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Bradykinin-Induced Potassium Current in Cultured Bovine Aortic Endothelial Cells

Margaret Colden-Stanfield*, William P. Schilling, Lourival D. Possani**, and Diana L. Kunze
Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030

Summary. Bovine aortic endothelial cells (BAECs) respond to bradykinin with an increase in cytosolic-free Ca^{2+} concentration, $[Ca^{2+}]_i$, accompanied by an increase in surface membrane K^+ permeability. In this study, electrophysiological measurement of K^+ current was combined with $^{86}Rb^+$ efflux measurements to characterize the K^+ flux pathway in BAECs. Bradykinin- and Ca^{2+}-activated K^+ currents were identified and shown to be blocked by the alkylammonium compound, tetrabutylammonium chloride and by the scorpion toxin, *noxiustoxin*, but not by apamin or tetraethylammonium chloride. Whole-cell and single-channel current analysis suggest that the threshold for Ca^{2+} activation is in the range of 10 to 100 nM $[Ca^{2+}]_i$. The whole-cell current measurements show voltage sensitivity only at the membrane potentials more positive than 0 mV where significant current decay occurs during a sustained depolarizing pulse. Another K^+ current present in control conditions, an inwardly rectifying K^+ current, was blocked by Ba^{2+} and was not affected by *noxiustoxin* or tetrabutylammonium chloride. Efflux of $^{86}Rb^-$ from BAEC monolayers was stimulated by both bradykinin and ionomycin. Stimulated efflux was blocked by tetrabutyl- and tetrapentyl-ammonium chloride and by *noxiustoxin*, but not by apamin or furosemide. Thus, $^{86}Rb^+$ efflux stimulated by bradykinin and ionomycin has the same pharmacological sensitivity as the bradykinin- and Ca^{2+}-activated membrane currents. The results confirm that bradykinin-stimulated $^{86}Rb^+$ efflux occurs via Ca^{2+}-activated K^+ channels. The blocking agents identified may provide a means for interpreting the role of the Ca^{2+}-activated K^+ current in the response of BAECs to bradykinin.

Key Words endothelial cells · K^+ channels · $^{86}Rb^+$ efflux · bradykinin · cytosolic-free Ca^{2+} · *noxiustoxin*

Introduction

Stimulation of vascular endothelial cells (VECs) by bradykinin (BK) results in the release of endothelium-derived relaxing factor (EDRF) suggested to be nitric oxide (Palmer, Ferrige & Moncada, 1987). Substantial evidence has now accumulated suggesting that a rise in cytosolic-free Ca^{2+} concentration $[Ca^{2+}]_i$, of VECs is necessary and sufficient for the production and release of EDRF (Zawadzki, Cherry & Furchgott, 1980; Singer & Peach, 1982; Long & Stone, 1985; Winquist, Bunting & Schofield, 1985). Of the EDRF-releasing agents tested to date, the majority have been shown to elevate $[Ca^{2+}]_i$ of VECs (Hallam & Pearson, 1986; Luckhoff & Busse, 1986; Rotrosen & Gallin, 1986; Colden-Stanfield et al., 1987; Morgan-Boyd et al., 1987). While the exact mechanism by which elevated $[Ca^{2+}]_i$ triggers the release of EDRF remains unknown, a variety of cellular events are undoubtedly affected by the rise in Ca^{2+}. Ca^{2+}-activated pathways could play an important role in the duration and/or magnitude of the release response.

Gordon and Martin (1983) demonstrated that potent releasing agents such as BK and the Ca^{2+} ionophore, A23187, produced an increase in $^{86}Rb^+$ efflux from VECs which may occur through Ca^{2+}-activated K^+ channels. Additional support for this hypothesis was obtained by Schilling et al. (1988) who showed that under a variety of conditions, $^{86}Rb^+$ efflux from cultured bovine aortic endothelial cells (BAECs) paralleled changes in $[Ca^{2+}]_i$ concentration as measured by fura-2 fluorescence. Colden-Stanfield et al. (1987) demonstrated that BK could activate large outward currents during whole-cell voltage-clamp recordings from VECs. These currents appeared to require $[Ca^{2+}]_i$ in that they were only observed if the intracellular solution used in the recording pipette lacked EGTA. A large, 150-pS K^+ channel which was both voltage- and Ca^{2+}-activated has been reported at the single-channel level in VECs (Fichtner et al., 1987). However, this channel activity was only observed in a low percentage (2 of 55) of the membrane patches examined. Suave et al. (1988) have reported the presence of a 40-pS K^+ channel in VECs. This channel ap-

* *Present address:* Department of Physiology, Defense Nuclear Agency, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20814.

** *Present address:* Departamento de Bioquímica, Centro de Investigacion Sobre Ingeníeria Genética y Biotechnologia, U.N.A.M., Apartado Postal 510-3, Cuernavaca 62270 Mexico.

pears to be activated by an ATP- or ADP-induced release of Ca^{2+} from internal stores and by Ca^{2+} influx from the extracellular space. To date, the only Ca^{2+}-activated whole-cell currents which have been reported in cultured endothelial cells from human umbilical vein (Bregestovski et al., 1988) appear to be due to activation of cationic channels. These investigators demonstrated an activation of inward current by exogenous histamine which could be mimicked by A23187.

In the present study, a pharmacological approach is used to characterize the Ca^{2+}-activated K^+ current in BAECs. We provide evidence that these channels are responsible for the BK-stimulated outward current and $^{86}Rb^+$ efflux previously reported. Additionally, blocking agents have been identified which should be useful in defining the functional role of the Ca^{2+}-activated K^+ channel in VECs.

Materials and Methods

Culture of Bovine Aortic Endothelial Cells

BAECs were harvested and cultured as previously described (Eskin et al., 1978). The culture medium was Minimal Essential Medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 μg/ml penicillin and first passage cells were cloned to eliminate contaminating smooth muscle cells. At confluency, the contact inhibited cobblestone monolayer typical of cultured BAECs was observed. The identification of this cell line as endothelial has been previously described (Colden-Stanfield et al., 1987). All experimental data were obtained from BAECs in their 4th to 10th passage and at 4–7 days postconfluency. Whole-cell current recordings were obtained from electrically isolated cells (trypsinized and allowed to attach to coverslips). Single-channel activity was monitored on postconfluent monolayers as well as subconfluent monolayers. All $^{86}Rb^+$ efflux experiments were performed on confluent monolayers grown in a 35-mm culture dish.

Measurement of Whole-Cell Membrane Currents

Intracellular whole-cell recordings of subcultured BAECs were obtained at room temperature with a List-EPC 7 amplifier (List Medical, Darmstadt-Eberstadt, FRG) using the tight-seal technique described by Hamill et al. (1981). Low resistance (2–5 MΩ) electrodes were fabricated from thin-walled glass (#8161 and #7052, Garner Glass). The indifferent electrode was a Ag-AgCl plug connected to the bath via a 150 mM KCl agar bridge. In all experiments ionic currents were recorded in physiological extracellular solution of the following composition (in mM): 137 NaCl, 5.4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 dextrose, and 10 HEPES, pH adjusted to 7.3 with NaOH. The pipette solution contained 145 mM KCl, 10 mM HEPES, pH adjusted to 7.3 with KOH, and 2.2 mM ethylene-glycol-*bis*(β-amino ethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) and sufficient $CaCl_2$ to yield calcium concentrations of either 10 nM or 1 μM.

