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Single-Channel Characterization of the Pharmacological Properties of the K(Ca^{2+}) Channel of Intermediate Conductance in Bovine Aortic Endothelial Cells

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Abstract. The pharmacological profile of a voltage-independent Ca^{2+}-activated potassium channel of intermediate conductance (IK(Ca^{2+})) present in bovine aortic endothelial cells (BAEC) was investigated in a series of inside-out and outside-out patch-clamp experiments. Channel inhibition was observed in response to external application of ChTX with a half inhibition concentration of 3.3 ± 0.3 nM ($n = 4$). This channel was insensitive to IbTX, but channel block was detected following external application of MgTX and StK leading to the rank order toxin potency ChTX > StK > MgTX >>IbTX. A reduction of the channel unitary current amplitude was also measured in the presence of external TEA, with half reduction occurring at 23 ± 3 mM TEA ($n = 3$). The effect of TEA was voltage insensitive, an indication that TEA may bind to a site located on external side of the pore region of this channel. Similarly, the addition of d-TC to the external medium caused a reduction of the channel unitary current amplitude with half reduction at 4.4 ± 0.3 mM ($n = 4$). In contrast, application of d-TC to the bathing medium in inside-out experiments led to the appearance of long silent periods, typical of a slow blocking process. Finally, the IK(Ca^{2+}) in BAEC was found to be inhibited by NS1619, an activator of the Ca^{2+}-activated potassium channel of large conductance (Maxi K(Ca^{2+})), with a half inhibition value of 11 ± 0.8 μM ($n = 4$). These results provide evidence for a pharmacological profile distinct from that reported for the Maxi K(Ca^{2+}) channel, with some features attributed to the voltage-gated $K_V1.2$ potassium channel.

Key words: Ca^{2+}-activated K^+ channel — Endothelial cells — Charybdotoxin — TEA — K^+ channel activator — d-Tubocurarine

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Introduction

Endothelial cells (EC) play an important role in the regulation of vascular tone by secreting a variety of vasoactive factors in response to hormonal and/or physical stimuli. These factors include vasorelaxing agents such as the endothelium-derived relaxing factor (putatively NO) [21] and vasoconstrictors such as endothelin-1 (and perhaps other endothelins), endoperoxides (PGH_2), thromboxane A2 and superoxyde anions. The production by the endothelium of vasodilators such as NO has been proven to be related to an increase in intracellular Ca^{2+}, resulting, in part, from an inositol 1,4,5 trisphosphate ($InsP_3$) mediated release of Ca^{2+} from internal stores coupled to an agonist-evoked Ca^{2+} influx. There is now supporting evidence that the Ca^{2+} influx in EC is affected by membrane potential, with a hyperpolarization leading to an augmentation and/or stabilization of the Ca^{2+} entry [1,7,33,42,45,55], and it is generally agreed that this augmentation arises from the increased electrical driving force acting on Ca^{2+} ions under these conditions [7,15,16,45,53,55]. Variations in membrane potential may originate from several factors, but a determinant role has been attributed to K^+-selective channels in several studies [2,15,33,45,53]. For instance, whole-cell patch recordings and microelectrode potential measurements on intact endothelium preparations have indicated that acetylcholine induces a biphasic hyperpolarization of the EC with an initial hyperpolarization related to the release of Ca^{2+} from internal stores, followed by a sustained hyperpolarization phase which required the presence of external Ca^{2+} [7,9,11,40]. It was argued that the activation of Ca^{2+}-activated K^+ channels (K(Ca^{2+})) was responsible for the membrane hyperpolarization in this case. These results, together with experiments in which internal Ca^{2+} and whole cell K^+ currents were recorded

simultaneously, further supported the proposal that the hyperpolarization of EC by agonists results from the activation of K(Ca^{2+}) channels [9,33,42,53,54]. An investigation of K(Ca^{2+}) channels in EC is therefore essential to fully understand the regulation of the Ca^{2+} signaling process in this case.

At least three different classes of K(Ca^{2+}) channels have so far been identified in EC. Maxi K(Ca^{2+}) channels with unitary conductance > 100 pS were found in rabbit aorta [52], human umbilical vein EC [45] and in pig coronary artery [4]. Channel activity appeared to be both Ca^{2+} and voltage dependent with an increased channel open probability at depolarizing potentials. Channel inhibition was observed in this case in EC from rabbit aorta exposed to ChTX (100 nM), external TEA (0.5–5 mM) or TBA (0.5–5 mM). This channel corresponds to the well-documented Maxi K(Ca^{2+}) channel reported in different types of cells and tissues [37]. An apamine-sensitive K(Ca^{2+}) of small conductance (9 pS) was also reported by Marchenco and Sage on EC from intact rabbit aorta [41]. This particular channel did not appear however to participate to the hyperpolarization evoked by acetylcholine in these cells. Finally, the presence of K(Ca^{2+}) channels of intermediate conductance (20–80 pS) (IK(Ca^{2+})) was also confirmed in several single-channel studies on vascular EC [14,41,53,54,60]. IK(Ca^{2+}) channels showed no significant voltage dependence and exhibited inward rectification in symmetrical salt solutions. In addition, single-channel evidence was provided in a previous patch-clamp study that stimulation of BAEC with bradykinin (BK) causes a hyperpolarization of the cell potential and the concomitant activation of IK(Ca^{2+}) channels [53]. These observations were interpreted as indicating a direct contribution of the IK(Ca^{2+}) channels to the BK-induced cell hyperpolarization. However, IK(Ca^{2+}) channels may not exclusively be involved in the Ca^{2+} response of EC to extracellular agonists linked to the phosphoinositide pathway. Recent data suggests that they may also play a role in cell proliferation [34,50]. This may explain the general observation that the presence of IK(Ca^{2+}) channels seems related to the cell metabolic state [46]. Despite these observations, the pharmacological profile of IK(Ca^{2+}) channels in EC still remains poorly documented.

In this work, we present a single-channel characterization of the pharmacological properties of IK(Ca^{2+}) channels present in BAEC. Our results essentially indicated that the IK(Ca^{2+}) in these cells show a toxin sensitivity profile characterized by ChTX > StK > MgTX >> IbTX, while being sensitive to external TEA (23 mM) and d-TC (4.4 mM) at elevated concentrations only. In addition, this IK(Ca^{2+}) channel could be blocked by internal application of the Maxi K(Ca^{2+}) channel opener NS1619 with half inhibition value of 11 μM.

