Bradykinin-Induced Increases in Cytosolic Calcium and Ionic Currents in Cultured Bovine Aortic Endothelial Cells

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The goal of the present study was to determine if voltage-sensitive calcium channels are present in bovine aortic endothelial cell plasmalemma and if they contribute to the rise in cytosolic calcium produced by bradykinin. After bradykinin (100 nM) exposure, endothelial cell associated fura-2 fluorescence peaked within 10–20 seconds and then declined to a steady level 2- to 3-fold above resting values. Pretreatment with lanthanum (20 μM) abolished the steady level produced by bradykinin but had little effect on the initial, transient rise in cytosolic calcium. Chelation of extracellular calcium with EGTA before addition of bradykinin resulted in a substantial decrease in the fura-2 transient and elimination of the long-lasting component. Nimodipine (3 μM) and nifedipine (1 μM) were without effect on either phase of the bradykinin-induced response. Moreover, elevation of extracellular potassium failed to produce a rise in intracellular calcium. With the use of the tight seal technique to voltage clamp the cells, inwardly rectifying and calcium-activated potassium currents were found to exist in the endothelial cells. Addition of bradykinin (100 nM) elicited a calcium-activated potassium current that was eliminated in the absence of intracellular potassium. No voltage-sensitive calcium currents were activated when the cells were exposed to 10 mM or 110 mM calcium chloride in the presence or absence of bradykinin. The binding of [3H](+)-PN200-110 to endothelial cell membrane preparations was 1–3 orders of magnitude lower than that observed in PC-12, GH4c1, or B1H1 cell membranes. Together, these results suggest that cloned bovine aortic endothelial cells lack voltage-sensitive calcium channels. Therefore, the changes in cytosolic calcium stimulated by bradykinin that are dependent on extracellular calcium must occur via some other calcium influx pathway. (Circulation Research 1987;61:632–640)

In 1980, Furchgott and Zawadzki1 discovered that acetylcholine produced a relaxation of precontracted aorta that required the presence of vascular endothelial cells. This relaxation occurred in response to a factor (or factors) released from the endothelial cell (endothelium-derived relaxing factor, EDRF). Since that time, the relaxation produced by several agents has been shown to depend on the presence of the vascular endothelium. The list of agents includes acetylcholine, histamine, adenosine triphosphate, adenosine diphosphate, the calcium ionophore A23187, substance P, thrombin, and bradykinin (BK).2 Evidence has accumulated which suggests that a rise in cytosolic calcium ([Ca]i) of the endothelial cell is involved in the release of EDRF produced by some agents, and it has been reported that the release of EDRF can be affected by both antagonists and agonists of the voltage-sensitive calcium channel.3 However, several recent studies have failed to discover effects of the calcium channel antagonists on endothelial-dependent responses in vascular smooth muscle.4–6 Thus, while the release of EDRF may be linked to a rise in [Ca]i of the endothelial cell, the role of voltage-sensitive calcium channels in this response remains controversial. The objective of the present study was to determine if voltage-sensitive calcium channels are present in the endothelial cell plasmalemma and if they contribute to the rise in [Ca]i, associated with stimulation by BK.

Materials and Methods

Culture of Bovine Aortic Endothelial Cells (BAECs)

BAECs were harvested and cultured as previously described except that the culture medium was Minimal Essential Medium supplemented with 10% fetal calf serum, 100 mcg/ml penicillin, and 100 mcg/ml streptomycin, and first passage cells were cloned into Microtest II plates (Falcon Labware, Houston, Tex.). All experimental data were obtained from BAECs in their 5th to 10th passage and at 1–10 days post-confluence. The contact inhibited cobblestone monolayer typical of cultured BAECs is shown in Figure 1. The presence of factor VIII related antigen confirmed that these were endothelial cells.10 Factor VIII-related antigen was demonstrated by indirect immunofluorescence,11 using antiserum to human factor VIII (Calbiochem-Behring, San Diego, Calif.), diluted 1:100, followed by FITC-conjugated antirab-
FIGURE 1. Phase micrograph of bovine aortic endothelial cells, fifth passage, 2 days postconfluency. Bar = 10 μm.

