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ORIGINAL ARTICLE

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Role of a Ca^{2+}-activated K^+ current in the maintenance of resting membrane potential of isolated, human, saphenous vein smooth muscle cells

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Abstract Calcium-activated potassium currents were studied in dissociated smooth muscle cells from human saphenous vein (HSV) using the patch-clamp technique in the whole-cell configuration. The average measured resting membrane potential (V_m) was –41±2 mV (n=39), when the cells were dialysed with an intracellular pipette solution (IPS) containing 0.1 mM ethyleneglycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (IPS-0.1 mM EGTA). When the EGTA concentration was increased to 10 mM (IPS-10 mM EGTA) V_m became significantly less negative: –13±2 mV (n=23, P<0.05). These results suggest that 10 mM EGTA reduces a calcium-dependent current involved in the maintenance of V_m. Depolarizing voltage steps up to +60 mV from holding potentials of –60 mV resulted in large (1–10 nA) time- and voltage-dependent outward currents. The amplitudes of total whole-cell current densities measured at voltages above –20 mV were significantly greater in the cells dialysed with IPS-0.1 mM EGTA than in those dialysed with IPS-10 mM EGTA. In the cells dialysed with IPS-0.1 mM EGTA, 0.1 mM tetraethylammonium chloride (TEA) and 50 nM iberiotoxin (IBTX), which selectively block large conductance Ca^{2+}-activated potassium channels (BK_{Ca}), diminished the total current recorded at +60 mV by 45±14% (P<0.05, n=5) and 50±6% (n=8, P<0.05), respectively. These blockers at the same concentrations did not affect the total current in cells dialysed with IPS-10 mM EGTA. When tested on intact HSV rings, both 0.1 mM TEA and 50 nM IBTX elicited vessel contraction. We conclude that BK_{Ca} channels present in HSV smooth muscle cells contribute to the maintenance of the V_m and sustain a significant portion of the total voltage-activated, outward current. Finally, BK_{Ca} channels appear to play a significant role in the regulation of HSV smooth muscle contractile activity.

Key words BK_{Ca} channels · Resting membrane potential · Vascular smooth muscle cells · Patch-clamp technique

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Introduction

Different types of potassium channels are found in vascular smooth muscle cells (VSMC), i.e. calcium-sensitive (K_{Ca}), voltage-dependent ($K_{v,}$), inwards rectifying (K_{IR}) and adenosine triphosphate-sensitive (K_{ATP}) channels [15]. Potassium channels are implicated in the genesis and regulation of resting membrane potential (V_m) and in this way are critically involved in the mechanism of VSMC contraction. Membrane potentials of smooth muscle cells have been measured in different vascular beds and the contribution of some K^+ channel types in the maintenance of V_m has been assessed. In particular, large-conductance Ca^{2+}-activated potassium channels (Maxi-K or BK_{Ca}), which commonly are present in different kinds of VSMC [5, 14], participate in the regulation of V_m in guinea-pig aortic cells [18], rabbit cerebral arteries [3], rat femoral arteries [2] and human pial artery cells [6].

Although many mechanical and electrophysiological studies have been performed to identify and characterize BK_{Ca} channels in VSMC from different animal tissues, little is known about the expression of these channels in human VSMC and their possible physiological and pathological roles.

Some functional roles for BK_{Ca} channels have been reported in human vessels. In pial arteries [6], charybdotoxin and tetraethylammonium (TEA) depolarize the membrane and increase both the amplitude and duration of action potentials and contractions, suggesting that BK_{Ca} channels are important determinants of VSMC V_m, excitability and tone in these vessels. In coronary artery

cells in culture [17], it has been shown that the 230-pS BK_{Ca} channel is activated by ATP acting via P2 purinoreceptors. This ATP-activated current is blocked by iberiotoxin (IBTX, 200 nM) and by strong buffering of intracellular Ca^{2+}, and was potentiated by the BK_{Ca} channel activator NS-1619 (30 μM).

Finally, in human saphenous vein (HSV) smooth muscle cells a BK_{Ca} single-channel conductance of 226 pS has been reported recently [24], although the contribution of these channels to the maintenance of the V_m in HSV smooth muscle cells has not been elucidated. In a cannulated preparation of HSV segments, vein diameter decreases significantly when the vessels are exposed to BK_{Ca} channel blockers such as IBTX [19]. This study shows that BK_{Ca} channel activity depends on the intraluminal pressure [19].