Materials and Methods

Cell Culture

The details of the BAE cell culture procedure and characterization have been described elsewhere [53]. The cells were tested with endothelial cell-labeling reagents or factor VIII antibodies (Daco, Santa Barbara, CA) and responded positively [31]. BAE cells were grown in Dulbecco's modified Eagle Medium (Gibco) supplemented with 10% newborn calf serum, 3.7 g/l $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin in humidified air with 5% CO_2 atmosphere at 37°C. Cells from serial passage 21–26 were reseeded on microscope coverslips to accommodate the superfusion chamber used for patch measurements.

Patch-Clamp Experiments

Characterization of the IK(Ca^{2+}) channel properties was carried out at the single-channel level using the patch-clamp technique in the inside-out or outside-out configuration with a List EPC7 amplifier. For inside-out experiments, the patch electrode and cytoplasmic-like solutions consisted in a standard 200 KCl medium containing (in mM): 200 KCl, 0.5 $MgCl_2$ buffered at pH 7.4 with 25 Hepes and 10 KOH, for a free Ca^{2+} concentration of approximately 3 μM (estimation based on fura-2 measurements). Unless specified otherwise, the voltage in the pipette was +60 mV throughout. The bathing solution for outside-out experiments was a standard 200 KCl medium, while the pipette contained (in mM): 200 KCl, 0.5 $MgCl_2$, 25.0 Hepes, 10.0 KOH, 1 EGTA and 0.92 $CaCl_2$ for a free Ca^{2+} concentration of 0.7 μM. The voltage in the patch pipette was maintained to −60 mV throughout. Patch pipettes were pulled from Pyrex capillaries (Corning 7040) using a David Kopf programmable pipette puller (Model 750) and used uncoated. The resistance of the patch electrode ranged from 4 to 10 MΩ. Current traces were recorded using a VR-10B digital data recorder (Instrutech, Great Neck, NY). For offline analysis, the signal was sampled at 1.0 kHz and filtered at 500 Hz with two low-pass four-pole Bessel filters (VVS 300B, Frequency Devices, Haverhill, MA) connected in series. Baseline drift was corrected through a multiple linear interpolation procedure. When required, the open-channel probability, P_o, was estimated from current amplitude histograms on the basis of a binomial distribution as presented elsewhere [44]. Time intervals were measured either on noise-free current records resulting from the application on selected current traces obtained experimentally of the Baum-Welch reestimation formulae [13], or using the double threshold procedure described in Bellemare et al., [5] on original current traces. The stationarity of the recorded signal was tested according to the criteria defined in Denicourt et al., [17]. The reduction in current jump amplitude caused by TEA and d-TC was fitted according to

$$\frac{I}{I_O} = \frac{1}{1 + \frac{[X]}{[X_0]} e^{-\frac{\alpha q V}{KT}}} \quad (1)$$

where I and I_0 are the current jump amplitudes measured with and without the blocking agent, $[X_0]$ the voltage independent concentration for half reduction, α the fractional electrical distance of the blocker binding site, q, the elementary charge, K and T the Boltzmann's constant and the temperature respectively. All the experiments were performed at room temperature.

Toxins

ChTX, IbTX, MgTX, and StK toxin were purchased from Alomone Labs (Jerusalem, Israel). TEA and d-TC were obtained from Sigma

and the Maxi-K(Ca^{2+}) opener NS1619 was purchased from RBI (Natick, MA). Each toxin was prepared as 1 μM stock solution in 200 mM KCl + 0.5 mM $MgCl_2$ + 25 Hepes and kept frozen until used. The final concentration of toxin for each experiment was obtained by diluting the appropriate amount of stock solution into 2 ml of the bathing medium which was then perfuse into the 200 μl patch-clamp chamber. This procedure could normally be carried out within less than 10 min. NS1619 was prepared in 0.1 M KOH and kept in the dark. The desired concentrations of NS1619 were achieved by diluting amounts of NS1619 stock solution directly into the bathing medium. TEA and d-TC were dissolved directly into the experimental solutions.

Abbreviations

ChTX: charybdotoxin; d-TC: d-Tubocurarine; EGTA: ethyleneglycol-bis-(B-amino-ethylether) N,N′-Tetra-acetic Acid; IbTX: iberiotoxin; MgTX: margatoxin; StK: stichodactyla toxin; TEA: tetraethylammonium; TBA: tetrabuthylammonium.

Results

Effects of K^+ Channel-Selective Toxins on IK(Ca^{2+}) in BAEC

ChTX constitutes a potent, but not truly specific, high affinity inhibitory agent (0.2–1 nM) of the Maxi K(Ca^{2+}) channels present in skeletal and vascular smooth muscle cells and in a variety of neuroendocrine tissues [25,27,43]. Figure 1*A* shows representative outside-out single-channel recordings of a IK(Ca^{2+}) channel in BAEC measured in control conditions and following bath application of 10 nM ChTX. As reported for the K(Ca^{2+}) channels of large conductance, the presence of ChTX in the bathing medium resulted in the appearance of silent periods of several seconds duration, occurring between bursts of channel activity. It is now generally agreed that these silent periods represent blocked time intervals [43]. There was no detectable change in unitary conductance, and channel inhibition was fully reversible within 2 min following washout of the toxin. The dose-response curve of the ChTX-induced inhibition is illustrated in Fig. 1*B*. Half inhibition was obtained at 3.3 ± 0.3 nM ($n = 4$), a value comparable to that reported for many of the K(Ca^{2+}) channels of large conductance identified so far. These results suggest therefore that a discrimination between Maxi K(Ca^{2+}) and IK(Ca^{2+}) channels in EC cannot be directly established from a ChTX-evoked variation in membrane potential alone, both channels being equally sensitive to the toxin over the same concentration range.