bit IgG (ICN ImmunoBiologicals, Cleveland, Ohio), diluted 1:20. Bovine aortic smooth muscle cell cultures provided negative controls. In addition, a second endothelial cell marker, angiotensin-converting enzyme (ACE), was determined by measuring the release of [3H]hippuric acid from [3H]hippuryl-glycyl-glycine,\textsuperscript{12} using the microvial assay system (Ventrex Laboratories, Portland, Me.) ACE activity was found to be 1.76 ± 0.21 U/mg protein when examined in membrane preparations isolated from cell cultures no more than 2 days postconfluency. This activity is low but not unexpected. ACE activity has been found to decrease during the growth phase of endothelial cells in culture and to increase dramatically when examined in cells cultured well past confluence.\textsuperscript{12} In line with this previous report, the ACE activity in our membrane preparation increased approximately threefold when the cells were cultured for an additional 7 days postconfluency.

Measurement of Whole Cell Membrane Potential and Currents

Intracellular whole cell recordings of subcultured BAECs were obtained at room temperature with a LIST-EPC7 amplifier (Darmstadt-Eberstadt, FRG) using the tight seal technique described by Hamill et al.\textsuperscript{13} Low resistance (2–5 MΩ) glass pipettes were pulled in two stages and fire-polished. The indifferent electrode was a Ag-AgCl plug connected to the bath via a 150-mM KCl agar bridge. Whole cell recordings were accomplished by first approaching the cell with the pipette and applying suction to obtain a cell-attached patch. Further suction resulted in disruption of the patch and access to the intracellular environment where the pipette solution readily exchanged with the cytoplasm of the cell. Currents were recorded in physiologic extracellular solution of the following composition (in mM): 137 NaCl, 5.4 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 glucose, and 10 HEPES, pH adjusted to 7.3 with NaOH (bath solution 1). The pipette solution contained 145 mM KCl, 10 mM HEPES, pH adjusted to 7.3 with KOH (pipette solution 1). In some experiments, 11 mM ethylene-glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) was also added to the pipette solution. In addition to recording whole cell currents, resting membrane potential was measured immediately on gaining access to the cell interior by the patch pipette. To identify and isolate calcium channel currents from other membrane currents, the following extracellular solution was used (in mM): 10 CaCl\textsubscript{2}, 5.6 KCl, 1 MgCl\textsubscript{2}, 10 glucose, 10 HEPES, 5 4-aminopyridine (4-AP), and 120 tetraethylammonium chloride (TEA), adjusted to pH 7.4 with KOH (bath solution 2). Pipettes were filled with an intracellular solution containing (in mM) 124 CsCl, 2 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 11 EGTA, 10 HEPES, pH 7.2 with CsOH (pipette solution 2). The response to BK was examined by addition of BK to the bath solution by manual pipetting or by pressure application from a micropipette placed near the cell. Both methods gave similar responses. Current traces were recorded on magnetic tape (Racal 4Ds recorder, Irvine, Calif.) at a
bandwidth of 5 kHz, later filtered at 1 kHz and digitized for computer analysis.

**Fura-2 Fluorescence Measurements of Intracellular Calcium Concentration**

[Ca] levels were monitored by measuring the fluorescence of fura-2. Endothelial cells were dispersed by incubation for 3 to 7 minutes in medium containing 0.25% trypsin and 1:5,000 ethylenediaminetetraacetic acid (EDTA) (GIBCO Laboratories, Gaithersburg, Md.). The cells (3 x 10^7) were centrifuged, washed, then incubated in 2 ml of a HEPES buffered HAM’s F10 medium containing 20 μM fura-2/AM. After 30 minutes of incubation at 37° C, the cells were diluted with 20 ml of HAM’s F10 medium and incubated for an additional 30 minutes. The cells were then centrifuged, washed with 20 ml of saline, placed in 20 ml of control saline, and stored at room temperature. Throughout the day, 2 ml aliquots were removed, centrifuged, resuspended in 1 ml of saline, and then transferred to a quartz cuvette. The cells (3–5 x 10^7/ml) were kept in suspension in the cuvette with a magnetic stirrer. Fluorescence was monitored at room temperature on a Perkin Elmer 650-10S spectrophotofluorometer (Norwalk, Conn.) at excitation and emission wavelengths of 342 and 498 nm, respectively. [Ca], was determined by using the equation:

$$[Ca] = \frac{(K_o)(F - F_{min})}{(F_{max} - F)}$$

The K_o for the Ca-fura-2 complex at room temperature is 135 nM. F is the fluorescence intensity emitted from intact cells, F_{max} is the maximum fluorescence obtained on disruption of the cells with 0.1% Triton X-100, and F_{min} is the minimum fluorescence obtained by a subsequent addition that results in a final solution that contains 15 mM K_2EGTA, 30 mM 3-(tris-(hydroxy-methyl)-methyl)-amino)-propanesulfonic acid (TAPS) buffer, pH 8.3. The autofluorescence from an equivalent number of cells that were not loaded with fura-2 was typically less than 10% of the F_{max}. The intracellular fura-2 concentration in these experiments was estimated to be 100 μM assuming a mean cell diameter of 15 μm. In a few rare experiments, addition of EGTA to intact cells caused a sharp decrease in fluorescence, indicating that a small amount of fura-2 was present in the extracellular solution, probably via leakage from damaged cells. In such experiments, F_{max} and F_{min} were appropriately reduced to reflect only the amount of fura-2 present internally. La^{2+} (20 μM) slightly increases F_{min} with no effect on F_{max}. Since no correction was made, the cytosolic calcium values reported in the presence of La^{2+} may be underestimates of the actual levels.

**Isolation of Membrane Preparations from BAECs**

The medium was aspirated from 10–20 100-mm culture dishes. Approximately 3 ml of ice-cold PBS containing 50 mM sodium phosphate and 150 mM NaCl, pH 7.4, were added to each dish. The endothelial cells were then removed from each dish by gently scraping with a rubber policeman. The suspended cells were placed in a 50-ml conical, plastic, culture tube and centrifuged at 100g for 10 minutes to pellet the cells. The supernatant was discarded, and the resulting cell pellets were resuspended in 45 ml of ice-cold 5 mM Tris/Cl, pH 7.4, at 2° C and allowed to sit on ice for 30–45 minutes. The cell lysate was homogenized in a glass tube with a motor driven Teflon pestle (10 times at 1,800 rpm). The cell homogenate was centrifuged at 1,500g for 20 minutes. The resulting pellets were discarded, and the supernatant was centrifuged at 48,400g for 20 minutes. The resulting microsomal pellet (M) was resuspended in approximately 1 ml of 10 mM Tris/Cl, pH 7.4, at 22° C. In some experiments, the cells were removed from the culture dishes by treatment with trypsin-containing culture medium for 3–7 minutes. The resulting cell suspensions were then treated as described above for cells isolated by scraping. Protein in the M preparation was determined by the method of Lowry with bovine serum albumin as the standard. The M preparation was stored at 2° C and used within 24 hours following the completion of isolation.

**Radioligand Binding Assays**

The binding of the calcium channel antagonist [H](+)PN200-110 was determined essentially as described previously. Aliquots of M preparation (0.2–0.3 mg protein) were added to reaction media (4 ml) at room temperature in glass tubes containing 150 mM KCl, 10 mM Tris/Cl, pH 7.4, and 0.3–0.4 nM [H](+)PN200-110. Nonspecific binding was determined by addition of 100 nM unlabelled nitrendipine to the reaction medium. All experiments were carried out in the dark and in duplicate.

Specific [H]ouabain was determined by the method of Inagaki et al at 37° C in the presence of 5 mM Tris/phosphate, 5 mM MgCl_2, and 50 mM Tris/Cl, pH 7.4, and 2 μM ouabain with [H]ouabain (100–200 cpm/pmol). Nonspecific binding was determined by including 1 mM unlabelled ouabain in the binding reaction.