Drugs were pressure applied to individual cells or added to the bath. Application was performed by imposing an air pressure of 4 kg/cm^2 to a pipette with a tip diameter of 1–2 μm via a picospritzer II (General Valve, Fairfield, NJ). Data were filtered at 2 kHz and digitized on-line at a sampling frequency of 5 kHz for subsequent computer analysis. Whole-cell recordings in the appropriate figures are shown without correction for capacitative currents.

Measurement of Single-Channel Currents

Single-channel activity was recorded in the cell-attached and inside-out patch configurations at room temperature using the technique of Hamill et al. (1981). For cell-attached patch experiments, the cells were bathed in the standard saline solution (*see above*). The pipette solution contained (in mM): 145 KCl, 1.6 EGTA, 0.8 $CaCl_2$, 10 HEPES, pH adjusted to 7.3 with KOH. In other experiments both the pipette and bath contained the standard saline solution. BK was applied by pressure ejection onto the cell. Inside-out patch experiments were performed under symmetrical K^+ conditions with 145 mM KCl in both the bath and pipette solutions. Appropriate concentrations of Ca^{2+} and EGTA were used to give concentrations of 10^{-8}, 10^{-7} or 10^{-6} M free Ca^{2+}. Data were filtered at 2–5 kHz and recorded on an FM tape recorder for subsequent digitization at a sampling frequency of 10 kHz. Single-channel currents were filtered further during the analysis using a 4-pole zero-phase digital filter. Unitary channels were measured using computer programs in which events were detected using a threshold discriminator (Lux & Brown, 1984). Amplitude and open-time distributions of single-channel currents were constructed from the digitized data.

Measurements of $^{86}Rb^+$ Efflux in BAECs

$^{86}Rb^+$ efflux was determined as previously described (Schilling et al., 1988). Briefly, $^{86}Rb^+$ (10–20 μCi) was added to confluent monolayers of BAECs in 35-mm culture dishes containing 2 ml of medium. Following equilibration of the cells with the isotope for 15–18 hr at 37°C, the medium was aspirated from the dish and the monolayer was immediately washed with approximately 3 ml of buffer (at room temperature) containing the following ionic composition (in mM): 140 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 10 HEPES. pH was adjusted to 7.4 with Tris base. The buffer was aspirated and the wash step repeated a total of three times. Following the final aspiration, 3 ml of the buffer was added to the dish and the cells were allowed to equilibrate for 5 min at which time duplicate 100-μl aliquots were withdrawn (time zero for all experiments) before addition of agonists. At each subsequent time point, duplicate samples were taken and placed in scintillation vials. After removal of the final aliquot for each efflux experiment, the remaining solution was rapidly aspirated and the monolayer was washed one time with fresh buffer. One ml of 0.5% NaOH was added to the dish for determination of the amount of radioactivity associated with the monolayer at the end of the flux experiment. All values are expressed as the percent isotope remaining associated with the monolayer relative to the value after the 5-min equilibration period. For each experimental condition tested, the efflux was determined on two dishes. The data points in each figure represent the average values obtained from these duplicate experiments. Where given in

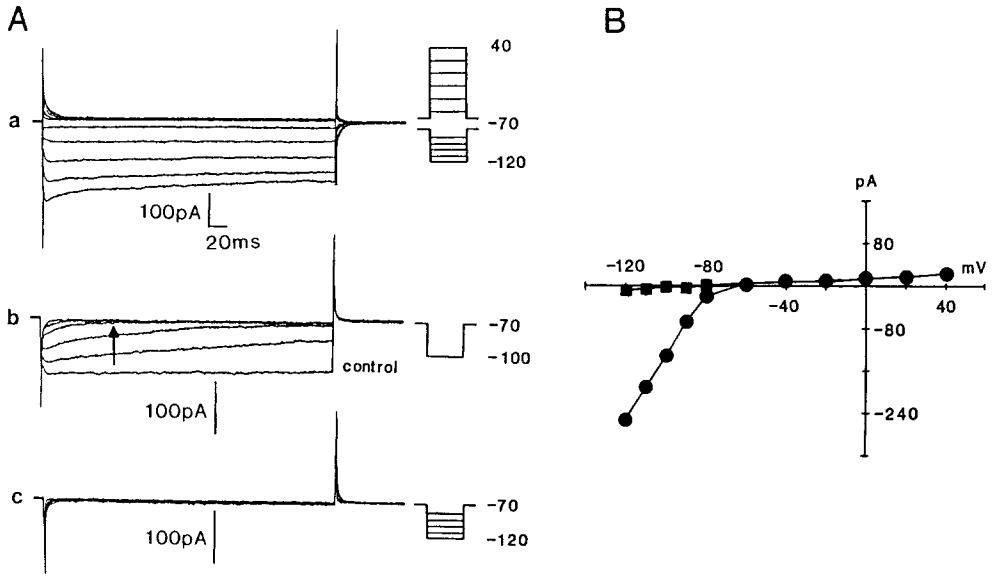

Fig. 1. Whole-cell recordings (A) of a family of currents depicting the inward rectifier K^+ current before (*a*), during (*b*), and after (*c*) exposure to externally applied 2 mM Ba^{2+}. The middle family of currents (*b*) represents the time-dependent block by Ba^{2+} of the rectifying current during six successive test pulses (arrow). The test pulses were 300-msec voltage steps from −70 to −100 mV delivered every 4 sec while the cell was perfused from a pipette containing 2 mM Ba^{2+}. The current-voltage relations for the peak current in the absence (●) and presence (■) of 2 mM Ba is shown in *B*. Current amplitude in this figure as well as all following figures illustrating whole-cell currents was measured 10 msec after the beginning of the voltage step

the text, *n* equals the number of monolayers tested. In those experiments where inhibitory agents were tested for effects on the efflux profiles, the agents were present at the indicated concentrations during the entire efflux protocol including the 5-min pre-equilibration period.

Preparation of *Noxiustoxin*

Noxiustoxin (NTX) was obtained from the venom of the Mexican scorpion, *Centruroides noxius* by the procedure previously described (Possani et al., 1981; Possani, Martin & Svendsen, 1982). Briefly, fractionation of the soluble venom on a Sephadex G-50 column yielded three major peaks designated I, II and III. Subfractionation of the material obtained in fraction II by ion exchange chromatography in carboxymethyl-cellulose resins, in 20 mM ammonium acetate buffer, pH 4.7, yielded 14 subfractions. Subfraction II-11 was further separated in the same ion exchange chromatographic support, in the presence of 50 mM Na^+-phosphate buffer, pH 6.0. The major chromatographic component obtained in this column was homogeneous by polyacrylamide gel electrophoresis and amino acid sequence analysis. It was named *Noxiustoxin* (NTX) and was shown to block K^+ channels in squid giant axon (Carbone et al., 1982) and to block Ca^{2+}-activated K^+ channels of skeletal muscle (Valdivia et al., 1988). In preliminary $^{86}Rb^+$ efflux studies in BAECs, soluble venom and fractions I, II and III were examined. The blocking activity was found in the venom and fraction II. Furthermore, the concentration of protein from venom and fraction II necessary to produce 50% inhibition of BK-stimulated $^{86}Rb^+$ efflux from BAECs suggested that NTX was the peptide responsible for blockade. All experiments reported in the present study employed the highly purified NTX, prepared as briefly described above.

