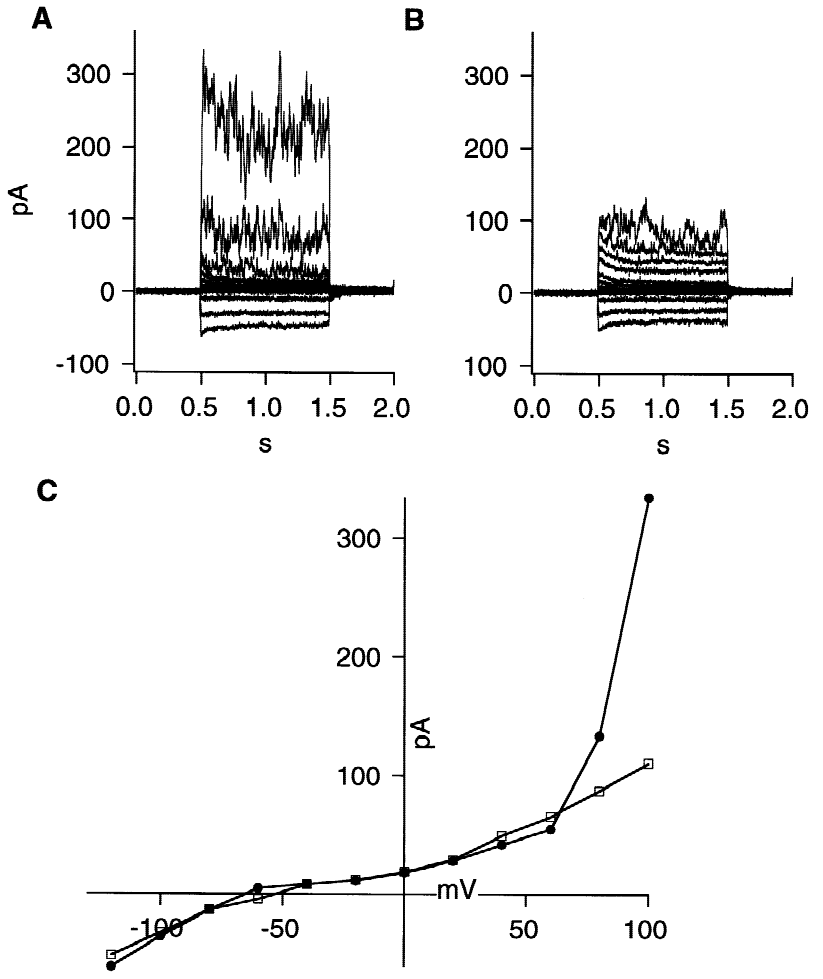

Fig. 3. Iberiotoxin-sensitive BKCA current from human capillary endothelial cell maintained in conditioned medium. (*A*) Current traces in response to voltage steps from −120 to +100 mV in 20 mV steps, from a holding potential of −60 mV, x axis: (s = time in seconds) for a HCEC in conditioned medium. Note the noisy outward traces for potentials above +40 mV indicative of BKCA channel activation (0.01 mM EGTA in pipette). (*B*) The current response from the same cell after application of 100 nM iberiotoxin. (*C*) The current-voltage plot for this cell before and after iberiotoxin.

did express the hSlo α subunit (35 cycles) (Fig. 4, lane 4) and had BKCA currents, supporting the hypothesis that de novo synthesis of hSlo protein is responsible for the appearance of the BKCA current.

If the whole cell current is indeed carried through the BKCA channel then this current should be activated by the Neurosearch compound NS1619, that is reported to be an opener of BKCA channels (Olesen et al., 1994). Figure 5 shows the effect of 33 μM NS1619 on a HCEC maintained in conditioned media. EGTA (5 mM) was added to the standard pipette solution to keep intracellular Ca^{2+} levels low. This particular cell exhibited little outward current even at depolarizing pulses of +100 mV but after application of NS1619 a large (4 nA) outward current could be elicited and this current was almost completely blocked by 100 nM iberiotoxin (Fig. 5*C*). The current-voltage plot shows that NS1619 opened the BKCA channel at all potentials positive to −10 mV. NS1619 (33 μM) gave variable results from cell to cell but in 15 cells tested showed an average increase in outward current of 300 ± 150%.