IbTX has been reported as a highly specific inhibitor of Maxi K(Ca^{2+}) channels in vascular smooth muscle cells [8]. The effect of IbTX on the IK(Ca^{2+}) channels in BAEC is shown in Fig. 2. The resulting current traces were obtained from outside-out patches in control conditions, and following exposure to 15 nM (Fig. 2*A*) and

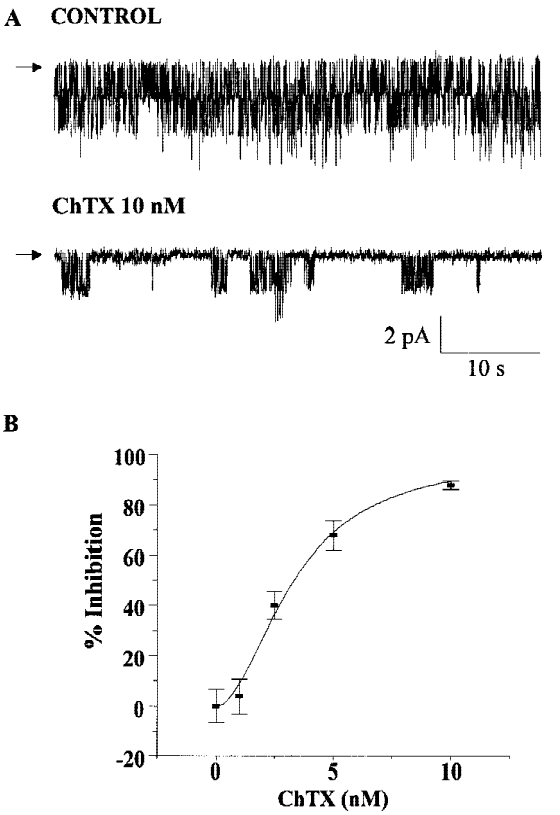

Fig. 1. Effect of ChTX on the IK(Ca^{2+}) channel in BAEC. (*A*) Outside-out single-channel records measured in symmetrical 200 mM KCl conditions. The free Ca^{2+} concentration in the electrode was 0.7 μM and the pipette potential was −60 mV throughout. The presence of ChTX resulted in the appearance of long silent periods, typical of a slow block process. There was no modification of the current jump amplitude. (*B*) Dose-response curve of the ChTX-induced IK(Ca^{2+}) channel inhibition as a function of the ChTX concentration. The percentage of inhibition at each ChTX concentration was computed from the channel open probability, P_o, measured in control conditions, and following application of the toxin. Each data point represents four different outside-out experiments. Half inhibition was estimated at 3.3 nM, indicating a high affinity binding of ChTX to the IK(Ca^{2+}) channel. Arrows indicate closed state current level.

100 nM (Fig. 2*B*) IbTX respectively. IbTX failed systematically to affect the IK(Ca^{2+}) channel activity at concentrations up to 100 nM, indicating a low binding affinity of the toxin to the IK(Ca^{2+}) in BAEC when compared to ChTX. Different results were obtained with StK, a peptide K^+ channel blocker of marine origin, which was shown to specifically inhibit the Ca^{2+}-activated $^{86}Rb^+$ influx in red blood cells [6] at a half inhibition concentration of 24.5 nM in normal saline conditions. It was proposed that the action of StK was mediated in this case by a Ca^{2+}-activated K^+ channel with conductance and gating properties similar to IK(Ca^{2+}) in BAEC [14,30,53, 54,60,63]. Figure 3*A* presents typical outside-out single-channel recordings in which the effect of 100 nM StK was tested on the IK(Ca^{2+}) of BAEC. The resulting in-

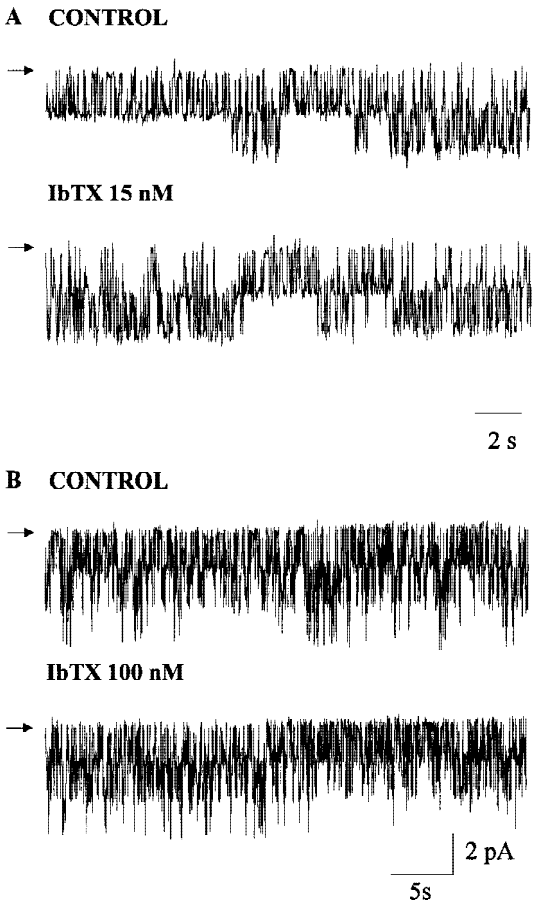

Fig. 2. Effect of IbTX on the IK(Ca^{2+}) channel in BAEC. (*A*) Typical outside-out current records measured in symmetrical 200 mM KCl, in control conditions, and following the addition of IbTX (15 nM) to the bathing medium. (*B*) Outside-out current records obtained from an other cell where 100 nM IbTX was added to the bathing medium. The pipette potential was equal to −60 mV throughout. Bath application of IbTX failed to affect the single-channel activity at concentrations up to 100 nM. Arrows indicate the closed state current level.

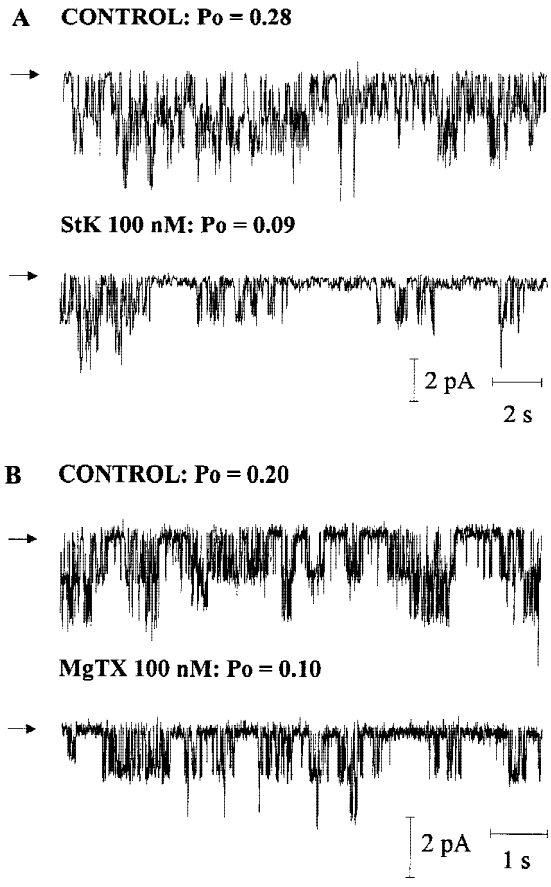

Fig. 3. (*A*) Effect of StK on the IK(Ca^{2+}) channel in BAEC. Representative outside-out current records measured in symmetrical 200 mM KCl in control conditions and following bath application of 100 nM StK. The pipette potential was equal to −60 mV throughout. The mean percentage of inhibition was estimated at 63 ± 5% (n = 4) under these conditions. (*B*) Effect of MgTX on the IK(Ca^{2+}) channel in BAEC. Experimental conditions as in *A*. At this concentration, MgTX caused a 47 ± 13% (n = 3). As observed with ChTX and StK, the addition of MgTX to the bathing solution resulted in the appearance of long closures, with no modification of the channel current jump amplitude. Arrows indicate the closed state current level.