Results

Activation and Inhibition of BAEC K^+ Currents

K^+ currents in BAECs were identified and studied in three forms: (*i*) as whole-cell currents; (*ii*) as single-channel current, and (*iii*) as the $^{86}Rb^+$ efflux.

Inward Rectifier K^+ Current

Initial experiments examined whole-cell currents under voltage clamp. With the pipette solution buffered to 10^{-8} M Ca^{2+}, an inward current was present when the membrane potential was hyperpolarized in 10-mV steps from a holding potential of −70 mV (Fig. 1*A*, trace set *a*). Outward current was negligible at potentials depolarized from −70 mV. Since the calculated E_K was approximately −80 mV, the current voltage relationship shown in Fig. 1 suggests that this inwardly rectifying current is predominantly carried by K^+. Addition of $BaCl_2$ (2 mM), a blocking agent of the inwardly rectifying K^+ current (Hagiwara et al., 1978; Standen & Stanfield, 1978), to the bath solution eliminated the current evoked during hyperpolarization (Fig. 1*A*, trace sets *b* and *c*). Similar results were obtained in four other cells. Exogenous administration of other K^+

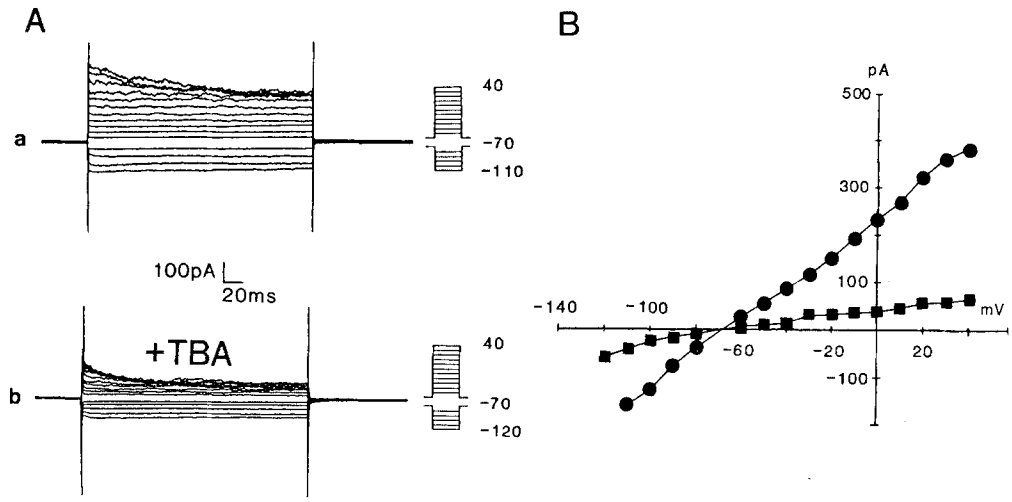

Fig. 2. Whole-cell current recordings in response to a series of depolarizing and hyperpolarizing pulses obtained in the absence (*A*, trace set *a*) and presence (*A*, trace set *b*) of 20 mM TBA in the bath solution. Each 250-msec pulse was separated by 4-sec intervals. Current-voltage relations are illustrated in *B* for the currents during the control period (●) and the currents during exposure to TBA 20 mM (■)

channel blockers such as the alkylammonium compounds, tetraethylammonium chloride, (TEA 12 mM) and tetrabutylammonium chloride (TBA 10 mM), or the scorpion toxin, NTX, had no effect on the inward current (*data not shown*).

Ca^{2+}-Activated K^+ Current

In order to identify Ca^{2+}-activated K^+ currents in BAECs, whole-cell currents were recorded with the pipette solution containing 10^{-6} M Ca^{2+}. Under these conditions, outward current was present in response to depolarizing voltage steps from a holding potential of −70 mV (Fig. 2*A*). The current reversed direction at potentials near the equilibrium potential for K^+. Under these ionic conditions no other ion had a similar equilibrium potential. The currents showed varying amounts of relaxation during depolarizing steps to potentials more positive than 0 mV. Currents evoked under these experimental conditions were not affected by external application of TEA (5–12.0 mM), 4-amino-pyridine (5 mM), or apamin (0.25–1.6 μM). However, TBA (5–20 mM) reduced the current during both hyperpolarizing and depolarizing steps (Fig. 2*A*, trace set *b*). TBA inhibition was obtained in four cells tested at 20 mM (75 ± 19% of the total outward current (included any leak current) at −60 to −40 mV). When K^+ in the pipette solution was substituted with Na^+, Cs^+, or N-methyl-D-glucamine (NMDG), outward current was not present suggesting that this current was selectively carried by K^+.

NTX had a blocking effect on the K^+ currents present in BAECs when the intracellular Ca^{2+} was buffered to 10^{-6} M. Figure 3 demonstrates the block of both inward and outward current by NTX leaving primarily the inwardly rectifying K^+ current. At potentials positive to −20 mV NTX (1 μM) reduced the total outward current by approximately 75%. In another experiment, control current at −20 mV was inhibited by 22% at 200 nM, 64% at 400 nM and 92% at 600 nM.

BK-Activated Current

In order to identify and characterize ionic currents activated by the rise in $[Ca^{2+}]_i$ produced by BK, $[Ca^{2+}]_i$ was buffered to 10^{-8} M using Ca^{2+}-EGTA and the response to BK was tested. In the absence of BK, the inwardly rectifying K^+ current was present, (Fig. 4*A*) with minimal outward current observed during depolarizing steps. Addition of BK (50 nM) to the bath activated additional current in response to both depolarizing and hyperpolarizing steps. When the membrane potential was clamped to values more positive than 0 mV, the current showed relaxation as did that in whole-cell recordings in which $[Ca^{2+}]_i$ was buffered to 10^{-6} M. This current was not blocked by TEA (10 mM). This current, however, was blocked by the addition of TBA to the bath (Fig. 4*A* and *B*). In two other experiments in which the effects of BK were examined during perfusion of the cell with a known concentra-