In a subset of experiments we examined the threshold for intracellular Ca^{2+} activation of K^+ current by simultaneous patch recording and Fura2 ratio imaging. Figure 6*A* shows a HCEC that was recorded from in this manner. The cell was maintained in conditioned media and recorded in normal extracellular solution. Intracellular solution was the standard pipette solution without EGTA and with 50 μM Fura2 acid. Intracellular Ca^{2+} was increased to the levels shown by bath application of 1 μM of the Ca^{2+} ionophore, ionomycin. The current-voltage ramps contain a noisy outward current that begins to develop at around 193 nM of intracellular Ca^{2+}. Figure 6*B* shows data from a different cell after application of the Ca^{2+} ionophore, ionomycin. The top panel shows the average calcium concentration at the times indicated. The bottom panel (*B*) plots the peak outward current level at the end of a 1-second ramp to +100 mV. Note that the activation of the noisy outward current tracks the increase in intracellular calcium. To establish the role of extracellular calcium entry in this process, we perfused the cell with extracellular solution containing very low calcium (2 mM EGTA and no calcium) in the continued presence of 1 μM ionomycin. This bath so-

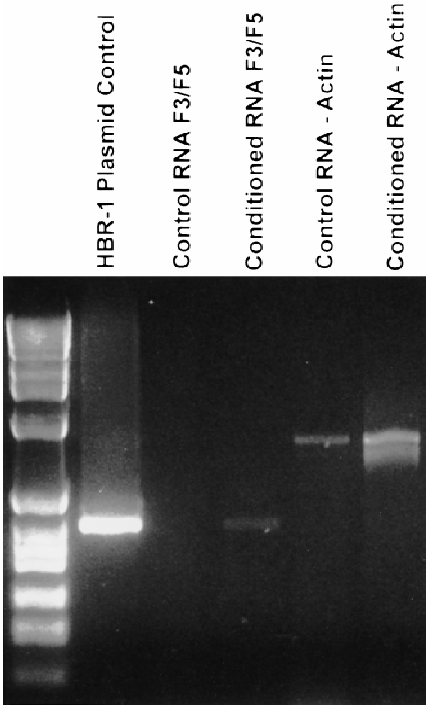

Fig. 4. Detection of hSlo message from conditioned HCEC. Lane 1: 1 Kb DNA ladder. Lane 2: positive control plasmid containing hSlo amplified with primers F3 and F5. F3 primer is the sense primer, F5 is the anti-sense primer. They amplify hSlo from base pairs 906-1317 (primer sequences:F3:5′CCATTTGGTGGAGAATTCAGGG 3′; F5: 5′GAGAGCTTCAAGCTCCAGGTTC 3′). Lane 3: RNA from Control HCEC cells amplified via RT-PCR with primers F3 and F5; lane 4: RNA from Conditioned HCEC cells amplified via RT-PCR with primers F3 and F5; lane 5: RNA from Control HCEC cells amplified via RT-PCR with Actin primers; lane 6: RNA from Conditioned HCEC cells amplified via RT-PCR with Actin primers. The Actin primers used were from Clontech.

lution reduced both intracellular calcium and peak outward current. The identity of this calcium-sensitive outward current was further characterized in Fig. 6*C* (same cell as *A*). Ionomycin was added at the arrow to produce a large increase in intracellular calcium and outward current. At about 400 sec 100 nM iberiotoxin was applied which blocked over 80% of the outward current at +100 mV but had little effect upon the intracellular calcium level. Perfusion with extracellular solution containing very low calcium (2 mM EGTA, no calcium) blocked the residual outward current and decreased the intracellular calcium levels to below 100 nM. Similar experiments in 2 additional cells gave an EC_{50} value of 213 ± 30 nM for half activation of maximal outward current at +100 mV (fit with Hill equation using increasing calcium and normalized corresponding current). Agonist induced calcium entry gave similar results for activation of BKCA current, with VEGF ($n = 3$) having an EC_{50} value of 239 ± 16 nM Ca^{2+} and histamine ($n = 1$) 195 nM Ca^{2+} for half activation of maximum outward current (+100 mV). Increases in intracellular Ca^{2+} in control unconditioned HCEC failed to elicit a BKCA current ($n = 12$).