hibition pattern was similar to that observed in the presence of ChTX (*see* Fig. 1), with the appearance of long silent periods interrupted by bursts of channel activity. Reductions of channel activity of 15 ± 3% (n = 4) and 63 ± 5% (n = 4) were obtained at StK concentrations of 50 nM and 100 nM respectively. The binding affinity of StK to the IK(Ca^{2+}) channel in BAEC appears therefore much lower than that measured for ChTX, but significantly higher than the half inhibition values expected for IbTX (Fig. 2). MgTX is also an other toxin which shares a significant degree of identity with ChTX (44%) and IbTX (41%). This toxin has been described as a selective inhibitor of voltage-gated K$^+$ channels, but appeared to be inactive against the Ca^{2+}-activated K$^+$ channel of intermediate conductance present in human T-lymphocytes [26,29]. Representative outside-out current traces recorded in control conditions and in the presence of MgTX (100 nM) are presented in Fig. 3*B*. At a concentration of 100 nM, MgTX caused a 47 ± 13% (n = 3) inhibition of the IK(Ca^{2+}) channel activity. This toxin is therefore a less potent IK(Ca^{2+}) channel blocking agent than both ChTX and StK, but constitute a better blocker than IbTX.

Effects of TEA and d-TC on IK(Ca^{2+}) in BAEC

Several studies have established that internal addition of the large organic cation TEA induces a slightly voltage dependent fast blockage of the Maxi K(Ca^{2+}) channels with characteristic K_d values ranging from 27–60 mM [25,37]. TEA applied externally caused however a more potent fast blocking action (K_d = 0.14 to 0.3 mM) with

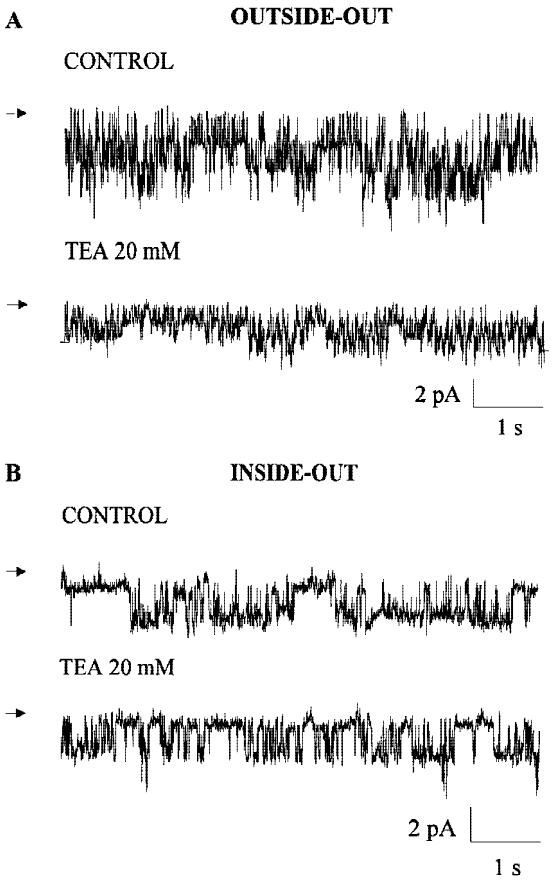

Fig. 4. Effect of TEA on the IK(Ca^{2+}) channel in BAEC. (*A*) Outside-out single-channel current records aimed at investigating the action of TEA when applied externally. Currents measured in symmetrical 200 mM KCl, at an applied voltage of −60 mV. The external addition of 20 mM TEA resulted in a clear decrease of the channel current jump amplitude with no significant changes in P_o. (*B*) In contrast, internal application of TEA failed to affect the unitary current jump amplitude under identical KCl (200 mM) and TEA (20 mM) conditions. Inside-out experiments carried out in symmetrical 200 mM KCl at a pipette potential of 60 mV. Arrows indicate the closed state current level.

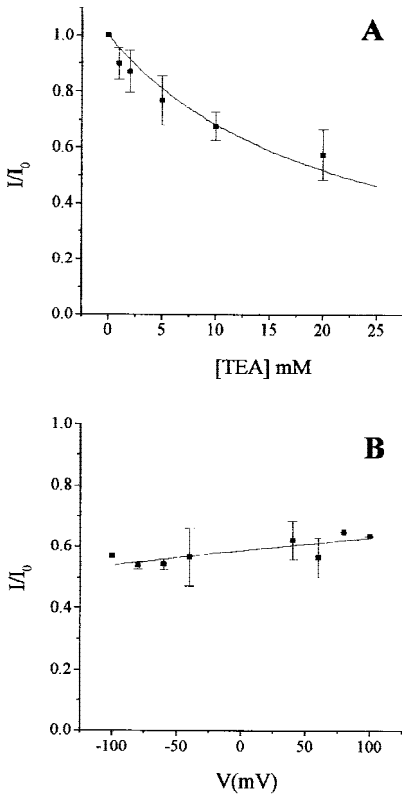

Fig. 5. (*A*) Dose-response curve of the normalized current jump amplitude as a function of the external TEA concentration. Results obtained from outside-out experiments performed in symmetrical 200 mM KCl at a constant pipette potential of −60 mV. The continuous curved was computed from Eq. (1), for a half inhibition concentration value at $V = 0$ mV of 23 ± 3 mM ($n = 3$). (*B*) Voltage dependency of the effect of TEA (20 mM) on the current jump amplitude. Results obtained from outside-out experiments performed in symmetrical 200 mM KCl at a fixed TEA concentration of 20 mM. Continuous line computed from equation (1) with $X_0 = 23$ mM and $\alpha = 0.045$ ($n = 3$). These results show that effect of external TEA is not significantly voltage dependent indicating a binding site located at the entrance of the channel external mouth.