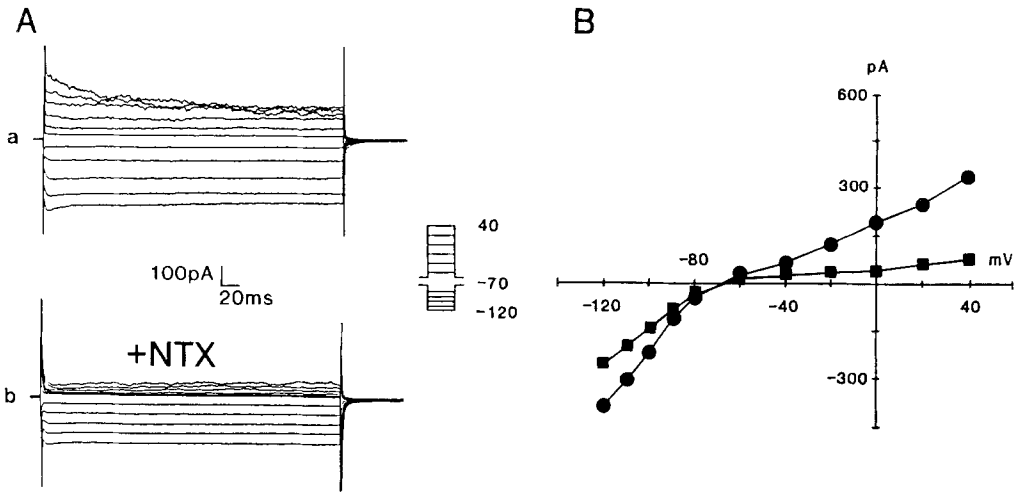

Fig. 3. Inhibition of Ca^{2+}-activated K^+ currents by 1 μM NTX. (*A*, trace *a*) Currents are illustrated under control conditions (10^{-6} M $[Ca^{2+}]_i$) as well as in the presence of externally applied NTX (trace *b*). Test pulses were 325 msec in duration and delivered every 4 sec. (*B*) Current-voltage relations are shown during the control period (●) and in the presence of NTX (■)

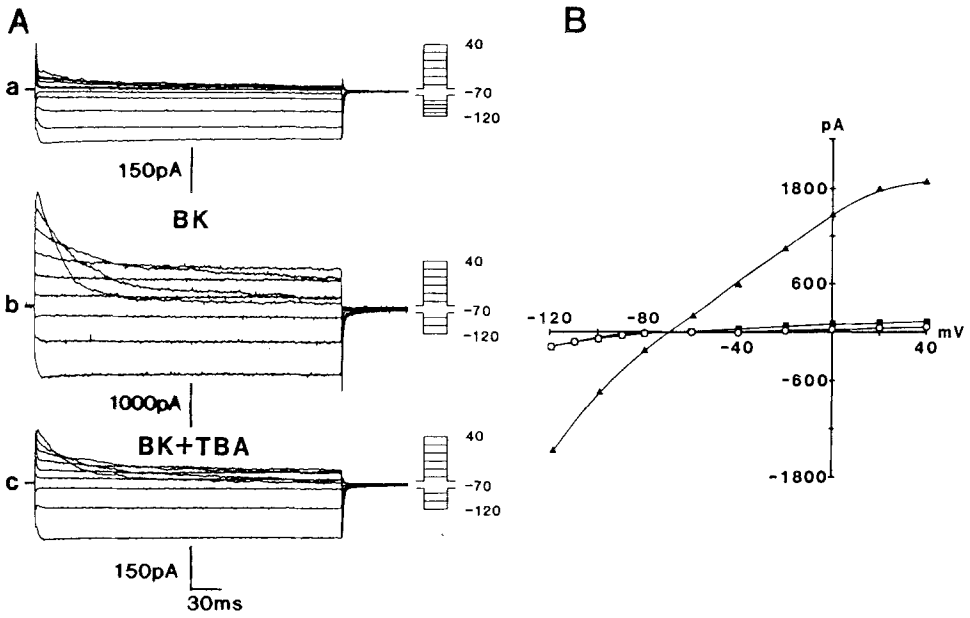

Fig. 4. Activation and inhibition of the BK-activated currents in BAECs. Intracellular Ca^{2+} was buffered to 10^{-8} M. (*A*, trace set *a*) Control whole-cell currents elicited holding at a membrane potential of −70 mV and stepping to +40 and −120 mV in 20- and 10-mV steps, respectively. Trace set *b*. Activation of outward as well as inward currents during BK (50 nM) exposure. Note change in current calibration in middle trace. Trace set *c*. Block of the BK-stimulated currents was accomplished by adding TBA to the bath to a final concentration of 5 mM. Each 325-msec test pulse was separated by 4-sec intervals. (*B*) The current-voltage relations for the peak current during the control period (○), during BK exposure (▲), and following addition of TBA (■)

tion of TBA, inhibition of the BK-induced current by TBA was observed (94% at 10 mM, 55% at 7 mM). No current was elicited by bradykinin when sodium, cesium or NMDG replaced potassium in the pipette solution. In sensitivity to TBA and voltage dependence, the BK-activated and Ca^{2+}-activated K^+ currents were similar. The fact that potassium current was elicited by bradykinin in spite of the presence of EGTA in the pipette solution suggests the buffering of Ca^{2+} by EGTA at the internal membrane surface is not sufficiently rapid to prevent the activation of the K^+ channels during cal-

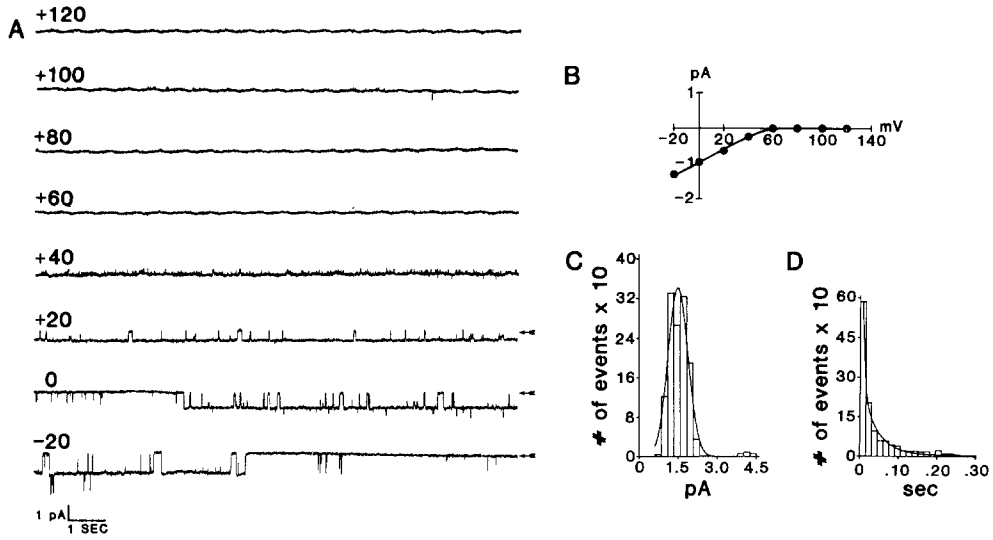

Fig. 5. (*A*) Inwardly rectifying K^+ channels from a cell-attached patch. The resting membrane potential was between −65 and −70 mV, the pipette contained 145 mM KCl and the E_K was estimated to be near 0 mV. The patch was depolarized from the resting potential by the potentials indicated. Data was filtered at 800 Hz. The arrow to the right of the individual records represents the closed level of the channel. (*B*) The current-voltage relation is shown with mV representing the potential applied to the patch. The amplitude and mean open-time histograms are illustrated in (*C*) and (*D*), respectively

cium influx or calcium release from intracellular stores.