By measuring whole-cell currents and V_m with the patch clamp technique in isolated HSV smooth muscle cells and by using specific BK_{Ca} blockers, we present first evidence that BK_{Ca} channels sustain a significant portion of the total voltage-activated, outward current and participate in the maintenance of the V_m in these cells. We also show that TEA and IBTX produce contraction of quiescent HSV rings, indicating that the BK_{Ca} channels contribute to the regulation of the contractile state of the vessel.

Materials and methods

Cell isolation procedure

Segments of HSV were obtained from 26 patients, aged 51–71 years undergoing aortocoronary bypass surgery. The vessels were placed in saline solution (NaCl 0.9% at 0–4 °C) and immediately transported to our laboratory. Cell isolation procedure was based on the method described by Klöckner [10]. Briefly, the sample vessel was cleaned of any residual connective tissue, cut in small strips and placed for 20 min in a dissociation medium (DM) containing (in mM): 140 NaCl, 5 KH_2PO_4, 5 $MgCl_2$, 20 glucose, 5 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, tris(hydroxymethyl)aminomethane salt (HEPES-TRIS), pH 7.4. The strips were then placed in DM with collagenase 2 mg/ml, papain 2 mg/ml, protease type I 0.5 mg/ml and dithiothreitol 2 mM for 60 min at 30 °C. After the incubation period the strips were washed with DM and single HSV smooth muscle cells were obtained by gentle dispersion of the treated tissue using a Pasteur pipette. The remaining tissue and the supernatant containing isolated cells were stored at room temperature until used.

Patch-clamp recording

HSV smooth muscle cells were allowed to settle onto the coverglass bottom of a 0.5 ml experimental chamber. The cells were observed with a mechanically stabilized, inverted microscope (Zeiss-Telaval3) equipped with a 40× objective. The chamber was perfused by gravity flow for 15 min at 0.5 ml·min^{-1}, with standard extracellular saline solution (SESS, composition below) before the patch-clamp experiment was started. Test solutions were applied through a multi-barrelled pipette positioned close to the cell investigated. After each experiment on a single cell, the experimental chamber was replaced by another one containing a new sample of cells. Only well-relaxed spindle-shaped smooth muscle cells were used for electrophysiological recording. Data were collected within 4–6 h after cell isolation. All experiments were performed at room temperature (approximately 20 °C).

The standard, tight-seal, whole-cell configuration of the patch-clamp technique [8] was used to current- and voltage-clamp single HSV smooth muscle cells. Pipettes were drawn from capillary glass (PG52165-4, WPI, Boca Raton, Fla., USA) on a two-stage vertical micropipette puller (PP-83, Narishige, Tokyo, Japan) and pipette resistances were 2–4 MΩ measured in SESS. Ionic currents were measured with an appropriate amplifier (Axopatch 200A, Axon Instruments, Foster City, Calif., USA). Whole-cell currents were filtered at 5 kHz, digitized (Digidata 1200, Axon Instruments) at a sample frequency of 20 kHz and stored on a computer hard disk for later analysis. Series resistances were 10–15 MΩ and were compensated electronically by 70–80%. Recordings were not corrected for leak subtraction or capacitive transients. Total cell membrane capacitance was estimated by integrating the capacitive current transient elicited by the application of 10-mV hyperpolarizing step pulses from a holding potential of –60 mV. The estimated membrane capacitance of HSV cells was 32.6±2 pF (n=35).

Solutions

The standard extracellular saline solution (SESS) used for recording ionic currents contained (in mM): 150 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 3.6 $CaCl_2$, 20 glucose, 5 HEPES-TRIS, pH 7.4. The composition of the intracellular pipette solution (IPS) containing 0.1 mM ethyleneglycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) was (in mM): 130 KCl, 5 ATP-Na_2, 1 $MgCl_2$, 10 glucose, 0.1 EGTA, 20 HEPES-TRIS, pH 7.3. Using a custom-made computer program that takes into account the concentration of salts, pH and temperature, we estimated an intracellular free$[Ca^{2+}]$ ($[Ca^{2+}]_i$) of 100 nM (assuming Ca^{2+} contaminant of other salts to be 50 μM). In experiments in which the EGTA concentration of the above-described IPS was augmented to 10 mM (IPS-10 mM EGTA) to buffer $[Ca^{2+}]_i$, the calculated free $[Ca^{2+}]_i$ was 1 nM.