a reduced voltage-sensitivity [37]. Figure 4 shows the effect of TEA on IK(Ca^{2+}) channels in BAEC, first (Fig. 4*A*) in outside-out experiments where TEA (20 mM) was applied to the membrane external surface, and in inside-out patch recordings in which TEA was added to the bathing solution (Fig. 4*B*). The results in Fig. 4*A* clearly indicate that the external application of 20 mM TEA caused a decrease of the unitary current jumps amplitude without modification of the channel open probability. Such a reduction was not observed when 20 mM TEA was added in inside-out patches to the cytosolic-like medium (Fig. 4*B*). The dose-response curve of current amplitude reduction measured at −60 mV as a function of the external TEA concentration is shown in Fig. 5*A*. The voltage dependence of the current ratio I/I_0 at a fixed TEA concentration of 20 mM is presented in Fig. 5*B*. The data were fitted to equation (1) with $X_0 = 23 \pm 3$ mM and $\alpha = 0.045 \pm 0.002$ ($n = 3$). On the basis of these results, it appears therefore that the IK(Ca^{2+}) channel in BAEC is substantially less sensitive to TEA than the Maxi K(Ca^{2+}) channels identified in smooth muscle cells, while displaying, as for the Maxi K(Ca^{2+}) channels, a weak voltage-dependent block ($\alpha < 0.1$) when TEA is applied to the external medium.

d-TC has been reported to affect the agonist-induced hyperpolarization observed in several vascular EC [4,41,62]. The current traces in Fig. 6*A* and *B* illustrate the differential effect of d-TC on the IK(Ca^{2+}) channel in BAEC depending on the patch-clamp configuration used. In Fig. 6*A*, the external addition of d-TC at concentrations up to 5 mM caused a clear reduction of the channel current jump amplitude, typical of a fast blocking pro-

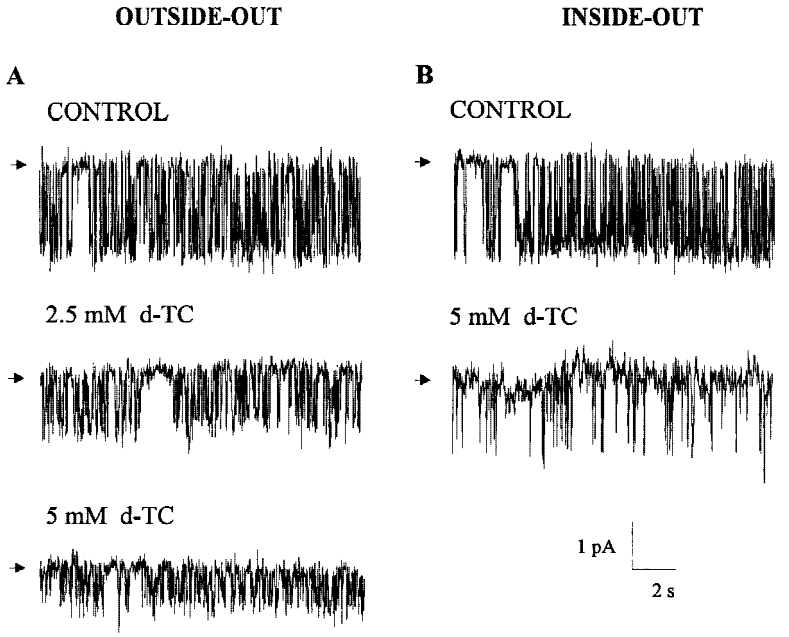

Fig. 6. Effect of d-TC on the IK(Ca^{2+}) channel in BAEC. (*A*) Outside-out current records measured in symmetrical 200 mM KCl solutions in control conditions and following addition of d-TC at 2.5 mM and 5.0 mM respectively. The pipette potential was −60 mV throughout. The external application of d-TC resulted in a dose-dependent decrease of the channel current jump amplitude. (*B*) In contrast, internal application of d-TC in inside-out experiments, led to the appearance of long silent periods with no significant change of the current unitary amplitude. Experiments performed in symmetrical 200 mM KCl, with a pipette potential = 60 mV. Arrows indicate the closed state current level.

cess. This effect was voltage independent. In contrast, when d-TC was applied internally, the single-channel activity measured over the same concentration range was characterized by the appearance of silent periods of several seconds duration, typical of a slow block of the channel by the drug (Fig. 6*B*). Figure 7 presents the dose-response curve of the effect of external d-TC on the current amplitude computed from four different recordings. Half inhibition was obtained at 4.4 ± 0.3 mM (n = 4) indicating a low affinity of the drug for the IK(Ca^{2+}) channel in BAEC [22,64].

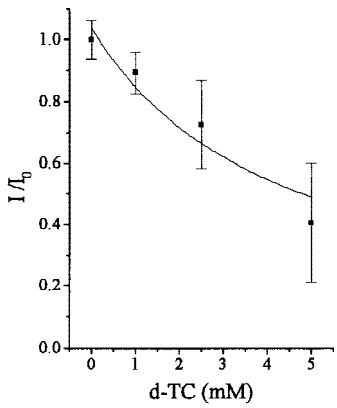

Fig. 7. Dose-response curve of the effect of external d-TC on the normalized unitary current amplitude of the IK(Ca^{2+}) channel in BAEC. Data points obtained from three different outside-out experiments performed in symmetrical 200 mM KCl. The pipette potential was maintained at −60 mV. The continuous curve was generated using Eq. (1) with X_o = 4.4 mM and α = 0.0.

Effects of the Potassium Channel Activator NS1619 on IK(Ca^{2+}) in BAEC

The benzimidazolone NS1619 has been proven to constitute a potent Maxi K(Ca^{2+}) channel activator in several cell types including human bronchial smooth muscle cells, human and bovine coronary artery smooth muscle cells, rat pancreatic B-cells, HIT-T15 cells, mouse cortical neurons and cerebellar granule cells [48]. The effect of NS1619 on the IK(Ca^{2+}) channel in BAEC was tested in a series of inside-out experiments in which NS1619 was applied at concentrations ranging from 10 to 100 μM. The recordings in Fig. 8 show that there was a clear decrease in single-channel activity following the addition of NS1619 to the bath, with a near complete inhibition at 100 μM (P_o = 0.06). The action of NS1619 was poorly reversible and involves both an increase of the channel mean closed time, coupled to a decrease of the channel open time. In addition, NS1619 at a concentration of 100 μM failed to affect the IK(Ca^{2+}) channel activity when applied to the membrane external surface in outside-out experiments (*data not shown*). The dose-response curve describing the effect of NS1619 on the IK(Ca^{2+}) open probability is illustrated in Fig. 9. Half inhibition was observed at 11 ± 0.8 μM (n = 4), a concentration value at which activation of Maxi K(Ca^{2+}) channel was reported [48]. The detailed analysis of the channel dwell time distributions shown in Fig. 10 reveals in addition that under control conditions, the IK(Ca^{2+}) channel kinetic behavior can be accounted for by a single open state coupled to two dominant closed states. The addition of NS1619 resulted in the appearance of a third closed state characterized by a slow time constant of 0.1