Single-Channel Currents

The similarity of the Ca^{2+}-dependent current and the BK-induced current led us to compare the single-channel activity produced by BK in cell-attached patches with the channel activity in inside-out patches exposed to various concentrations of Ca^{2+}. Under control conditions of a saline bath and high K^+ pipette solutions, single-channel activity was recorded in 42 out of 44 cell-attached patches (Fig. 5) when no potential was applied to the patch (thus, the patch potential was the cell resting potential, approximately −65 mV), the currents were inward. With step depolarization from the resting potential, single-channel amplitude declined and was undetected at potentials positive to +60 mV, a value close to the predicted E_K of 0 mV with high K^+ solution in the pipette. Single-channel conductance of this inwardly rectifying channel was 21 ± 3 pS near the resting membrane potential (n = 4). This channel appears to be responsible for inward rectifying current seen in the whole-cell current records.

Using the same ionic conditions as above, a second, distinct channel which, contrary to the channel described above, opened at potentials both depolarized and hyperpolarized from E_K was sometimes observed (14 of 31 cell-attached patches). The open probability of this channel increased when BK (50–200 nM) was pressure ejected onto the cell (10 out of 14 patches tested). An example is shown in Fig. 6*A*. The current-voltage relationship was inwardly rectifying with a single-channel conductance of 38 pS (larger than the channel described above) calculated over the voltage range where the current was inward and 16 pS when the net current flow was in the outward direction. The current reversed direction at approximately 65 mV depolarized from the resting potential. Since the membrane potential was expected to be −60 to −70 mV (Colden-Stanfield et al., 1987) this would be a reversal near 0 mV, the value expected for nearly symmetrical K^+ solutions. The single-channel open-time histogram was biexponentially fit with time constants (τ) of 1.6 and 11.1 msec. In four cell-attached patches where the single-channel activity activated by BK was examined at several different voltages, the conductance was 39 ± 6 pS in the inward direction and 18 ± 5 pS in the outward direction. The mean open time was fit with taus of 1.24 ± 0.6 msec (range 0.7–2.2) and 10.0 ± 5 msec (range 6–18). When the pipette contained 5.4 mM KCl instead of 145 mM KCl, the reversal potential for current through this channel was shifted to the left (E_{rev} = −10 mV, applied voltage) as shown by the current-voltage relationship and was close to the resting membrane potential (Fig. 6*D*). The conductance at applied potentials from 0 to depolarizing 50 mV was 20 pS. Replacing the KCl with a K-asparate pipette solution did not

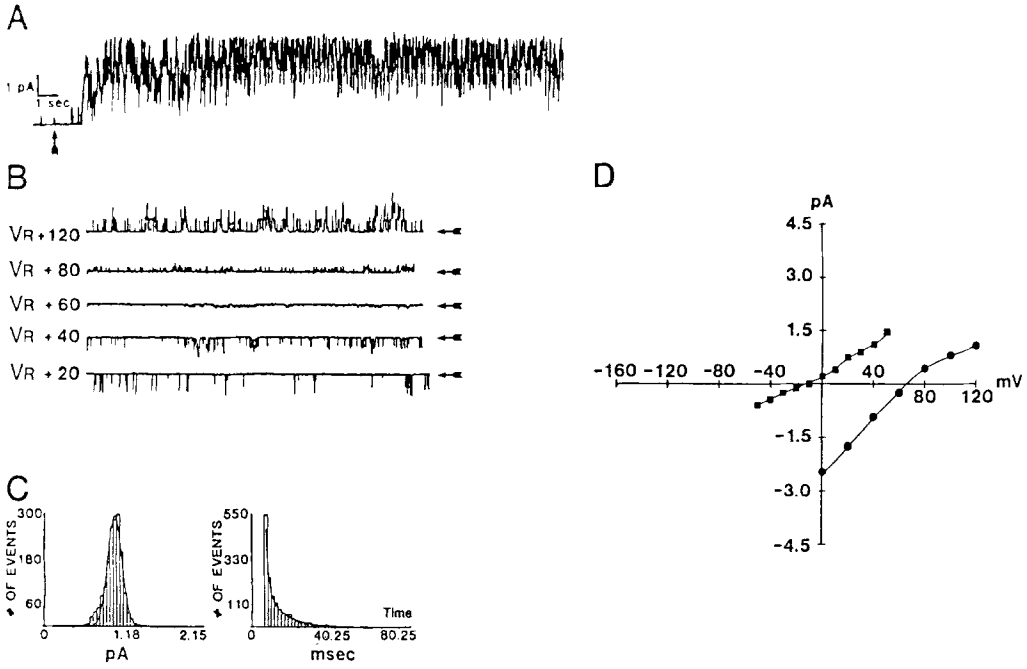

Fig. 6. (*A*) Channel activity from a cell-attached patch elicited in response to BK (200 nM). This patch contained at least five channels, as indicated by superimposed open events. A potential of +120 mV was applied to the patch. Activity was evoked by application of BK at the arrow. (*B*) Example of single-channel activity taken 2–6 min following BK at the applied potentials indicated from same patch. Closed level (arrow) indicated to right of each trace. (*C*) Amplitude and open-time distributions calculated from records taken at +120 mV applied potential. (*D*) The current-voltage relations for the cell shown in *B* where the pipette contained 145 mM K^+ (●) and for a cell-attached patch from another cell where the pipette contained 5.4 mM K^+ (■). The potential plotted on the abcissa is the potential applied to the patch

affect channel activity or the reversal potential indicating that this channel is not permeable to chloride ions.

The sensitivity of the K^+ channels to $[Ca^{2+}]_i$ was determined in inside-out patches under symmetrical K^+ conditions. With 10^{-6} M $[Ca^{2+}]_i$ single-channel current reversed direction at approximately 0 mV (Fig. 7). In separate experiments substituting the intracellular (bath) solution with a Na-isethionate solution containing 10^{-6} M Ca^{2+} abolished the outward single-channel activity (three of three patches tested) indicating that Na^+ is not permeable through this channel. Again, this channel appears to be impermeable to Cl^- since substituting the KCl in the pipette solution with a K-aspartate solution did not affect E_{rev}. In symmetrical K^+ solutions the single-channel conductance was inwardly rectifying and, in the example illustrated, was calculated to be 21 pS in the outward direction and 48 pS in the inward direction. The open-time distribution at −40 mV was fit with a single tau of 11.8 msec. When the bath solution was changed to one containing only 10^{-8} M Ca^{2+}, channel activity disappeared (Fig. 7*C*). Increasing the Ca^{2+} of the bath solution to 10^{-7} M did not elicit channel activity in this particular patch. However, returning the free Ca^{2+} concentration to 10^{-6} M reactivated channels in the patch. Single-channel activity was present in 29 out of 30 patches when the intracellular surface was bathed with 10^{-6} M Ca^{2+}. Six of these patches were studied when $[Ca^{2+}]_i$ was reduced to 10^{-7} M Ca^{2+}. Four of the six retained activity. In five of seven patches examined, reduction of Ca^{2+} from 10^{-6} to 10^{-8} M eliminated channel activity. In the two other patches only infrequent channel openings (two open events/min) were observed. This is consistent with the whole-cell current experiments where not more than 50 pA of outward current (including leak current) was present when the pipette contained 10^{-8} M Ca^{2+}, even at potentials as depolarized as +20 mV. The mean open time for channels studied in four other inside-out patches examined at membrane potentials between −30 and −40 mV were 1.8 ± 1.1 msec and 11.9 ± 3 msec. The conductances were 39 ± 5 pS in the inward and 18 ± 2 pS in the outward direction. Thus, the single-channel conductance was similar to that observed for channels activated by the application of bradykinin.