Tension measurements in HSV rings

HSV rings (2–3 mm) were cut and attached isometrically to a force transducer (Letica TRI-201), the output signal from which was amplified and recorded using a data acquisition board (DT16EZ, Data Translation, Marlboro, Mass., USA) and software (Labtech Notebook Pro, Labtech, Andover, Mass., USA) and stored for further analysis. The preparations were then submerged in a glass chamber containing Krebs Ringer bicarbonate solution (KRBS), bubbled with 5% CO_2 and 95% O_2, at 37 °C. The composition of KRBS was in mM: 130 NaCl, 4.7 KCl, 24 $NaCO_3H$, 1.17 Na_2PO_4H, 1.16 $MgSO_4$, 1.6 $CaCl_2$ and 11 glucose, pH 7.4. A passive tension of 2 g was applied, and the rings were allowed to equilibrate for 60 min, changing the solution in the chamber every 20 min. To test the contractile response of the vein, the rings were exposed to a solution containing a high KCl concentration prepared by substituting 80 mM KCl for 80 mM NaCl. The force developed was 33 g per gram wet tissue (n=12). To test the effects of BK_{Ca} channels blockers on intact tissue, aliquots of a stock aqueous solution of TEA 200 mM and IBTX 60 μM were added to the experimental chamber (30 ml) to achieve final concentrations of 0.1 mM and 50 nM, respectively. The contractile effects of these BK_{Ca} channels blockers will be expressed as a percentage of the KCl contractions.

Reagents

TEA, IBTX, EGTA and ATP-Na_2 were purchased from Sigma (St. Louis, Mo., USA). All other reagents were analytical grade. Fresh aliquots of stock aqueous solutions of TEA or IBTX were added to the extracellular solutions on the day of the experiment.

Statistics

The results are expressed as means±SEM. Student's t-test, for paired or unpaired samples as appropriate, was used to establish the significance of differences between means. P<0.05 was accepted as significant.

Results

Electrophysiological properties of HSV smooth muscle cells

The V_m of HSV smooth muscle cells was measured in the current-clamp mode in the whole-cell configuration. In cells superfused with SESS and dialysed with the IPS-0.1 mM EGTA, V_m was –41±2 mV (n=39), whereas in cells dialysed with IPS-10 mM EGTA V_m was significantly less negative:-13±2 mV (n=23, P<0.05).

Figure 1A shows typical currents elicited in response to a series of test potentials ranging from –50 to +60 mV applied in 10-mV increments from a holding potential of –60 mV. This protocol was performed in cells dialysed with IPS-0.1 mM EGTA (Fig. 1A left panel) or with IPS-10 mM EGTA (Fig. 1A right panel). Figure 1B shows current/voltage (I/V) relations in cells dialysed with IPS-0.1 mM EGTA (n=13) or with IPS-10 mM EGTA (n=13). Mean total current densities (amplitude of end pulse currents measured at 340 ms divided by the cell membrane capacitance) are shown as a function of V_m. Total current densities, measured at potentials between –20 and +60 mV, were significantly lower when intracellular Ca^{2+} was strongly buffered with 10 mM EGTA compared with the 100 nM free-$[Ca^{2+}]_i$ (IPS-0.1 mM EGTA). These results, taken together with the observed shift of V_m to more positive values in the IPS-10 mM EGTA dialysed cells, could indicate the presence of a calcium-dependent potassium current in HSV smooth muscle cells. To test whether this current is a large-conductance Ca^{2+}-activated K^+ current (IBK$_{Ca}$) type, the effects of two specific blockers of this current were tested.

Effects of BK$_{Ca}$ channel blockers

Low concentrations of TEA (0.1–1 mM) and IBTX (20–200 nM) have been shown previously to be effective BK$_{Ca}$ channels blockers in different types of vascular myocytes including rabbit portal vein [14], rabbit cerebral arteries [3], rat femoral arteries [2], human coronary artery [17], human saphenous vein [24], and cloned human BK$_{Ca}$ channels (hSlo) expressed in *Xenopus laevis* oocytes [7, 12].