A transient outward current was seen alone in 24.7% ($n = 85$) of all HCEC maintained in conditioned media. Another group of conditioned cells contained both the transient outward current and the noisy outward current (22.3%). Figure 7 shows that the transient outward current could be blocked by 3 mM 4-aminopyridine (4AP). This was true for 5 cells tested, with 3 mM 4AP blocking most of the transient outward current (Fig. 7*B*). The 4AP sensitive current was obtained by subtraction of the currents in the absence and presence of 4AP. This fast transient component is shown in Fig. 7*C*. A slower transient current can still be seen in Fig. 7*B* that was insensitive to 4AP block. This current was seen inconsistently in conditioned cells expressing transient current and was identified as an inactivating Cl current. The current could be blocked by substitution of external Cl with N-methylsulfonate or by 100 μM of the Cl channel blocker NPPB. Due to the infrequent and inconsistent pattern of induction seen with this current it was not studied in any greater detail and was not present at all in a second batch of HCEC conditioned as before.

Figure 8*A* shows a cell expressing both transient and noisy outward current. The transient outward current (A current) was fully inactivated when the cell was held at −20 mV (Fig. 8*B*). The time constant for inactivation was determined for the subtracted current traces from holding potentials of −60 and −20 mV. The subtracted current traces of 20, 40 and 60 mV with their individual fits are shown in Fig. 8*C*. The inactivation at all potentials between 20 and 80 mV was well fitted with a single exponential. The average mean inactivation time constant at 20 mV was 33 ± 4 msec and decreased to 25 ± 3 msec at 80 mV ($n = 7$). The inactivation time constants were therefore only weakly voltage dependent.

The steady-state activation and inactivation of this transient outward current was characterized with a voltge clamp prepulse protocol before and after 4AP block and the results for five cells are plotted in Fig. 8*B*. Mean steady-state half activation occurred at +12.0 ± 1.8 mV with half inactivation at −40.0 ± 1.6 mV ($n = 5$). A window current was present between −30 and 0 mV with a peak at −17.8 ± 4.6 mV and a value of 12.6 ± 2.8% of the maximum normalized current (values determined from the overlap region of Boltzman fits to activation and inactivation curves). Recovery from inactivation using a two-pulse protocol to +40 for 100 msec and holding at −60 mV required 4 seconds for complete recovery from inactivation ($n = 3$, *not shown*).

The overall profile for the outward currents induced in 85 HCEC by conditioning of their media is as follows: 24.7% contained only the transient outward current, 23.5% contain only the noisy outward current, and