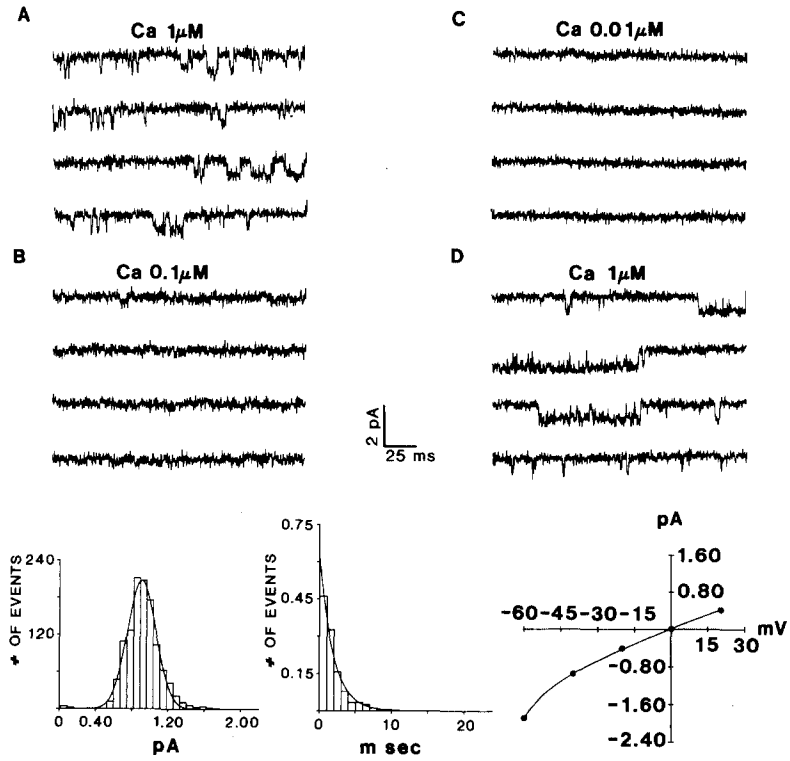

Fig. 7. K^+ channels in an inside-out patch hyperpolarized 40 mV as Ca^{2+} in the bath solution is changed from 10^{-6} to 10^{-8}, 10^{-7} and back to 10^{-6} (*A*, *B*, *C* and *D*, respectively). The pipette and bath solutions contained 145 mM K^+. The amplitude and open-time distributions were calculated from data when the cell was hyperpolarized 40 mV and are shown with the single-channel current-voltage relationship. The data were filtered at 1 kHz

Effect of K^+ Channel Inhibitors on $^{86}Rb^+$ Efflux from BAEC Monolayers

Measurement of whole-cell currents or single channels in the inside-out configuration using the tight-seal technique may lead to washout of a component necessary for the expression of a particular current. Therefore, $^{86}Rb^+$ efflux was measured in these cells to attempt to identify any component of K^+ permeability which might have been lost in the whole-cell or single-channel measurement. In addition, comparison of the pharmacological sensitivity of the BK-activated K^+ channels observed electrophysiologically with the $^{86}Rb^+$ efflux would provide evidence for the involvement of the Ca^{2+}-activated K^+ channels in this response.

As previously reported (Schilling et al., 1988), $^{86}Rb^+$ efflux from BAEC monolayers is linear with time in the absence of agonist agents (Fig. 8). The mean ± SEM rate coefficient obtained from 13 monolayers was 0.0126 ± 0.0011 min^{-1}. Upon addition of BK (50 nM), the $^{86}Rb^+$ efflux rate coefficient increased 10-fold to 0.130 ± 0.0084 min^{-1} (n = 10) (Fig. 9). A variety of K^+ channel inhibitors were tested for effects on BK-stimulated $^{86}Rb^+$ efflux. As seen in Fig. 8, alkylammonium K^+ channel inhibitors caused a dose-dependent inhibition of the BK-stimulated $^{86}Rb^+$ efflux. The rank order of potency was tetrapentyl- > tetrabutyl- > tetraethylammonium chloride. At the concentrations shown, these compounds had no effect on basal $^{86}Rb^+$ efflux. At concentrations above 1 mM, however, tetrapentylammonium-chloride (TPA) caused a dramatic increase in the basal efflux rate suggesting a detergent-like effect on the plasmalemma of the BAEC. Similar increases in basal efflux have been observed with low concentrations of saponin (*data not shown*). The dose-response curve for TBA is shown in Fig. 9. The IC_{50} was approximately 7 mM.

BK increases $[Ca^{2+}]_i$ of the BAEC by release of Ca^{2+} from the internal pools, as well as Ca^{2+} influx from the extracellular space (Colden-Stanfield et al., 1987; Schilling et al., 1988). The results of Gordon and Martin (1983) and Schilling et al. (1988) suggest that the increase in $^{86}Rb^+$ efflux produced by BK occurs through Ca^{2+}-activated K^+ channels. Thus, in addition to K^+ channel blockage, the alkylammonium compounds could inhibit $^{86}Rb^+$ efflux by a number of indirect mechanisms. These compounds may inhibit BK binding to its receptor, inhibit some step leading to release of Ca^{2+} from internal stores, or they may inhibit the influx of Ca^{2+} from the extracellular space. To test these possibilities, the effect of TBA on ionomycin-stimulated $^{86}Rb^+$ efflux was examined (Fig. 10). At the concentration employed (100 nM), ionomycin produced an increase in $^{86}Rb^+$ efflux comparable to that obtained with BK. The ionomycin-stimulated efflux was in-