The effects of TEA 0.1 mM on representative current traces evoked by membrane depolarization to +60 mV from –60 mV recorded in myocytes dialysed with IPS-0.1 mM EGTA or IPS-10 mM EGTA are shown in Fig. 2A and B, respectively. In HSV smooth muscle cells dialysed with IPS-0.1 mM EGTA, TEA 0.1 mM significantly reduced the control total end pulse current elicited by

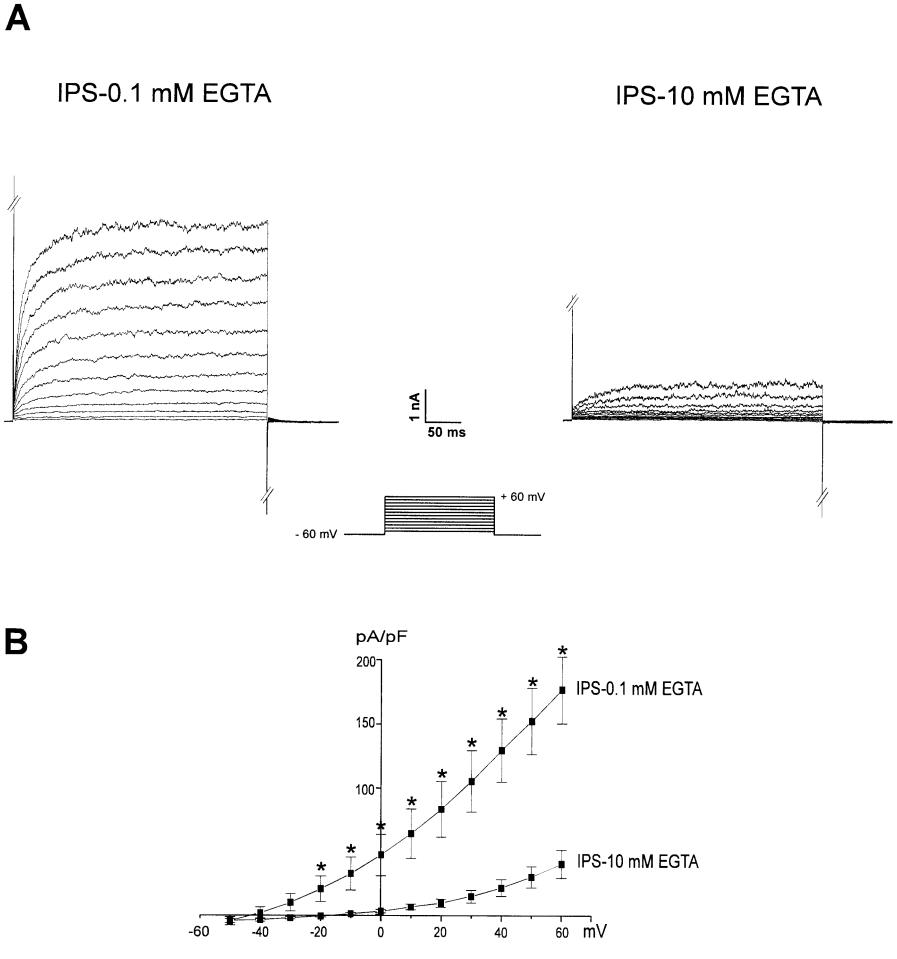

Fig. 1 **A** Typical, whole-cell, voltage-activated currents in human saphenous vein (HSV) smooth muscle cells dialysed with an intracellular pipette solution (*IPS*) containing 0.1 mM ethyleneglycolbis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (*EGTA*, *left panel*) or 10 mM EGTA (*right panel*). **B** Current/voltage (I/V) curves for the total end pulse current density. Values shown are means±SEM in IPS-0.1 mM EGTA (n=13) and in IPS-10 mM EGTA (n=13). *P<0.05 by paired Student's t-test

a depolarizing step to +60 mV (Fig. 2C). After 5 min TEA washout, the current returned to the control amplitude in two cells, while three cells failed to recover the initial total current amplitude. Conversely, when the same concentration of TEA was tested on HSV smooth muscle cells dialysed with IPS-10 mM EGTA, this blocker did not affect the control total current (Fig. 2C).