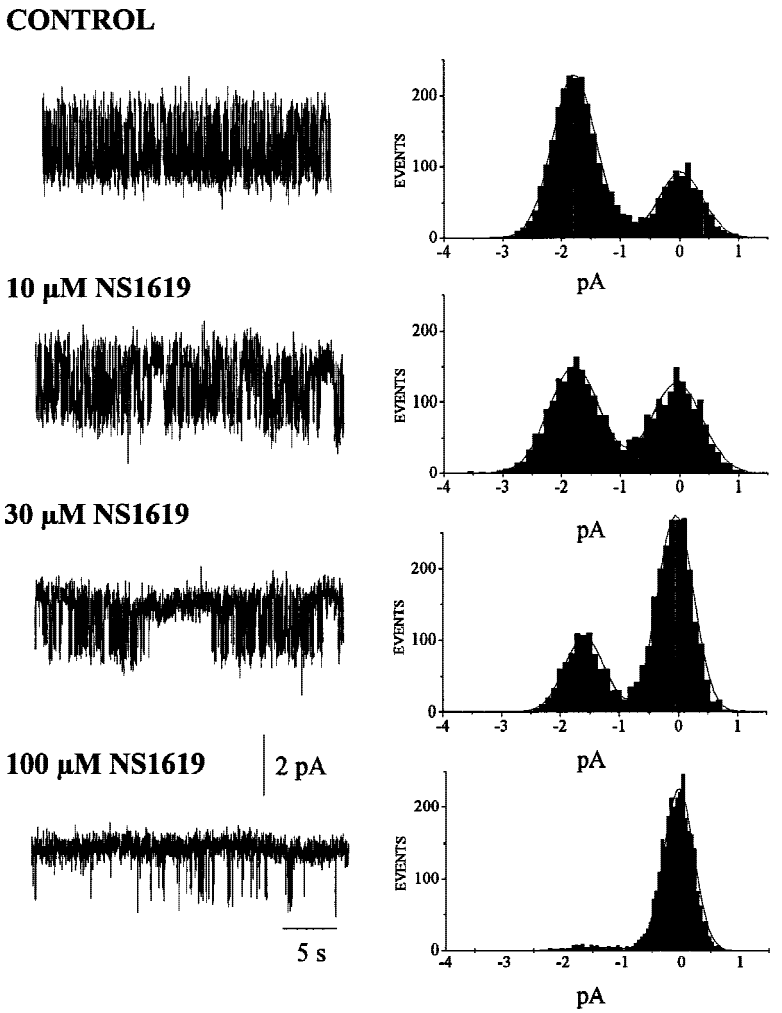

Fig. 8. Effect of the Maxi K(Ca^{2+}) channel activator NS1619 on the IK(Ca^{2+}) channel in BAEC. Inside-out single channel recordings obtained in symmetrical 200 mM KCl. The pipette potential was 60 mV throughout. Near complete inhibition of channel activity was observed at a NS1619 concentration of 100 μM. There was no significant changes in the channel unitary amplitude.

sec at 10 μM NS1619 (*data not shown*) and 0.6 sec at 30 μM (Fig. 10) respectively. The appearance of this new state led to a significant increase of the channel mean closed time with values of 7 msec, 13 msec, 77 msec and 140 msec at 0, 10, 30 and 100 μM NS1619 respectively. There was also a reduction of the channel mean open time from 18 msec in control conditions to 9 msec and 6 msec in 30 and 100 μM NS1619 respectively. These combined effects account for the decrease in P_o observed at increasing NS1619 concentration values.

Discussion

K(Ca^{2+}) have been divided into three main classes on the basis of their single-channel conductance [10,25,37]: large conductance (100–250 pS) K(Ca^{2+}) channels that are inhibitable by nanomolar concentrations of IbTX and ChTX [8,43], apamine sensitive small conductance K(Ca^{2+}) characterized by an unitary conductance of less than 20 pS [36] and K(Ca^{2+}) channels of intermediate conductance (20 pS to 80 pS). IK(Ca^{2+}) channels have recently been cloned from human T lymphocytes (hKCa4) [38], human pancreas (hIK1) [35] and HeLa cells (this laboratory; *unpublished results*). The resulting protein comprises 427 amino acids with six transmembrane segments S1-S6, and a pore motif between S5 and S6. The amino acid sequence of the IK(Ca^{2+}) channels identified so far appeared 42–45% identical and 50–55% conserved compared to the K(Ca^{2+}) channels of small conductance (SK). These structural informations provide therefore strong evidence that IK(Ca^{2+}) channels are more closely related to SK than Maxi K(Ca^{2+}) channels. The present work provides new single-channel results on the IK(Ca^{2+}) channels in BAEC for which a detailed pharmacological profile was unknown to this date. Evidence was herein presented indicating that despite a similar sensitivity to ChTX, Maxi K(Ca^{2+}) and IK(Ca^{2+}) channels differ markedly in their pharmacological specificity. For instance, the IK(Ca^{2+}) channel was insensitive to IbTX and responded to TEA at concentrations 100 times higher than that used for Maxi K(Ca^{2+}) inhibition.

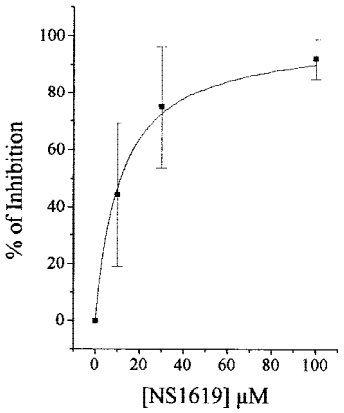

Fig. 9. Dose-response curve of the inhibitory effect of internal NS1619 on the K(Ca^{2+}) channel activity. Data points obtained from three different inside-out experiments performed in symmetrical 200 mM KCl. The pipette potential was maintained equal to 60 mV throughout. The percentage of inhibition was calculated according to $100(1 - P_o(X)/P_o(0))$ where $P_o(X)$ is the channel open probability measured at a given concentration X of NS1619, and $P_o(0)$, the channel open probability measured in the absence of NS1619. The continuous line was obtained by curve fitting the data to $100X/(X + X_o)$ with $X_o = 11 \pm 0.8$ μM ($n = 3$).