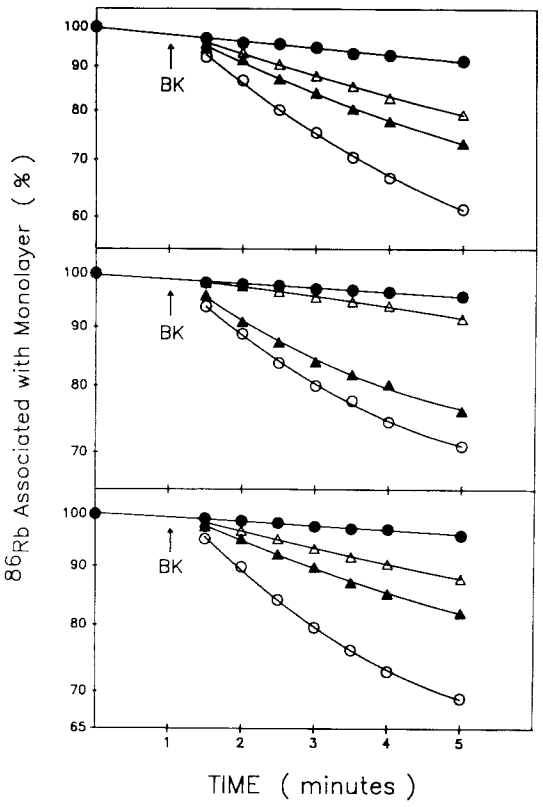

Fig. 8. Effect of alkylammonium compounds on BK-stimulated $^{86}Rb^+$ efflux in BAEC monolayers. Each panel shows the $^{86}Rb^+$ efflux under control basal conditions (●) and following the addition of BK (50 nM, ○). Upper panel: The effect of BK in the presence of 5 mM (▲) and 10 mM (△) TBA. Middle panel: The effect of BK in the presence of 20 mM TBA (△) and 20 mM TEA (▲). Lower panel: The effect of BK in the presence of 10 mM TBA (▲) and 150 μM TPA (△)

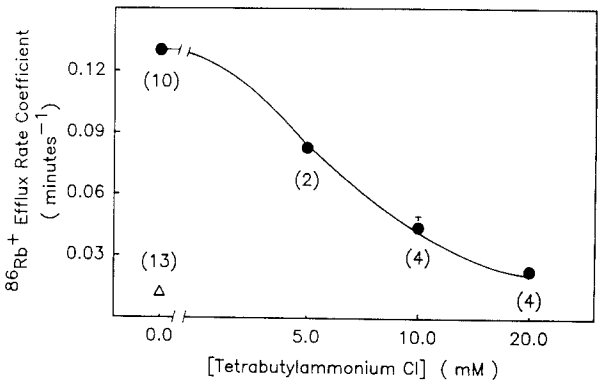

Fig. 9. Effect of TBA on the BK-stimulated increase in $^{86}Rb^+$ efflux. The $^{86}Rb^+$ efflux rate coefficient was obtained from the slope of the linear regression line of plots as shown in Fig. 8 during the first 2 min following BK addition (●). The basal $^{86}Rb^+$ efflux rate coefficient determined in the absence of BK is shown (△). The values represent the mean ± SEM. Where not shown, the standard error was less than the size of the symbol employed. The values in parentheses are the number of monolayers tested

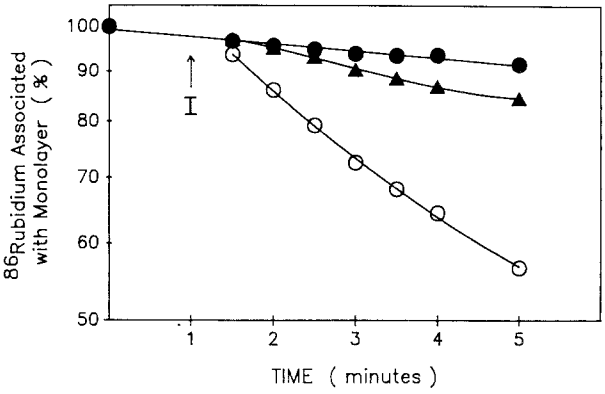

Fig. 10. Effect of TBA on ionomycin-stimulated $^{86}Rb^+$ efflux in BAEC monolayers. Basal $^{86}Rb^+$ efflux (●) and efflux following the addition of 100 nM ionomycin in the absence (○) and presence (▲) of 10 mM TBA are shown

hibited approximately 80% by 10 mM TBA. Thus, TBA has a similar potency on both BK- and ionomycin-stimulated $^{86}Rb^+$ efflux. Furthermore, TPA (0.15 mM) and TBA (10 mM) had no effect on basal $[Ca^{2+}]_i$ as determined using the fluorescent Ca^{2+} indicator, fura-2 (*data not shown*). These results clearly indicate that the alkylammonium compounds act directly on the efflux pathway rather than at some intermediate step leading to a rise in $[Ca^{2+}]_i$.

The effect of NTX on both BK- and ionomycin-stimulated $^{86}Rb^+$ efflux from BAEC monolayers is shown in Fig. 11. NTX (200 nM) produced a potent inhibition of both responses consistent with a direct action of this agent on the $^{86}Rb^+$ efflux pathway. As with the alkylammonium compounds, NTX was equipotent on both the BK- and ionomycin-stimulated $^{86}Rb^+$ efflux. Furosemide (1 mM) produced less than 10% inhibition of BK-stimulated $^{86}Rb^+$ efflux suggesting that ion movement via the Na^+, Cl^-, K^+-cotransporter is not directly involved in the efflux of Rb^+. Additionally, apamin (1 μM) did not affect basal or BK-stimulated $^{86}Rb^+$ efflux (n = 2).

Discussion

Bradykinin, a potent endothelium-dependent vasodilator, produces a change in $[Ca^{2+}]_i$ of cultured vascular endothelial cells (Colden-Stanfield et al., 1987; Schilling et al., 1988; Schilling, Rajan & Strobl-Jager, 1989; Schilling, 1989) which is thought to be necessary and sufficient for the release of endothelium-derived relaxation factor. The rise in $[Ca^{2+}]_i$ also increases surface membrane K^+ perme-

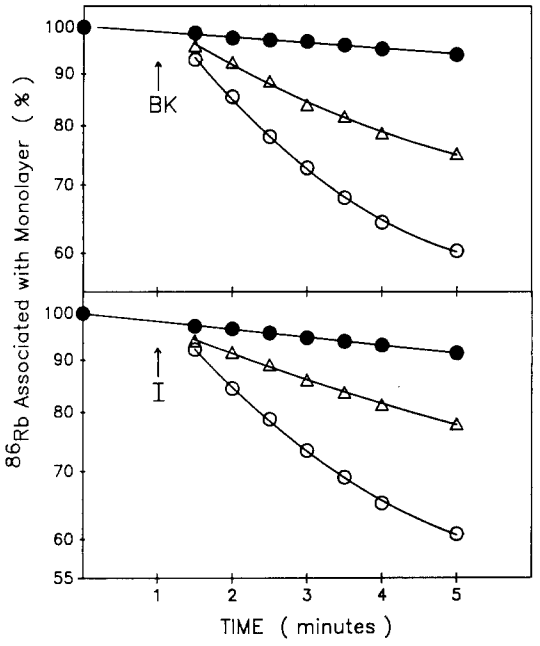

Fig. 11. Effect of NTX on BK- and ionomycin-stimulated $^{86}Rb^+$ efflux in BAEC monolayers. Basal $^{86}Rb^+$ efflux (●) and efflux following the addition of 50 nM BK (upper panel) or 100 nM ionomycin (lower panel) in the absence (○) and presence of 200 nM NTX (△) are shown

ability (Schilling et al., 1988). In previous experiments we found that BK increased $^{86}Rb^+$ efflux in a manner dependent upon the change in $[Ca^{2+}]_i$ and that similar increases in $^{86}Rb^+$ efflux could be obtained with low concentrations of Ca^{2+} ionophores (Schilling et al., 1988). In electrophysiological experiments it was found that BK-induced a K^+ current which was also found to be dependent upon the rise in $[Ca^{2+}]_i$ (Colden-Stanfield et al., 1987). In the present study, a pharmacological approach was used to provide conclusive evidence that (*i*) the BK-induced membrane current is a Ca^{2+}-activated K^+ current and (*ii*) the majority of the BK-stimulated $^{86}Rb^+$ efflux occurs via this channel mechanism.