**Results**

**Effect of Bradykinin on Cytosolic Calcium**

The resting [Ca], in dispersed BAECs was determined by measurement of the fluorescence intensity of the calcium indicator, fura-2. Basal [Ca], in control saline (bath solution 1) was found to be 64 ± 7 nM (mean ± SEM of 18 measurements). Addition of BK (100 nM) resulted in a rapid increase in [Ca], that peaked within 10–20 seconds and then declined with time to a steady level that was 2 to 3 times above the resting concentration at time 3 minutes (Figure 2, trace A). On incubation of the endothelial cells with 20 μM LaCl_3 (La^{3+}) for 2 minutes prior to the addition of BK, the initial, transient rise was still present; however, the long-lasting component was completely eliminated (Figure 2, trace B). Incubation of the cells in nominally calcium-free saline or chelation of extracellular calcium with EGTA resulted in a substantial decrease in
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Further evidence that BAECs lack voltage-sensitive calcium channels. The possibility remains, however, that BK opens receptor-operated calcium channels present in the surface membrane of the BAECs.

Patch-Clamp Recording of BAEC Membrane Potential and Currents

Resting membrane potential, which was monitored in current-clamp and voltage-clamp modes with bath and pipette solutions 1, was found to be $-68 \pm 5$ mV (mean $\pm$ SEM of 25 measurements). The calculated $E_K$ was $-83$ mV, suggesting that $K^+$ permeability is a major determinant of the resting membrane potential of the BAECs under these conditions. Similar results were seen in initial isolates, dividing or confluent cells, and in cells with and without trypsin treatment.

Measurement of whole cell membrane currents using solutions containing the normal extracellular ionic composition (bath solution 1) and with $[Ca]_o$ buffered with 11 mM EGTA (pipette solution 1) revealed only an inwardly rectifying $K^+$ current (Figure 3). This current was blocked by addition of barium chloride (1 mM) to the bath solution (Figure 4). Even when BK was added to the extracellular solution, no additional currents were observed. If $[Ca]_o$ was not buffered with EGTA, exogenous BK (100 nM) produced an outward current. This outward current was not present in the absence of intracellular $K^+$, suggesting that BK stimulated calcium-activated $K^+$ currents in BAECs (Figure 4A and 4B).

Evaluation of membrane currents under ionic conditions designed to suppress $K^+$ channel activity and increase the likelihood of detection of calcium channel currents (bath and pipette solutions 2) failed to reveal any inward current (total 38 cells) over the range of potentials that would be expected to activate voltage-dependent calcium channels in nerve or muscle (Figure 5). When $(-)$BAY K 8644, a calcium agonist, was added to the bath in concentrations up to 1 $\mu$M, no currents were elicited ($n=5$ cells). Addition of BK to the extracellular solution, again, produced no current ($n=8$ cells tested). Calcium currents also were not produced when the same experiments were performed in 110 mM CaCl$_2$ or BaCl$_2$ extracellular solution ($n=9$

Table 1. Effect of Calcium-Free Saline and Dihydropyridines on Bradykinin Induced Increases in Cytosolic Calcium Levels

<table>
<thead>
<tr>
<th>Condition</th>
<th>Basal level</th>
<th>Peak</th>
<th>3 min</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$56 \pm 6$ nM</td>
<td>$259 \pm 16$ nM</td>
<td>$128 \pm 9$ nM</td>
<td>4</td>
</tr>
<tr>
<td>Calcium-free</td>
<td>$41 \pm 9$ nM</td>
<td>$95 \pm 18$ nM*</td>
<td>$47 \pm 11$ nM*</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>$55 \pm 7$ nM</td>
<td>$247 \pm 14$ nM</td>
<td>$125 \pm 13$ nM</td>
<td>3</td>
</tr>
<tr>
<td>Dihydropyridine</td>
<td>$53 \pm 3$ nM</td>
<td>$219 \pm 25$ nM</td>
<td>$106 \pm 5$ nM</td>
<td>3</td>
</tr>
</tbody>
</table>

For the calcium-free condition, cells were in nominally calcium-free ($n=2$) or EGTA containing saline for 2 to 5 minutes before addition of BK. Dihydropyridine-treated cells were in 5 $\mu$M nimodipine for 2 minutes ($n=1$) or 1 $\mu$M nitrendipine for 5 minutes before addition of BK. The cytosolic calcium levels are expressed as the mean $\pm$ SEM.* Mean values that are significantly different from controls at the 0.05 level (Student's $t$ test).
cells). These electrophysiologic results suggest that BAECs lack voltage-dependent calcium channels.