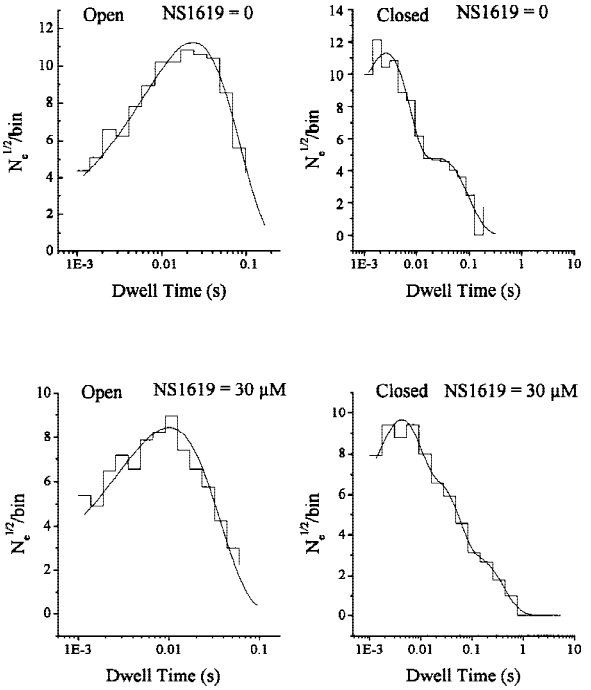

Fig. 10. Effect of NS1619 on the IK(Ca^{2+}) channel dwell time distributions. Examples of dwell time distributions where time intervals were bined according to a logarithmic scale. The open time distribution was fitted to a single exponential function while a minimum of two exponentials and three exponentials respectively were required to account for the closed time interval distribution in control conditions and following the addition of NS1619. The mean open time was equal to 18 msec in the absence of NS1619, and to 8 msec in 30 μM NS1619. The characteristic times for the closed state distribution corresponded to 2.5 and 26 msec in control conditions and to 3.5 msec, 28 msec and 0.6 sec in the presence of 30 μM NS1619. N_e represents the number of intervals collected per bin.

More remarkable is the observation that the Maxi K(Ca^{2+}) channel activator NS1619 caused a decrease rather than an increase of the IK(Ca^{2+}) channel activity. In addition, MgTX, a toxin which failed to block Maxi K(Ca^{2+}) channels, and the marine toxin StK which appeared as a potent inhibitor of voltage-gated K^+ channels, were both effective in reducing the IK(Ca^{2+}) channel open probability. Finally, our results showed a differential inhibitory action of d-TC whether the drug was applied to the internal or external surface of the membrane.

ChTX was regarded originally as a specific inhibitor of Maxi K(Ca^{2+}) channels. More recent works have indicated however that the voltage-gated $K_V1.2$ and $K_V1.3$ channels are also very sensitive to the peptide [28,57]. The presence of ChTX-sensitive IK(Ca^{2+}) has also been reported in a variety of cell preparations. For instance, dissociation constants of 5.4 nM and 3–4 nM have been measured in human red cells [6] and human T-lymphocytes [29] respectively. These values are in agreement with the data presented in Fig. 1*A* where half inhibition of the IK(Ca^{2+}) channel in BAEC was observed at 3.3 nM. ChTX was less effective however in blocking IK(Ca^{2+}) channels present in HL-60 cells [61], in *ras*-transformed fibroblasts [34] or in the endothelium of intact rat aorta [41]. In the latter case 50% inhibition was achieved at 137 nM ChTX only. In addition, ChTX failed to cause at concentrations up to 1 μM a significant reduction of the IK(Ca^{2+}) channel activity in the epithelial cell line T_{84} [58]. The results in Fig. 1 and the observations of Marchenko and Sage, [41] on intact aortic endothelium confirm therefore that the Ca^{2+}-dependent K^+ permeability of EC can be affected by ChTX, but a ChTX related depolarization of the cell potential in this case can not be used to discriminate Maxi K(Ca^{2+}) channels from IK(Ca^{2+}).

One of the main findings of this work is the absence of IbTX inhibition despite a 68% sequence identity with ChTX [8]. The results in Fig. 2 support the findings reported on human red cells where IbTX appeared ineffective at inhibiting the influx of $^{86}Rb^+$ activated by Ca^{2+} [6]. Because IK(Ca^{2+}) channels are IbTX insensitive, the observation of IbTX-related effects on EC is likely therefore to reflect the contribution of Maxi K(Ca^{2+}) channels to the electrophysiological response of EC. In contrast, the marine toxin StK caused a significant inhibition of the IK(Ca^{2+}) channel in BAEC (Fig. 3*A*). This toxin has been reported to block rat brain K^+ channels which are primarily of the $K_V1.2$ type and the human Jurkat T-lymphocyte $K_V1.3$ channel [51]. Interestingly, the single-channel recordings in Fig. 3*B* show that the

IK(Ca^{2+}) channel in BAEC was also inhibitable by MgTX, a specific blocker of $K_V1.3$ channel. However, the ID_{50} value measured in the present case is 1000 higher than that reported for $K_V1.3$ inhibition. MgTX was also shown to affect the Ca^{2+}-activated $^{86}Rb^+$ influx in human red cells, but the toxin was clearly less potent than ChTX and StK, in agreement with the patch-clamp results in this work [6].

The results in Figs. 4 and 5 provide evidence for a low binding affinity of external TEA to IK(Ca^{2+}) channels compared to Maxi K(Ca^{2+}). The half inhibition concentration derived from the data in Fig. 5*A* is within the reported range of values obtained for the SK1 channel [59] and for the IK(Ca^{2+}) channels identified in human T-lymphocytes [29], *ras*-transformed fibroblasts [34], HeLa cells [18], T_{84} epithelial cells [58]. Among the voltage-gated K^+ channels, $K_V1.1$ channels show a high affinity to TEA with an IC_{50} value of 0.3 mM, whereas the closely related $K_V1.2$ channels appeared weakly blocked (IC_{50} = 150 mM) [39]. In the latter case, site directed mutagenesis experiments have shown that mutation of Val^{381} to Tyr, leads to a TEA affinity equivalent to that of $K_V1.1$ [32]. Similarly, substituting in the Ca^{2+}-activated SK1 channel a Val at position 355 by a Tyr increased sensitivity to external TEA from 14.6 mM to 0.3 mM [59]. Maxi K(Ca^{2+}) channels which are characterized by a high affinity to external TEA, also contain a Tyr residue at the C-terminal portion of the pore region [3]. Our results on the effects of TEA, ChTX and IbTX on the IK(Ca^{2+}) channel in BAEC provide therefore indirect evidence that the overall architecture of the other vestibule of the IK(Ca^{2+}) channel in BAEC can be better accounted for by a SK type than a Maxi K(Ca^{2+}) type channel structure. Interestingly, the amino acid sequence obtained for the IK(Ca^{2+}) channels cloned from human T lymphocytes (hKCa4) [38] and human pancreas (hIK1) [35] show a Val at position 257 in the C-terminal portion of the pore region. This finding is compatible with the present observation of a low TEA sensitivity for the IK(Ca^{2+}) channel in BAEC.