Comparison of BK- and Ca^{2+}-Activated Membrane Currents

Whole-cell currents observed at both depolarized and hyperpolarized potentials were activated by BK or by raising the internal Ca^{2+} to 10^{-6} M. This current was carried by K^+ since it was not present if the pipette contained Na^+, Cs^+, or NMDG instead of K^+ and it was not affected by replacement of chloride with aspartate. The kinetics of the current were such that the current increased linearly with depolarization steps to 0 mV, but showed relaxation at more depolarized test potentials. An A-current or transient outward current, blocked by 4-AP, has been described in a population of cultured BAECs (Takeda, Schini & Stoeckel, 1987). The outward current observed in the present study was not blocked by 5 mM external 4-AP or by apamin. Exogenous TEA (12 mM) also had no inhibitory effect on this current. However, two other compounds, TBA and NTX did inhibit the current. NTX has been shown to inhibit the maxi Ca^{2+}-activated K^+ channel in membranes derived from transverse tubules of skeletal muscle incorporated into planar lipid bilayers with a K_d of 450 nM (Valdivia et al., 1988) and has also been shown to block the delayed rectifier current in squid axon (Carbone et al., 1982, 1987). The results of the present study clearly indicate that the BK-induced whole-cell current is essentially identical in, time course, voltage sensitivity, K^+ selectivity and pharmacological responsiveness when compared with the Ca^{2+}-activated whole-cell currents. The $^{86}Rb^+$ efflux data further supported this conclusion as TBA blocked both BK- and ionomycin-stimulated $^{86}Rb^+$ efflux.

Comparison of BK- and Ca^{2+}-Activated Single Channels

In cell-attached patches, BK activated a K^+ channel which fell into the intermediate size classification when compared to the Ca^{2+}-activated K^+ channels described in other preparations (for review *see* Castle, Haylett & Jenkinson, 1989). Based on single-channel conductance and the rectification properties, it appears to be the same channel as that activated by ATP in BAECs (Sauve et al., 1988). It resembles K^+ channels also described in HeLa cells (Sauve et al., 1986) canine tracheal epithelia (Welsh & McCann, 1985) red blood cells (Grygorczyk, Schwarz & Passow, 1984) and human macrophages (Gallin, 1989). Ionomycin activates similar inward-rectifying Ca^{2+}-activated K^+ channels in thymocytes and lymphocytes (Mauhaut-Smith & Schlichter, 1989).

Stimulation of channel activity in response to exogenous BK in cell-attached patch experiments indicates that BK is activating these channels via an intracellular second messenger as has been recently described for the ATP-activated channel (Sauve et al., 1988). Channels of similar conductance and open-time distribution to those elicited by BK in cell-attached patches were activated by Ca^{2+} during inside-out experiments. These findings suggest that $[Ca^{2+}]_i$ is the likely candidate for the second messenger. Basal $[Ca^{2+}]_i$ in these cells is approximately 65 nM as measured by fura-2 fluorescence at 22°C (Colden-Stanfield et al., 1987; Schilling et al., 1988).

BK increases $[Ca^{2+}]_i$ in these cells to approximately 300 nM. Since the threshold for Ca^{2+} activation appears to lie between 10 and 100 nM, the channel is in the threshold range for activation in the basal state. This is consistent with the $^{86}Rb^+$ efflux data which shows that basal flux is little affected by TBA. Furthermore, while channels with characteristics of the Ca^{2+}-activated channel were observed in 46% of the cell-attached patches prior to application of BK, the probability of an open event was very low. Thus, the BK-induced channels are not open or open only rarely under basal, resting conditions.

Comparison of BK-Stimulated Currents and $^{86}Rb^+$ Efflux

Although the electrophysiological results clearly indicate that the BK-induced membrane current occurs via Ca^{2+}-activated K^+ channels, the physiological role for these channels remains unknown. Previously it was shown that both BK and A23187 could increase $^{86}Rb^+$ efflux from vascular endothelial cells suggesting a Ca^{2+}-dependent mechanism (Gordon & Martin, 1983; Schilling et al., 1988). However, studies by Brock et al. (1986) have shown that BK activates the furosemide-sensitive Na^+, K^+, Cl^--cotransporter and that this may occur via a Ca^{2+}-dependent mechanism since the transporter was also stimulated by ionomycin. Thus, to determine the contribution of the Ca^{2+}-activated K^+ channel to the $^{86}Rb^+$ efflux observed, the sensitivity of the flux to the identified K^+ channel blockers was examined. The increase in $^{86}Rb^+$ efflux stimulated by both BK and ionomycin was sensitive to the same blocking agents as both the BK-induced and Ca^{2+}-induced membrane currents. The flux was inhibited by the alkylammonium ions TBA and TPA but little affected by TEA or by apamin. Additionally, the efflux was potently inhibited by NTX but unaffected by furosemide, the inhibitor of the Na^+, K^+, Cl^--cotransporter. The concentration range of the blocking agents necessary for inhibition of the currents was similar to that necessary for the inhibition of $^{86}Rb^+$ efflux. These results provide strong evidence that the majority of both the BK- and ionomycin-stimulated $^{86}Rb^+$ efflux occurs via Ca^{2+}-dependent K^+ channels.

The purpose for the Ca^{2+} activation of K^+ channels in endothelial cells is not clear. Recent studies have suggested that BK produces a hyperpolarization of the membrane which may be important for the magnitude and time course of stimulated Ca^{2+} influx (Schilling, 1989). Alternatively, the increase in K^+ permeability of the surface membrane could lead to pronounced changes in the K^+ concentration within the interstitial space between the endothelial cells and the underlying smooth muscle. Similar Ca^{2+}-dependent increases in K^+ permeability with concomitant release of K^+ has been shown to be important for agonist-induced changes in Cl^- secretion from epithelial tissues (Petersen & Maruyama, 1984). Such a mechanism could also account for the increase in activity of the Na^+, K^+, Cl^--cotransporter seen in endothelial cells if a substantial change occurs in the transmembrane K^+ gradient. Loss of cell K^+ could produce osmotic changes giving rise to cell shrinkage and alterations in monolayer permeability. The use of the specific K^+ channel antagonists identified in the present study may prove useful in defining the role of these Ca^{2+}-activated K^+ channels in vascular physiology.

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