To test the possibility that BAECs possess surface membrane calcium channels that are activated by the change in [Ca\textsuperscript{2+}], associated with stimulation by BK, membrane currents were recorded without buffering the [Ca\textsuperscript{2+}]. Again, BK failed to activate any inward calcium currents when recorded from BAECs bathed in extracellular solution 2, despite the absence of EGTA in the pipette (n = 5 cells tested).

Similar results have been obtained in BAECs allowed to grow in culture for 3–8 days postconfluency and in acutely isolated endothelial cells obtained from bovine and canine aortas.

**Measurement of Dihydropyridine Calcium Channel Antagonist Binding in Isolated BAEC Membrane Preparations**

Membrane preparations were obtained by hypo-osmotic cell lysis followed by differential centrifugation. This procedure yielded 1.5–2 mg of membrane protein when isolated from fifteen 100-mm culture dishes containing confluent endothelial cell monolayers. The specific binding of ouabain to the Na,K-ATPase pump was used as an indicator for the presence of surface membrane in this microsomal preparation. Ouabain binding was 8.98 ± 0.9 pmol/mg protein, a value similar to that obtained for smooth muscle sarcolemma preparations.

The specific binding of the dihydropyridine calcium antagonists has been used as an indicator of the presence of voltage-sensitive calcium channels in both membrane preparations and in whole cells from a variety of species and tissue types, including many cell lines grown in culture (for a recent review, see Triggle and Janis\textsuperscript{18}). The binding of (+)PN200-110, a specific high affinity ligand for the dihydropyridine binding site, was found to be 5.1 ± 2.1 fmol/mg protein in the endothelial cell membrane preparation when measured at a free (+)PN200-110 concentration of 0.3–0.4 nM. This level of binding is at the limit of detection and required the use of high (>200 mg) protein per assay. Furthermore, specific binding represented only 10% of the total binding observed under these binding conditions. Binding was examined in membrane preparations obtained from cells removed from the culture dishes by trypsinization and by scraping, with similar (+)PN200-110 binding results for each. As a positive control, the binding of (+)PN200-110 to membranes isolated from BC\textsubscript{1}H\textsubscript{1} cells, a clonal cell line with skeletal muscle characteristics, was measured. At a free (+)PN200-110 concentration of 0.4 nM, specific binding was 75.2 fmol/mg protein; specific binding represented 70% of the total binding observed under these conditions.

![FIGURE 3. Whole cell recordings of BAECs. Cells were bathed in solution 1 with the pipette containing pipette solution 1 and 11 mM EGTA. Cells were held at a membrane potential of −70 mV. Hyperpolarizing steps to −140 mV and depolarizing steps to +80 mV were applied. Inset shows the current-voltage relation. The currents in this and following figures are shown before correction of capacitative currents.](image-url)
Figure 4. Whole cell recordings of BAECs. Cells were bathed in solution 1, and the pipette contained solution 1. The bath contained 1 mM BaCl₂ to block the inwardly rectifying potassium current. Cells were held at a membrane potential of —70 mV. Hyperpolarizing steps to —100 mV and depolarizing steps to +40 mV were applied to obtain the control response on the left (A). BK (100 nM) added to the bath elicited outward current on step depolarization, as shown on the right (B); 14 of 15 cells responded to BK addition. The inset shows the current-voltage relation before (△) and after (▲) BK addition.

Figure 5. Whole cell recordings of BAECs. Cells were bathed in bath solution 2, and the pipette contained pipette solution 2. No voltage-dependent calcium currents were produced when the cells were held at a membrane potential of —70 mV and step depolarized to +80 mV or step hyperpolarized to —140 mV. The inset illustrates the current-voltage relation.
Discussion

The release of relaxing factors from vascular endothelial cells in response to agonists requires the presence of extracellular calcium. In the present study, experiments were designed to test the hypothesis that calcium crosses the surface membrane of endothelial cells via voltage-sensitive calcium channels. Three lines of evidence suggest that this hypothesis is false. First, patch-clamp measurements of whole cell currents indicated that inwardly rectifying and calcium-activated K+ channels appear to be the only functional channels evident in the BAEC membrane, even when measured under ionic conditions specifically designed to reveal calcium channels. Second, while the change in fluorescence of BAEC-associated fura-2 indicates that a long-lasting increase in [Ca2+], produced by BK was dependent on the presence of extracellular calcium, both nimodipine and nitrendipine, calcium channel antagonists, were without significant effect on the BK-induced response. Furthermore, elevation of extracellular K+, a condition that depolarizes the membrane and opens voltage-sensitive calcium channels in many cell types, failed to produce a change in [Ca2+]. Third, measurement of specific high-affinity dihydropyridine binding in isolated membrane preparations from BAECs was extremely low and at the technical limit of detection for currently available calcium channel ligands. Taken together, these results demonstrate that the changes in BAEC [Ca2+]i associated with stimulation by BK are not the result of calcium influx through voltage-sensitive calcium channels.