d-TC has been reported to reduce the hyperpolarization evoked by a Ca^{2+} ionophore or bradykinin in cultured guinea-pig [11] and pig coronary artery endothelial cells [62]. d-TC has also been found to reduce the current jump amplitude of a IK(Ca^{2+}) present in the endothelium of intact rat aorta [41]. This latter effect was observed at concentrations >1 mM, in agreement with the results presented in Figs. 6 and 7 of this work. In addition, because the reduction in unitary current amplitude illustrated in Fig. 7 appeared voltage-insensitive, the binding site of d-TC to IK(Ca^{2+}) is likely to be located on the external side of the pore region. The single-channel recordings in Fig. 6 also indicate that d-TC caused a differential effect depending on the patch-clamp configuration used. D-TC applied externally induced a reduction of the unitary current jump amplitude typical of a fast blocking process whereas, addition of d-TC in inside-out recordings resulted in a slow blocking action. These observations suggest a stronger binding of d-TC to the channel inner than external pore region. Block of Maxi K(Ca^{2+}) channels by d-TC on the cytoplasmic side has been reported by Egan et al., [20] in rat olfactory bulb neurons and by Baron et al., [4] in pig coronary artery endothelial cells. However, near complete channel inhibition was measured in these cases at d-TC concentrations <100 μM. Similarly, results obtained on small-conductance K(Ca^{2+}) channels have provided evidence for an external d-TC block at concentrations ranging from 5 μM for the SK2 channel to 300 μM for the SK1 channel [36,49]. Interestingly, the double mutations E330D plus H357N of the SK1 channel shifted the sensitivity to that of SK2 [59]. The finding that the IK(Ca^{2+}) channel in BAEC is insensitive to external d-TC at concentrations where total block of the d-TC sensitive SK1 or SK2 channels was measured (<1 mM), argues for different structures in the external pore region between these two types of Ca^{2+}-activated channels. This conclusion is further supported by the recent publication of the amino acid sequence for the IK(Ca^{2+}) channels hKCa4 [38] and hIK1 [35] where a Gly^{259} and an Asn^{232} are now substituting for the His/Asn at position 357 and the Glu/Asp at position 330 in SK1/SK2 respectively [35]. These differences may partly explain the low affinity of the IK(Ca^{2+}) in BAEC to d-TC compared to for both SK1 and SK2 channels. The results in Fig. 7 agree however with the low sensitivity to external d-TC reported for an IK(Ca^{2+}) channel measured in intact rat aorta [41]. Both channels share several key pharmacological features such as poor d-TC sensitivity and lack of apamine-related inhibition, while displaying different affinities to ChTX. The absence of an apamine block in both cases supports the close correlation established in SK1 and SK2 channels between apamine and d-TC sensitivity [59]. The d-TC results suggest therefore that the IK(Ca^{2+}) channel in intact rat aorta corresponds to the IK(Ca^{2+}) channel in BAEC or to a closely related structure.

A major observation of the present work is the inhibitory action of the Maxi K(Ca^{2+}) activator NS1619. NS1619 is known to activate a variety of Maxi K(Ca^{2+}) channels including the cloned dSlo, mSlo and hSlo α-subunits [47]. There are indications however that this compound can block L-type Ca^{2+} currents as well as K_{ATP} and delayed rectifier currents in freshly isolated rat portal vein smooth muscle cells [19]. A 40% inhibition of K_V currents was also observed in HIT-T15 at 10 μM NS1619 [47]. In hypothalamus neurons, NS1619 caused the activation of a Maxi K(Ca^{2+}) channel, but failed to affect the K_{ATP} channels present in the same neurons [56]. Our results clearly indicate a reduction in single-

channel activity in inside-out recordings where NS1619 was applied into the bathing medium (Fig. 8). Half inhibition was observed at 11 μM (Fig. 9) and there was no significant change in the current unitary amplitude. An analysis carried out using an algorithm based on the Baum-Welch re-estimation formulae [13] confirmed that NS1619 caused a marked increase in the channel mean closed time, coupled to a less important reduction of the channel mean open time. For instance, the channel mean closed time and mean open time changed from 7 and 18 msec in control conditions to 13 and 12 msec at 10 μM NS1619 and to 77 and 8 msec at 30 μM NS1619 respectively. The analysis presented in Fig. 10 confirmed in addition that the presence of NS1619 resulted in the appearance of an additional closed state characterized by a slow time constant. The fact that the time constant of the channel open state decreased at increasing NS1619 concentrations suggests that NS1619 binds to the channel while being in the open state. But, the observation that the time constant of the additional closed state varied as a function of the NS1619 concentration, argues against an action of NS1619 that can be modeled in terms of a simple block of the open channel by NS1619. Because benzimidazolones are highly lipophilic compounds, the fact that no significant effect of NS1619 was observed over a 2-min period in outside-out experiments is unexpected. Our results do not therefore support an inhibitory action that would be mediated by an unspecific effect on the membrane structure, but rather suggest that NS1619 may interact with an hydrophobic domain located on the cytoplasmic moiety of the IK(Ca^{2+}) channel. Such an interaction may in turn lead to modifications of the channel kinetic properties as shown in Fig. 10, and which can not be accounted for by a simple block of the channel pore region.

Conclusions

This work provides evidence at the single-channel level for a pharmacological profile for the IK(Ca^{2+}) channels in BAEC that is distinct from Maxi and small-conductance K(Ca^{2+}). IK(Ca^{2+}) channels have been involved in a variety of cellular processes including cell proliferation [34,50], electrical response to mechanical stimulation [23,24], cell volume regulation [6,12], salt secretion [58] and internal Ca^{2+} signaling [29,53]. A specific pharmacological profile for the IK(Ca^{2+}) may provide not only additional tools to further evaluate the contribution of these particular channels to the electrophysiological response of EC to Ca^{2+}-mobilizing agonists, but also structural informations to better characterize the unique properties of these channels.

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