Figure 3 shows the effects of the more selective BK_{Ca} channel blocker, IBTX (50 nM), on families of outward currents evoked by the same voltage protocol as in Fig. 1. These currents were recorded in cells dialysed with IPS-0.1 mM EGTA. Representative current traces shown in Fig. 3A, left and middle panels, were recorded before and after exposure of a myocyte to IBTX 50 nM. IBTX substantially reduced the amplitude of voltage-activated outward currents. IBTX also reduced the amount of current noise, a result that is consistent with BK_{Ca} channel blockade. Figure 3 A, right panel, depicts the difference current obtained by subtracting the currents recorded in the presence of the blocker (middle panel) from those measured in its absence (left panel). These IBTX-sensitive difference currents exhibit voltage- and time-dependent activation, no inactivation, and high current noise level. These properties are characteristic of a typical IBK_{Ca}. The blocking effects of IBTX on families of outward currents were statistically significant at all potentials ranging between –10 to +60 mV, as is depicted in the mean *I*/*V* curves in Fig. 3B. IBTX blocked 50±6% (n=8, P<0.05) of the total end pulse current recorded at +60 mV. This is

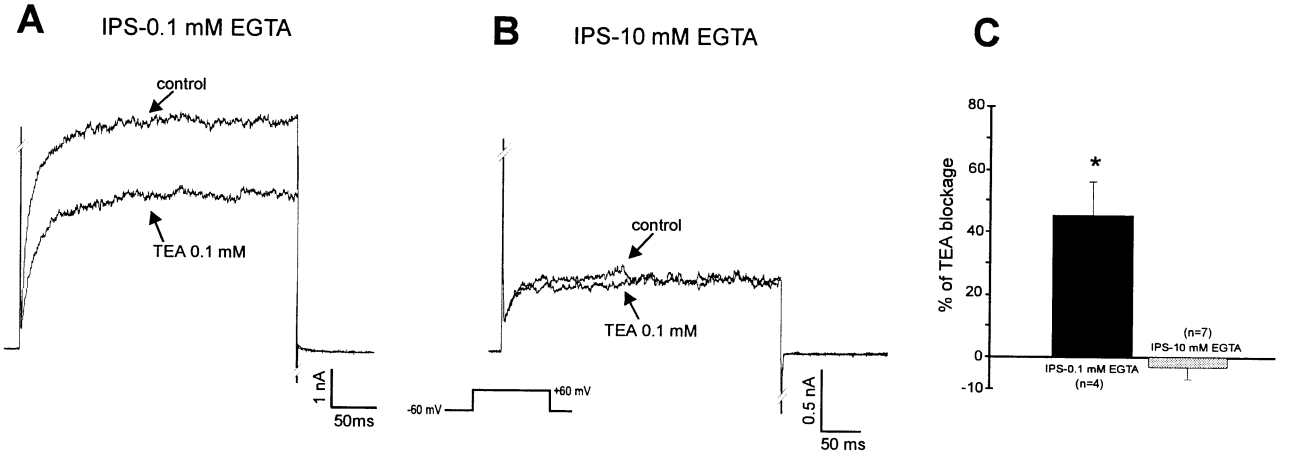

Fig. 2A–C Effects of tetraethylammonium (*TEA*, 0.1 mM on total, whole-cell, voltage-activated, outward current elicited by a depolarizing step to +60 mV from a holding potential of –60 mV in HSV smooth muscle cells. Cells were dialysed with IPS-0.1 mM EGTA (**A**) or 10 mM EGTA (**B**). **C** Summarized data showing the effect of TEA 0.1 mM in both conditions. Values are means±SEM and are expressed as a percentage of the blockade produced by TEA 0.1 mM. **P*<0.05 by unpaired Student's *t*-test