Calcium channel agonists and antagonists have been used by others to implicate voltage-sensitive calcium channels in the release of EDRF, although results from these experiments are contradictory. Verapamil and nifedipine were used by Singer and Peach to inhibit partially the endothelial-dependent A23187- and methacholine-induced relaxation of rabbit aortic rings. Using a "cascade" assay method, in which tension was recorded from a canine coronary arterial ring denuded of endothelium and perfused with the effluent from a femoral artery with intact endothelium, Rubanyi et al. have demonstrated that low concentrations of BAY K 8644 (5 pM—2.5 nM) present in the femoral artery superfusate produced a relaxation of the precontracted bioassay ring. Addition of nitrendipine (0.05—1 nM) to the superfusate at the same locus prevented the BAY K 8644-induced relaxation of the bioassay ring, suggesting that calcium influx through voltage-dependent calcium channels in endothelial cells may enhance EDRF release. However, these results may be species dependent. Similar experiments performed by Spedding et al. failed to reveal relaxant effects of BAY K 8644 on rat aorta preparations, and these authors conclude that calcium entry via voltage-operated channels is not the trigger for EDRF release. Consistent with the present study is a report from Schoeffter and Miller, which shows that the calcium channel antagonists verapamil, nifedipine, diltiazem, and bepridil did not affect endothelial-dependent acetylcholine-stimulated vascular smooth muscle cyclic guanosine monophosphate levels in a rat aortic preparation. Furthermore, several other studies failed to find an effect of calcium channel antagonists on endothelial-dependent responses. A recent report, Peach et al. demonstrated that a putative calcium channel agonist, maitotoxin, could produce relaxation in precontracted rabbit aortic rings with an intact endothelium. However, this response was not blocked by equilibration of the tissue with verapamil prior to the addition of maitotoxin (see Figure 3 of Peach et al.), suggesting that this action of maitotoxin was not via calcium channels in endothelial cells. These authors conclude that most of the agonist-stimulated divalent cation influx is via a mechanism that is not sensitive to calcium channel blockers. Finally, Hallam and Pearson found no effect of nifedipine, diltiazem, or D600 at concentrations up to 20 μM on the ATP-stimulated changes in fura-2 fluorescence in piglet endothelial cells.

The reasons for these discrepancies in reported findings are not obvious. Clearly, the evaluation of drug effects on intact tissue is complex. These drugs could have effects at the level of the endothelial cell or at the smooth muscle, or they may influence the half-life and/or the effect of EDRF once released from the endothelial cell. Likewise, studies that employ cultured endothelial cells are complicated by growth variables, such as cell passage number, days postconfluence, and culture medium composition. With regard to these cell culture variables, similar electrophysiologic results have been obtained in the present study when measurements were made on acutely isolated bovine and canine aortic endothelial cells and on cells cultured for various days postconfluence. However, that some specific growth factor necessary for the expression of functional calcium channels is absent from the culture medium employed is a possibility that cannot be eliminated.

Membranes isolated from cultured BAECs have an extremely low density of high-affinity dihydropyridine calcium channel antagonist binding sites when compared with membranes isolated from several cell lines known to possess calcium channels. For example, specific (+)PN200-110 binding experiments in GH3 cell membranes yielded a Bmax of 800 fmol/mg protein and a Kd of 92 pM (D. Rampe, D. Triggle, and A.M. Brown, unpublished observations). Likewise, (+)PN200-110 binding to membranes from BCh1 cells, a cell line with skeletal muscle characteristics, exhibits a Bmax of 150—300 fmol/mg and a Kd of approximately 300 pM (D. Rampe and W.P. Schilling, unpublished observations), and it has been reported that PC-12 cell membranes have a Bmax of 30—45 fmol/mg and a Kd of 40—79 pM. Thus, the value of 5.1 fmol/mg obtained in the BAEC membranes was 6—10 times lower than that of the PC-12 cells and several orders of magnitude lower than that of the BCh1 and GH3 cells.