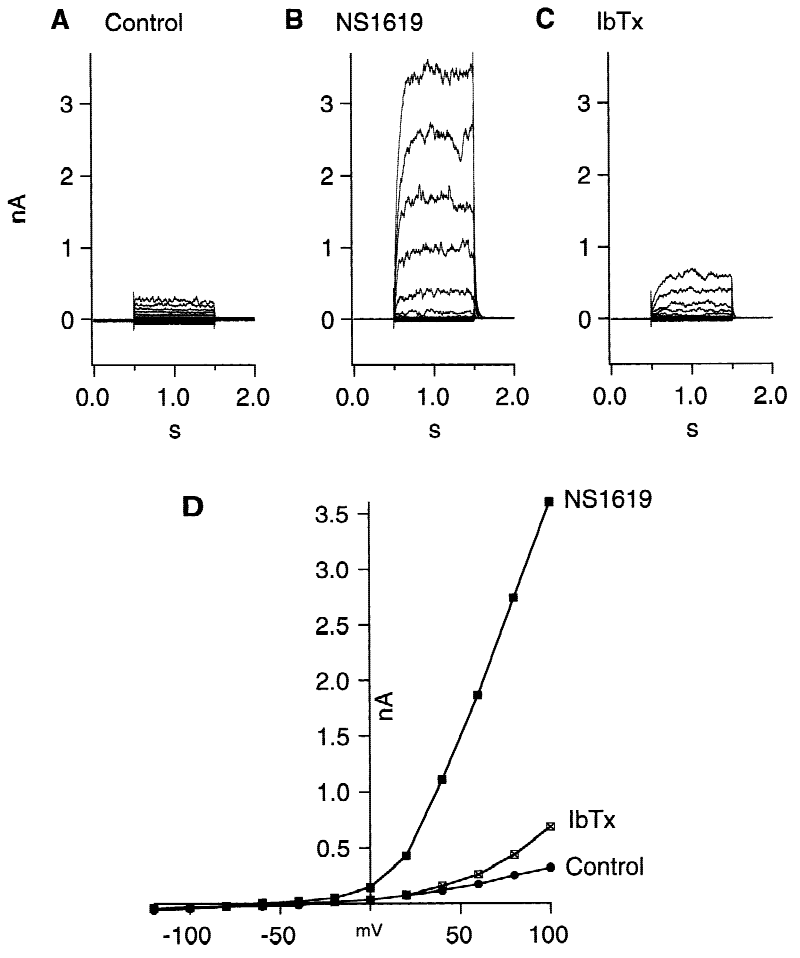

Fig. 5. The iberiotoxin-sensitive BKCA current in HCEC from conditioned media is activated by NS1619. (*A*) Current response to voltage pulses from −120 to 100 mV from a holding potential of −60 mV, x axis: (s = time in seconds). (*B*) The same cell after application of 33 μM NS1619. There is a large increase in noisy outward current and a zero current shift toward a more hypoerpolarized potential. (*C*) Block of the noisy outward current activated by NS1619 with 100 nM iberiotoxin. (*D*) Current-voltage relationships before NS1619, after NS1619 and in the presence of NS1619 plus iberiotoxin.

22.3% contain both the noisy and transient currents. The remainder of the cells (29.5%) exhibited outward currents as seen in HCEC without conditioned media (n = 120). We attempted to block the currents induced with conditioned media by incubating with various mixtures of known blockers of endothelial cell signaling pathways. We incubated cells that were already expressing BKCA and/or A type currents following conditioning, with 10 μM of the NOS inhibitor N(5)-(1-iminoethyl)-L-ornithine (L-NIO) and 10 μM indomethacin, an inhibitor of cyclo-oxygenase for five days. Four out of six control conditioned cells expressed a BKCA and/or A current and all 5 of the drug-treated cells contained BKCA and/or A current. We therefore included an inhibitor of lypo-oxgenase (1 μM meclomen) along with 10 μM L-NIO and indomethacin and incubated HCEC already expressing BKCA and/or A current. Drug containing media was replaced everyday and at 7 days 1 out of 4 drug-treated cells expressed BKCA and/or A current whereas 3 out of 4 control conditioned cells contained these currents. This suggested that the lypo-oxygenase pathway may be involved but was inconclusive.

We therefore investigated this further by examining the effects of 10 μM L-NIO, 10 μM indomethacin, and 1 μM meclomen on unconditioned cells not expressing BKCA and/or A currents that were then allowed to condition their media in the continued presence of the drugs. The media of the control and the drug-treated cells were changed every 2 days and the cells were maintained on this protocol for 24 days. Four out of five of the drug treated cells expressed BKCA and/or A current and 3 out of 5 control conditioned cells expressed these currents. Taken together the data suggest that the induction of BKCA and A current in HCEC is not mediated through endothelial NOS, cyclo-oxgenase, or lypo-oxygenase pathway.

Discussion

The ability of endothelial cells to condition their media and the effects of this conditioned medium on endothelial cell and smooth muscle cell physiology has been previously reported (Dodge, Lu & D'Amore, 1993; Sakuda et al., 1992; Scott & Merrilees, 1987). To date, media conditioning effects on endothelial ion channels