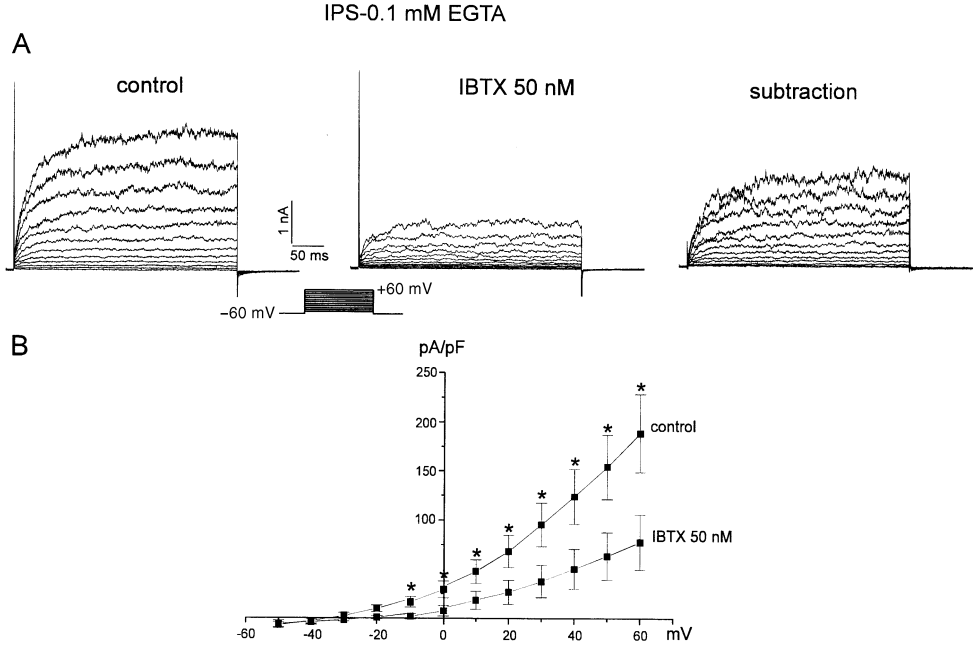

Fig. 3A, B Effect of iberiotoxin (*IBTX*, 50 nM) on whole-cell currents in HSV smooth muscle cells dialysed with an IPS containing 0.1 mM EGTA. **A** Representative families of superimposed currents elicited by depolarizing steps from –50 to +60 mV from a holding potential of –60 mV. Currents were recorded before (*control*) and after (*IBTX 50 nM*) IBTX exposure. Difference currents (*subtraction*) representing IBTX-sensible component of total currents. **B** Mean *I*/*V* curves obtained from four cells before and after IBTX exposure. Current amplitudes were measured at 340 ms from 360-ms voltage steps. Data are expressed as means±SEM, **P*<0.05 by paired Student's *t*-test

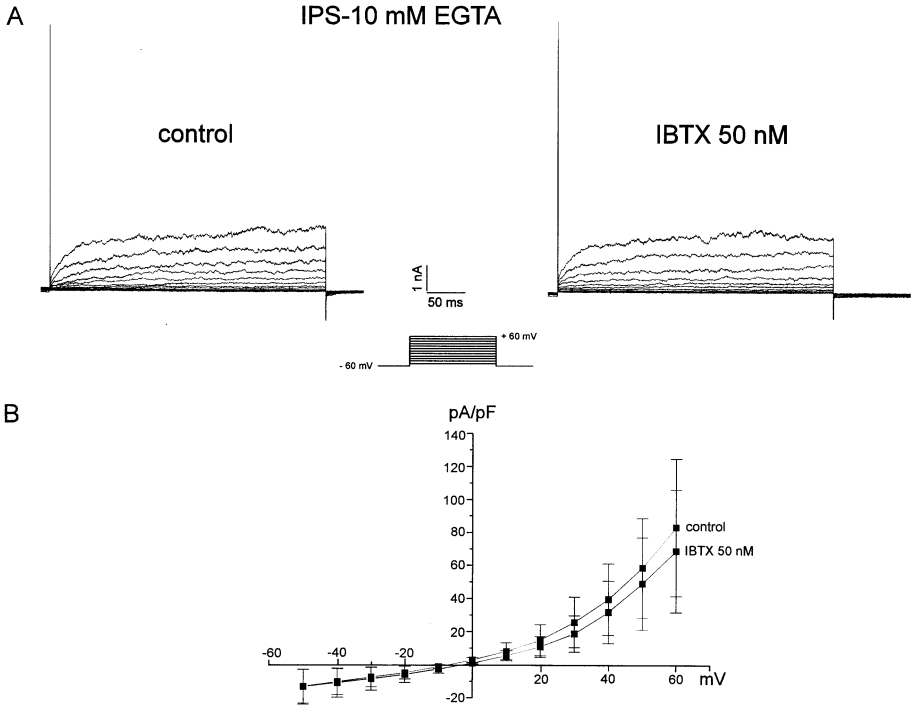

Fig. 4A, B Effect of IBTX 50 nM on whole-cell currents in HSV smooth muscle cells dialysed with an IPS containing 10 mM EGTA. **A** Representative families of superimposed currents elicited by depolarizing steps from –50 to +60 mV from a holding potential of –60 mV. Currents were recorded before and after about 7 min perfusion with IBTX. **B** Mean *I*/*V* curves obtained from four cells before and after about 7 min perfusion with IBTX. Current amplitudes were measured at 340 ms from 360-ms voltage steps. Data are expressed as means±SEM

close to the value observed with TEA 0.1 mM in the cells dialysed with IPS-0.1 mM EGTA (Fig. 2C).