No significant effect of nitrendipine on the BK-induced changes in cytosolic calcium were observed in the BAECs as measured by fura-2. A large and significant decrease in the BK-stimulated cytosolic calcium channel agonist binding sites when compared with membranes isolated from several cell lines known to possess calcium channels. For example, specific (+)PN200-110 binding experiments in GH3 cell membranes yielded a Bmax of 800 fmol/mg protein and a Kd of 92 pM (D. Rampe, D. Triggle, and A.M. Brown, unpublished observations). Likewise, (+)PN200-110 binding to membranes from BCh1 cells, a cell line with skeletal muscle characteristics, exhibits a Bmax of 150—300 fmol/mg and a Kd of approximately 300 pM (D. Rampe and W.P. Schilling, unpublished observations), and it has been reported that PC-12 cell membranes have a Bmax of 30—45 fmol/mg and a Kd of 40—79 pM. Thus, the value of 5.1 fmol/mg obtained in the BAEC membranes was 6—10 times lower than that of the PC-12 cells and several orders of magnitude lower than that of the BCh1 and GH3 cells.

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Ca2+ change was seen in calcium-free buffer (Table 1), confirming the role of extracellular Ca2+ in this response. The observed decrease in the peak level of cytosolic Ca2+ may reflect depletion of intracellular stores under this condition. The fura-2 experiments clearly indicate that the late phase of elevated [Ca2+] produced by BK is not observed in the presence of La3+ (Figure 2) and is dependent on the presence of extracellular calcium (Table 1).

These observations must be reconciled with the lack of a demonstrated mechanism of calcium influx through membrane channels. The possibility still exists that BK opens receptor-activated calcium channels in the plasma-membrane. These may require an intracellular messenger that has been diluted by equilibration with the pipette solution during the whole cell recording. Sodium/calcium exchange has also been suggested by Winquist et al.26 and by Schoeffter and Miller27 as a possible mechanism for endothelial-dependent acetylcholine-induced relaxation of rat aortic rings.

To date, resting membrane potential has been the only electrophysiologic property measured in vascular endothelial cells. With the use of the glass microelectrode technique, measurement of the resting membrane potential of different vascular endothelial cells has been reported to range from -8 mV to -41 mV.26,27 The technique employed in the present study offers an advantage over the usual recording method with glass microelectrodes because it minimizes the leakage due to cell penetration with the microelectrode. A resting membrane potential of -68 mV was obtained in BAECs measured under conditions where internal K+ was 145 mM and intracellular free calcium was less than 10-7 M. This finding suggests that the resting membrane potential is primarily determined by the permeability of the membrane to K+ ions (E\textsubscript{k} = -83 mV). A value of intracellular potassium of 130 mM has been reported in BAECs.26 This gives a calculated E\textsubscript{k} of approximately -80 mV in 5.4 mM K+. If the equilibration of the cytoplasm with the pipette solution alters or eliminates an ion channel that would otherwise contribute to the resting potential, this effect would not be reflected in the membrane potential measured here.

Gordon and Martin28 have demonstrated that BK (0.1-100 nM) dose-dependently relaxed precontracted pig aortic strips. These investigators also showed that BK increased 86Rb efflux from cultured pig aortic endothelial cells, and they speculated that this increase was dependent on a rise in [Ca2+], and subsequent activation of Ca2+-activated K+ channels. With the use of an electrophysiologic measurement, our study has shown that BK produced an outward current consistent with the activation of K+ channels by the rise in [Ca2+]. These measurements represent the first description of ionic currents in vascular endothelial cells. The functional role of the calcium-activated K+ current produced by BK and/or its modulation by cellular second messengers is, at present, unknown. The continued biochemical/electrophysiologic approach should prove useful in this regard.

References


**KEY WORDS** • bradykinin • fura-2 • currents • endothelial cells • voltage-sensitive calcium channels
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