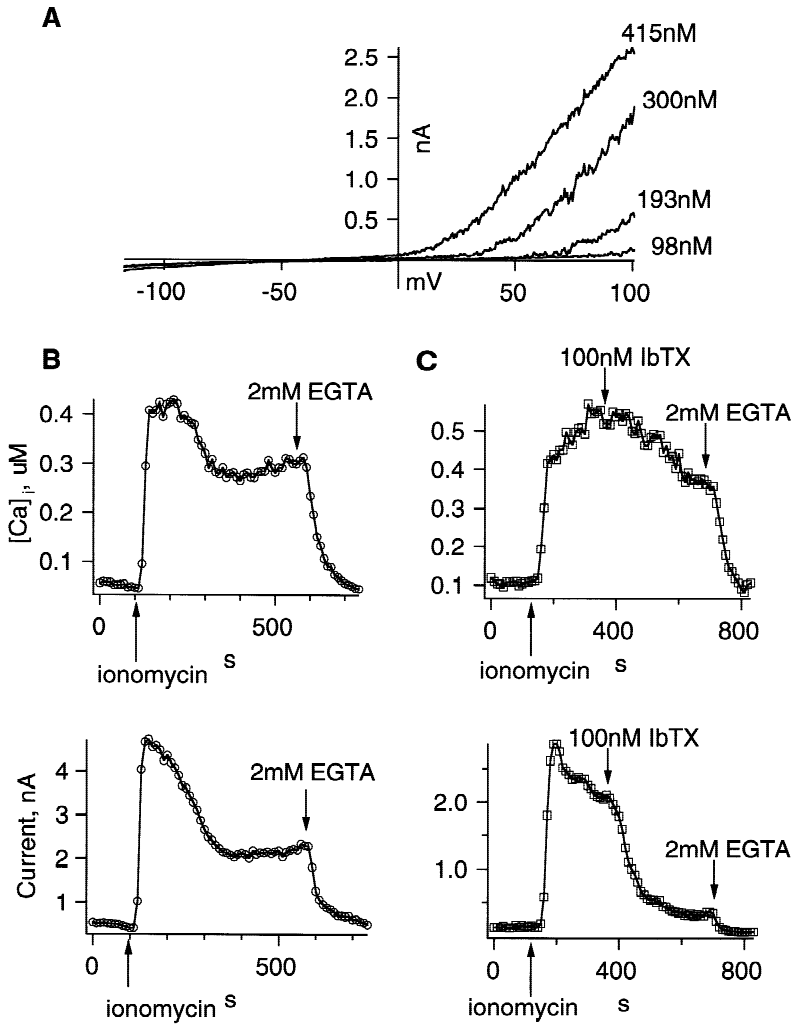

Fig. 6. Correlation of BKCA current with intracellular Ca^{2+} levels. (*A*) Current responses to 1 sec voltage ramps from −120 to +100 mV before and after application of 1 μM ionomycin to increase intracellular Ca^{2+} levels to the values indicated. Intracellular Ca^{2+} monitored with Fura2 ratio photometry. Intracellular pipette solution contains 50 μM Fura2 acid. (*B*) Intracellular Ca^{2+} on the top graph and peak outward current at +100 mV on the bottom graph for a different cell after application of 1 μM ionomycin. When the intracellular Ca^{2+} and corresponding outward current reach steady-state; the external solution is switched to one containing nominally free Ca^{2+} and 2 mM EGTA. This produced a fall in both intracellular Ca^{2+} levels and outward current as a result of the loss of Ca^{2+} entry. (*C*) Data from the same cell as in *A* but on a slower time scale. Intracellular Ca^{2+} is plotted on the top graph and peak outward current at +100 mV on the bottom graph. 100 nM iberiotoxin was added at 400 sec and produced a dramatic block of the BKCA current induced by Ca^{2+} entry through ionomycin.

have not been described. We observed an alteration in the ionic conductances of human capillary endothelial cells (HCEC) when they are allowed to condition their own media. Specifically before conditioning, HCEC contain only an inward rectifier, delayed rectifier, and a noninactivating chloride current (Fig. 1 and Jow, Numann & Colatsky, 1996). After conditioning, the cells acquire a Ca^{2+}-activated K current (BKCA) which is blocked by iberiotoxin, and a transient outward current (A current) blocked by 4AP. Substitution of conditioned media from human capillary endothelial cells to similar cultures maintained in nonconditioned media quickly induced (within 2 days) the BKCA and A type outward currents in the unconditioned cells. Endothelial cells are known to produce and release a variety of growth factors such as platelet-dereived growth factor (PDGF) and fibroblast growth factor (FGF) as well as prostacylin, endothelin, plasminogen activator inhibitor-1 (PAI-1), and nitric oxide (NO) Inagami et al., 1995. These as well as other unknown factors released into the media by the human capillary endothelial cells may be inducing the ionic currents described. Our results help to explain some of the variations in ionic currents reported by different groups studying the ion channels in cultured endothelial cells (Nilius et al., 1997; Revest et al., 1992).

Calcium-activated potassium channels have been described from a variety of endothelial cell preparations (Nilius et al., 1997) including rabbit aortic (Rusko et al., 1992; Sakai, 1990) and bovine aortic endothelial cells (Colden-Stanfield et al., 1990; Fichtner et al., 1987; Sauvé et al., 1990; Sauvé et al., 1988). Only two of these studies have characterized these currents at the whole cell level from unstimulated cells (Rusko et al., 1992; Sakai, 1990) where they see peak currents near 300 pA for a 100 mV depolarization. Pharmacological characterization showed that the channels were blocked by low levels of TEA, TBA, and charybdotoxin. Many investigators have failed to find evidence for Ca^{2+} activated K currents in similar endothelial cell preparations (Nilius et al., 1997; Revest et al., 1992).