Figure 4A shows representative families of currents recorded in a myocyte dialysed with IPS-10 mM EGTA before (left panel) and after (right panel) IBTX treatment. Average data recorded under these conditions are presented in the *I*/*V* curves in Fig. 4B where it can be observed that IBTX 50 nM did not significantly affect total current in these cells.

The effect of IBTX 50 nM was also tested on V_m in current-clamp mode. Figure 5A shows a typical experiment in which the application of the toxin depolarized HSV smooth muscle cells dialysed with IPS-0.1 mM EGTA; cells were depolarized from a mean of –46.2±3.4 to –17±2.3 mV (n=6, P<0.05). In three cells this effect was partially reversed by washing out the IBTX while three other cells could not be washed. These data are in agreement with the observation that cells dialysed with 10 mM EGTA were more depolarized than those dialysed with 0.1 mM EGTA. When IBTX 50 nM was tested on the V_m of cells dialysed with IPS-10 mM EGTA we did not observe any changes in V_m (–13±4 vs –14±6 mV, n=4, N.S.).

To examine the action of BK_{Ca} channel blockers on intact tissue, HSV rings were mounted in an apparatus for determination of force developed by the vascular segment. Figure 5B shows the time course of the increase of resting tone in HSV rings exposed to TEA 0.1 mM (upper tracing) or IBTX 50 nM (lower tracing). The time delay observed in the onset of IBTX-induced contraction compared with that evoked by TEA could be due to a different rates of diffusion of these drugs throughout the intact tissue. The presence of several tissue layers in the intact vessels could also explain the much slower IBTX-

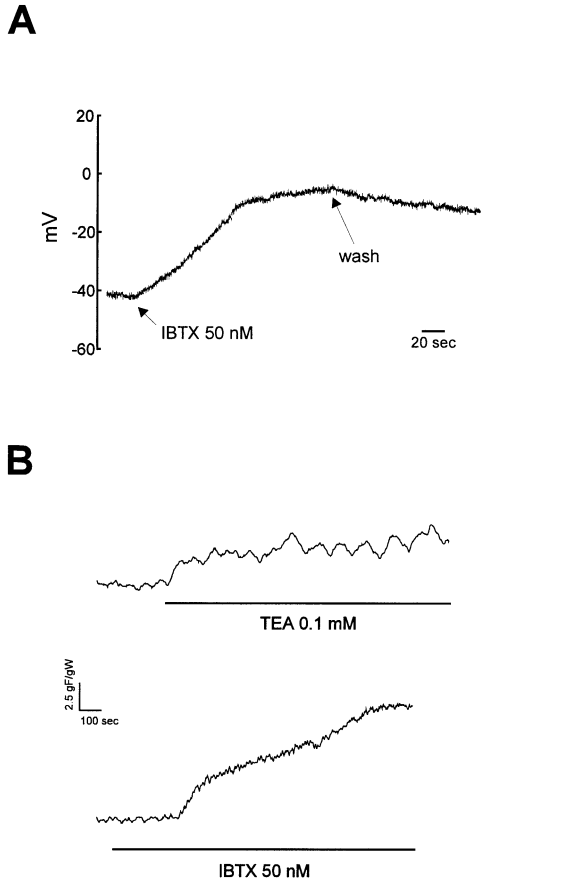

Fig. 5 A Depolarizing effect of IBTX 50 nM on resting membrane potential of a isolated HSV smooth muscle cell dialysed with an IPS containing 0.1 mM EGTA. **B** Contractions induced by TEA 0.1 mM (*upper tracing*) and IBTX 50 nM (*lower tracing*) in quiescent HSV rings

induced onset of contraction compared to the depolarizing effect of this drug in isolated single myocytes. The vessels exposed to 0.1 mM TEA or 50 nM IBTX produced an increase in force amounting to 18±7% (n=4) and 16±5% (n=7) of the previous KCl 80 mM contractions, respectively. In five out of seven veins IBTX 50 nM produced periodic contractions attaining a maximum force of 40±13% of the previous KCl 80 mM contractions (n=5). These effects were observed after 10–30 min of the drug application and were not reversible by IBTX washout.

Discussion

Several features, such as absence of time-dependent inactivation, sensitivity to internal [Ca^{2+}] and blockade by low concentrations of TEA and IBTX allow us to identify the current studied in isolated HSV smooth muscle cells as the IBK$_{Ca}$ type. The functional role of BK$_{Ca}$ channels in the maintenance of V_m in HSV smooth muscle cells and in the regulation of vascular tone is discussed in this paper.

The values of V_m that we report are similar to those measured in other types of isolated VSMC using the patch-clamp technique with an intracellular solution containing 0.1 mM EGTA, i.e. rabbit coronary artery cells (–32.2 mV) [11], rabbit portal vein myocytes (–48.7 mV) [13] and human coronary artery cells in culture (–32 mV) [17]. However, using intracellular microelectrodes, a V_m of –69.5 mV has been measured in HSV smooth muscle cells of intact vessels [21]. It must be taken into account that in intact vessels many factors may modify steady-state V_m, such as the release of endothelial factors or endogenous neurotransmitters, which may hyperpolarize vascular smooth muscle cells.

Our data show that in isolated HSV smooth muscle cells dialysed with IPS-0.1 mM EGTA (free $[Ca^{2+}]_i$ about 100 nM) the V_m was significantly more negative than when the cells were dialysed with 10 mM EGTA (about 1 nM free $[Ca^{2+}]_i$). If BK$_{Ca}$ channels were involved in the maintenance of V_m in HSV smooth muscle cells, the observed shift of V_m to more positive values in the presence of a low $[Ca^{2+}]_i$ could be explained by reduced activation of these channels [5]. Moreover, the significantly higher amplitude of the total current density found with high $[Ca^{2+}]_i$ (0.1 mM EGTA) could indicate the presence of a BK$_{Ca}$ current that is absent when the $[Ca^{2+}]_i$ is low (EGTA 10 mM). It has been reported that a high intracellular EGTA concentration (i.e.10 mM) minimizes the contribution of IBK$_{Ca}$ to total outward current in other types of VSMC, i.e. canine pulmonary artery [16], rat pulmonary artery [22], rat mesenteric artery and human pulmonary and mesenteric arteries [1]. Consistently, an outward current sensitive to 0.1 mM TEA and 50 nM IBTX was observed only when the $[Ca^{2+}]_i$ was not strongly buffered. Current-clamp experiments carried out under these conditions also showed that V_m was depolarized by the specific BK$_{Ca}$ channel blocker IBTX (50 nM), achieving values similar to those obtained by buffering intracellular calcium.

We can not rule out the possibility that other Ca^{2+}-dependent currents or mechanisms contribute to the changes in V_m and total current density when $[Ca^{2+}]_i$ is changed, but the results obtained with the specific blockers of BK$_{Ca}$ current clearly suggest that at least one of the components of the total current is IBK$_{Ca}$. This current is active in the range of potentials that we have found as an average value of V_m for HSV smooth muscle cells, which makes it feasible that these channels participate in the maintenance of the V_m, as we have observed. The pharmacological sensitivity of this current is similar to that described for both native BK$_{Ca}$ channels from HSV smooth muscle cells [24] and macroscopic currents from homogeneous populations of cloned human BK$_{Ca}$ channel (hSlo) expressed in oocytes [7, 12]. In addition, quiescent rings of the same vessels used to obtain isolated smooth muscle cells contracted in the presence of BK$_{Ca}$ blockers (TEA and IBTX); therefore, the depolarization elicited by these blockers allows an influx of calcium that produces the vessel contraction. These results demonstrate the participation of BK$_{Ca}$ channels in the maintenance of the contractile state of the HSV.

Potassium channels activated by intracellular Ca^{2+} and depolarization have been found in different VSMC and several studies have described the importance of BK$_{Ca}$ in controlling V_m and in regulating vascular smooth muscle contraction [3, 6, 23]. These channels are also targets for physiological vasoconstrictor and vasorelaxant agents which induce their inhibition or activation [4, 20]. At the moment we are aware of only three reports suggesting functional roles for BK$_{Ca}$ channels in HSV smooth muscle cells. First, noradrenaline-induced contraction of HSV rings is potentiated by blockade of BK$_{Ca}$ channels [24]; second, BK$_{Ca}$ and K$_v$ channels have been shown to be involved in the counterregulation of myogenic tone of HSV [19] and finally BK$_{Ca}$ may be involved in the nitric oxide-dependent vasodilatation of serotonin-induced contraction in HSV rings [9].

Our results allow us to conclude that the BK$_{Ca}$ channels present in HSV smooth muscle sustain a significant portion of the total voltage-activated current, contribute to the maintenance of the resting potential, and participate in the regulation of the muscle contractile response.

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