We find that HCEC maintained in normal media also have no Ca^{2+}-activated K^+ currents and are insen-

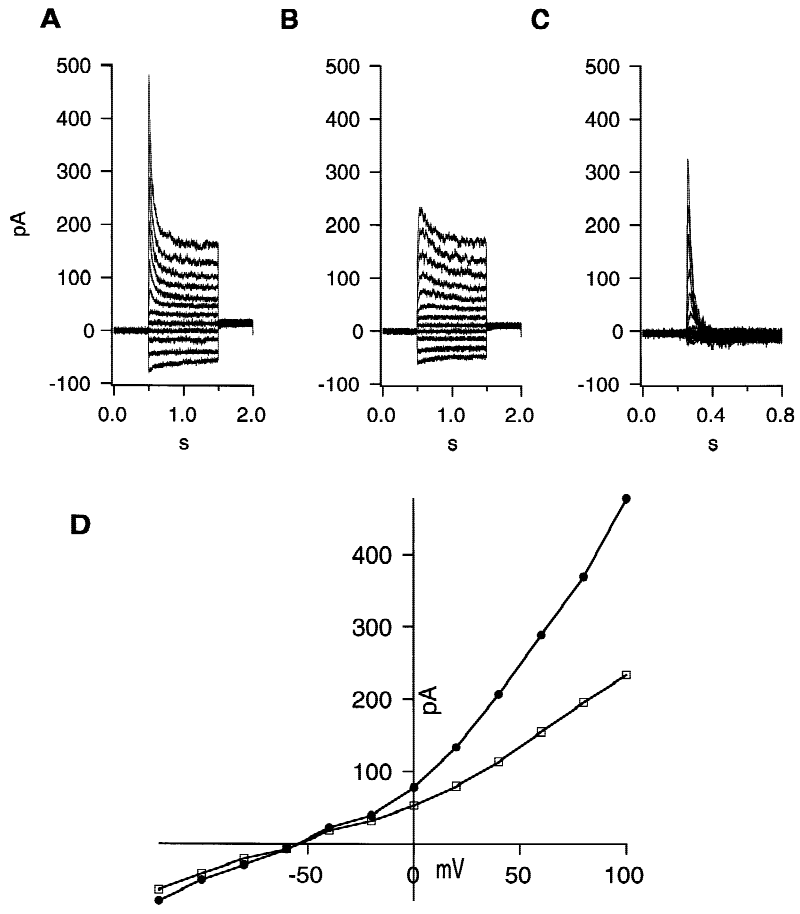

Fig. 7. The transient outward current seen in 24.7% of the cells from conditioned media can be blocked by 4-aminopyridine (4AP). (*A*) Current traces from a HCEC maintained in conditioned medium. The cell displays a prominent transient outward current at potentials above 0 mV (voltage from −120 to +100 mV in 20 mV steps, holding potential = −60 mV), x axis: (s = time in seconds). (*B*) The same cell after application of 3 mM 4AP. The 4AP blocked most of the transient outward current in this cell. (*C*) The difference current before and after 3 mM 4AP illustrates the fast kinetics of the 4AP sensitive component of the transient outward current. (*D*) Current-voltage relation of the transient outward current in the absence of 4AP (close circles) and in the presence of 4AP (open squares).

sitive to iberiotoxin. However after growing in conditioned media for four days or more we were able to detect a large Ca^{2+}-activated K current (467 ± 8 pA at +100 mV, $n = 27$) in over 50% of all cells. This current was blocked by 100 nM iberiotoxin and enhanced by NS1619. Simultaneous fura ratio imaging and whole cell voltage clamp after addition of ionomycin showed a strong correlation between intracellular calcium levels and iberiotoxin sensitive outward currents. The Ca^{2+} sensitivity of different BKCA channels varies over a wide range depending on the tissue and species used (Latorre et al., 1989). BKCA in guinea-pig endocardial endothelial cells (Manabe et al., 1995) and *Necturus* Enterocytes (Sheppard et al., 1991) show activation at >100 nM Ca^{2+} compared to BKCA in frog and toad muscle (Berger, Grygorcyk & Schwarz, 1984) and rabbit smooth muscle cells (Benham et al., 1985) which activate at 10 nM $[Ca^{2+}]_i$. Activation of BKCA in HCEC was observed at 150 nM $[Ca^{2+}]_i$. Half-maximal activation of BKCA in rabbit aortic endothelial cell was reported as approximately 1 μM (Rusko et al., 1992). In HCEC Hill equation fits of the rising phase of intracellular calcium after ionomycin and the corresponding peak outward current gave an EC_{50} of 213 ± 30 nM for Ca^{2+} activation of iberiotoxin sensitive outward current at +100 mV ($n = 3$).

The rate of calcium entry through the plasma membrane is modulated by the driving force for calcium. Membrane depolarization by elevation of extracellular K^+ or voltage clamp reduces the agonist-stimulated Ca^{2+} influx in vascular endothelial cells (Lasky et al., 1990; Luckhoff & Busse, 1990). Currents such as BKCA will tend to provide a large voltage gradient for calcium entry by hyperpolarizing the cell. The activation of BKCA channels concomitant with the receptor-mediated increases in intracellular calcium is important in maintaining the driving force for calcium entry (Adams et al., 1989). Endothelial cells secrete both the endothelial-derived relaxing factor (EDRF), likely to be nitric oxide (NO), and the constricting factor endothelin. The mechanisms of secretion is unclear, but NO secretion has been shown to be modulated by changes in membrane potential and $[Ca^{2+}]_i$ (Luckhoff & Busse, 1990).

A rapidly activating, transient outward K^+ current elicited by depolarizing steps has been described in cultured endothelial cells obtained from bovine aorta (Takeda, Schini & Stoeckel, 1987), bovine pulmonary artery (Silver & DeCourseky, 1990), and rabbit corneal endo-

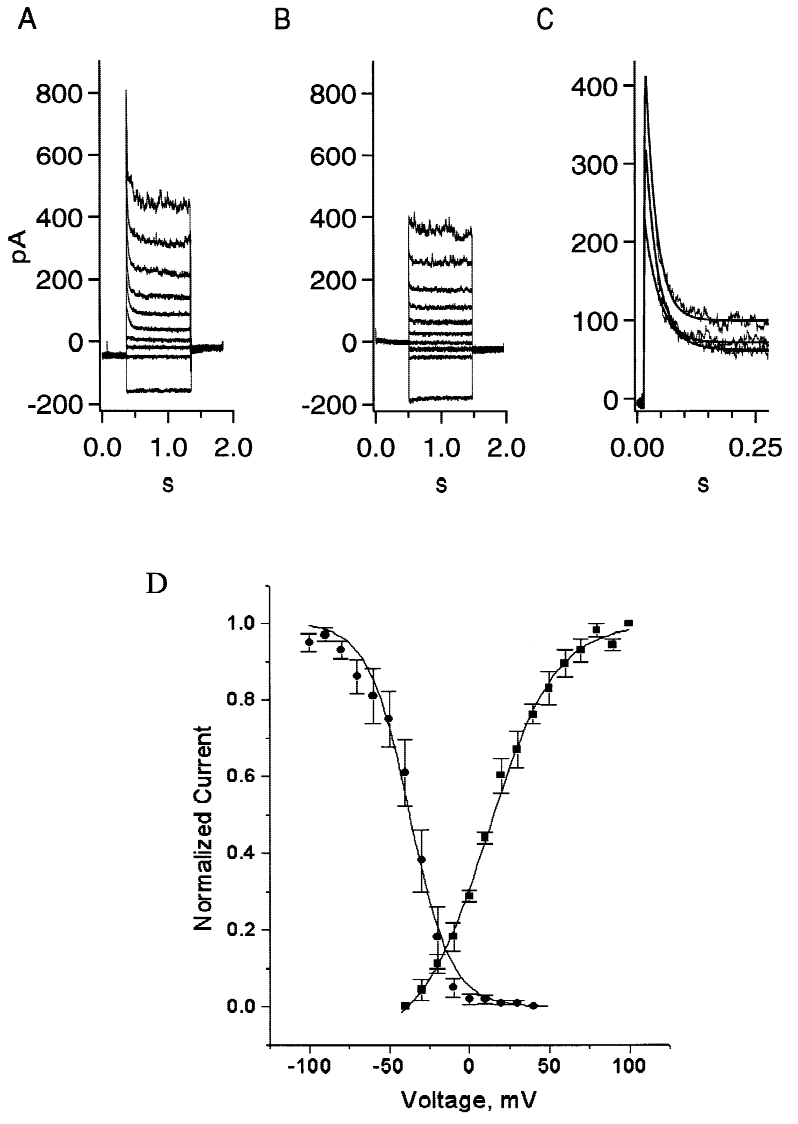

Fig. 8. The inactivation time course of transient outward current. (*A*) Current traces elicited from −120 to 100 mV with 20 mV steps at a holding potential of −60 mV. (*B*) The same voltage pulses with a holding potential of −20 mV, x axis: (s = time in seconds). (*C*) The subtracted current traces at 20, 40 and 60 mV and the single exponential fits overlapping the current traces. (*D*) Steady-state activation and inactivation conductance-voltage plots for the transient outward current seen in HCEC maintained in conditioned media. Peak conductance calculated from difference traces before and after 3 mM 4AP. For steady-state activation the cell is held at −80 mV for 500 msec then pulsed from −80 to +100 mV in 10 mV steps. For steady-state inactivation the cell is held at −60 mV and a 500 msec prepulse applied from −100 to +40 mV in 10 mV steps. A test pulse is immediately given to +80 mV for 1 sec. Boltzman fits to steady-state activation and inactivation give a mean half activation of 12.0 ± 1.8 mV and mean half inactivation of −40.0 ± 1.6 mV (n = 5). The average slope value for activation was 17.5 ± 1.1 and the slope for inactivation was 10.4 ± 1.5. The window current arising from the overlap region of the two Boltzmans had a peak at 17.8 ± 4.6 mV with 12.6 ± 2.8% of the maximum conductance (n = 5).

thelium (Watsky, Cooper & Rae, 1992). In the rabbit corneal endothelium, 94% of all cells contained an A type current with peak currents ranging between 70–200 pA. The $V_{1/2}$ of inactivation was 3.5 mV and $V_{1/2}$ of inactivation −42 mV compared to 12 and −40 mV for steady-state activation and inactivation in these HCEC. The data for our steady-state activation and inactivation curves clearly show the presnce of a significant window current with a peak near −18 mV and an amplitude of 12.6% of the maximum outward current. Watsky et al. (1992) also report a window current around −25 mV but this is only true for the fitted Boltzmann and is not apparent from the data points themselves. The A current they observed inactivated with two time constants of 18 and 92 msec, and was voltage independent. The A current in HCEC inactivate with a single exponential ranging from 33 ± 4 msec for a 20 mV test pulse and 25 ± 3 msec for a +80 mV test pulse. The A current in rabbit corneal endothelial cells requires 350 msec to completely recover from inactivation produced by a test pulse to +85 mV. This is in sharp contrast to the 4 seconds required for full recovery of A current inactivation in our HCEC (n = 3). This suggests that the A current in these HCEC may be similar to the cloned A current Kv1.4 (Engel, Rabba & Schild, 1996; Roeper, 1997). Taken together these differences suggest that the A current in HCEC is not the same as that seen in rabbit corneal endothelium.

In vascular preparations, Takeda (1987) saw an A-type current in about one-third of all cultured bovine aortic endothelial cell. The current is activated near −10 mV, as is ours is inactivated by depolarizing prepulses, and is blocked by 5 mM 4AP. They suggest that the appearance of the A current may be linked to time in culture and that it is most often present in cells from older primary and secondary cultures. This correlates well with our observation that the A current is only pres-

ent in cells allowed to condition their media. Takeda (1987) changed the media of their cultures every 2–3 days and as the density of the cells increases in the culture, one can easily see how the media could become conditioned. Unlike our results in HCEC, they did not report any additional outward currents from endothelial cells expressing A currents. This difference may reflect true differences in bovine aortic and human capillary endothelial cells or in culturing conditions.

Himmel et al. (1994) have shown that inhibitors of the cyclo-ocygenase pathway block the calcium-sensitive ionic currents activated by bradykinin in bovine aortic endothelial cells. It therefore seemed reasonable to test the effects of a cyclo-ocygenase inhibitor, as well as other blockers of known endothelial cell signaling pathways such as nitric oxide and lypo-oxgenase, on the induction of the ionic currents. We found that the induction of BKCA and/or A current was not inhibited by incubating the cells with 10 μM N(5)-(1-iminoethyl)-L-ornithine (L-NIO), a potent inhibitor of endothelial NOS, plus 10 μM indomethacin, a cyclo-oxgenase inhibitor, plus 1 μM meclomen, a lypo-oxgenase inhibitor either before or after current expression. We therefore conclude that other modulatory factors secreted by the endothelial cells must be mediating the effects seen on ion channel expression.

The changes in ionic currents seen in this study after conditioning of media suggest that human capillary endothelial cells are extremely sensitive to their external environment and can alter their phenotype in response to external cues.

The physiological relevance of the changes in ion channels in this study is unknown, but these changes would likely modulate the response of these cells to agonists. The plasticity of these cultured human endothelial cells suggest that media and culturing conditions must be carefully controlled when studying the physiology of these cells.

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