Block of large conductance Ca^{2+}-activated K^+ channels in rabbit vascular myocytes by internal Mg^{2+} and Na^+

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1. We studied the biophysical properties of single large conductance (>200 pS in symmetrical K^+ pipette and bath solutions) Ca^{2+}-activated K^+ (BK_{Ca}) channels of rabbit portal vein and coronary arterial smooth muscle cells using the cell-attached and inside-out variants of the patch-clamp technique (at 22 °C).

2. The unitary conductance of BK_{Ca} channels recorded in cell-attached patches with K^+ concentrations in the range 5·4–140 mM was significantly lower than that predicted on the basis of the conductance measured in inside-out patches with symmetrical K^+ pipette and bath solutions (140 mM) and the constant field equation. In cell-attached patches from cells bathed in depolarizing medium (140 mM) with 5·4 mM K^+ in the pipette solution, BK_{Ca} channels were difficult to detect in the physiological range of membrane potentials (approximately −50 mV). Unitary currents were smaller at all voltages in the range −50 to 0 mV and the i–V relationship exhibited strong inward rectification at potentials >0 mV. These channels were unequivocally identified as BK_{Ca} channels due to their sensitivity to caffeine (10 mM) and iberiotoxin (20 nM), and their non-stationary kinetic properties.

3. Exposure of the cytoplasmic side of excised patches to [Mg^{2+}] in the range 0–15 mM produced two effects on BK_{Ca} channel activity: the slope conductance and open probability were reduced and enhanced, respectively, in a concentration-dependent manner by this cation. The Mg^{2+}-induced reduction in conductance exhibited weak voltage dependence.

4. Application of 20 mM Na^+ to the internal face of BK_{Ca} channels recorded in the inside-out configuration produced a flickery block at potentials ⩾+20 mV resulting in reduced unitary current amplitudes and strong inward rectification of the i–V relationship. Exposure of inside-out patches to a combination of 20 mM Na^+ and 2 mM Mg^{2+} further reduced unitary current amplitude to a level similar to the algebraic sum of the effect of each cation in isolation.

5. We conclude that Ca^{2+}-dependent K^+ channels of vascular smooth muscle cells display a lower unitary conductance when recorded under physiological conditions than that previously estimated on the basis of their behaviour in excised membrane patches. Our data indicate that the decreased permeation through BK_{Ca} channels may be partly attributed to block by intracellular Mg^{2+} and Na^+, which appear to interact with distinct binding sites along the inner side of the pore.

Among the various sarcolemmal K^+ channels described so far in smooth muscle cells, large conductance voltage- and Ca^{2+}-dependent K^+ (BK_{Ca}) channels have been best characterized in terms of their biophysical properties, pharmacology, and regulation by classical signal transduction mechanisms. Inside-out patch experiments performed under symmetrical K^+ conditions (~140 mM) across the membrane revealed a typical conductance of ~250 pS for these channels (for review see Nelson & Quayle, 1995). The channel displays voltage-dependent activation kinetics but does not inactivate. It is generally accepted that the BK_{Ca} channel is primarily activated by voltage, and intracellular calcium ions serve to sensitize the channel to transmembrane voltage by shifting its activation relationship to more negative potentials (Carl & Sanders, 1989) by binding to an allosteric site located on the inner face of the

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membrane. Tetraethylammonium (TEA; K_d, ~300–500 μM), charybdotoxin (CTX; K_d, ~10 nM) and iberiotoxin (IBTX; K_d, ~1–10 nM) have been used to separate BK_{Ca} from other K^+ channels and characterize its contribution to whole-cell macroscopic currents, although TEA (Gelband & Hume, 1992; Leblanc, Wan & Leung, 1994) and CTX (Leblanc *et al.* 1994) were also shown to inhibit delayed rectifier K^+ channels in some preparations.

Numerous studies have reported that Ca^{2+}-dependent K^+ channels are ubiquitously and densely distributed in smooth muscle cell membranes. The BK_{Ca} channel has been implicated in the repolarization of all-or-none electrical events in phasic, as well as bursting, smooth muscle cells, and maintenance of negative resting membrane potential ($V_{m,rest}$) in arterial myocytes, especially at high arterial transmural pressures (Brayden & Nelson, 1992) or elevated $[Ca^{2+}]_i$ (Miller, Morales, Leblanc & Cole, 1993; Leblanc *et al.* 1994; Nelson & Quayle, 1995).

In a recent preliminary study (Morales, Cole, Remillard & Leblanc, 1995), we reported that CTX-sensitive Ca^{2+}-sensitive K^+ current ($I_{BK(Ca)}$) recorded in a physiological K^+ gradient (pipette, 5·4 mM; bath, 140 mM) in cell-attached patches from rabbit portal vein and coronary arterial smooth muscle cells exhibited a much lower conductance (~20 pS at 0 mV, assuming a $V_{m,rest}$ of −50 mV) than that predicted by the constant field equation (estimated conductance at 0 mV, ~141 pS). A possible explanation for this observation is that one or several intracellular blocking particles may limit efflux of K^+ through the channel. Magnesium is the most abundant divalent cation in the intracellular medium. Besides serving as an important cofactor for numerous enzymatic reactions, Mg^{2+} is known to block a variety of K^+ channels, including cardiac ATP-dependent K^+ channels (Horie, Irisawa & Noma, 1987) and inward rectifier K^+ channels (Matsuda, Saigusa & Irisawa, 1987), delayed rectifier K^+ channels in renal smooth muscle cells (Gelband, Ishikawa, Post, Keef & Hume, 1994), and Ca^{2+}-dependent K^+ channels in skeletal muscle (Ferguson, 1991), neurons (Forsythe, Linsdell & Stanfield, 1992), and, more recently, cerebral arterial smooth muscle cells (Zhang, Puil & Mathers, 1995). In addition to its effect on the permeation properties of BK_{Ca} channels, Mg^{2+} has also been shown to affect the gating of BK_{Ca} channels by enhancing the effect of Ca^{2+} (Golowasch, Kirkwood & Miller, 1986; Trieschmann & Isenberg, 1989; Zhang *et al.* 1995).

Internal application of sodium ions produces a flickery block of BK_{Ca} channels in chromaffin cells which reduces unitary currents through BK_{Ca} channels ($i_{BK(Ca)}$) and causes apparent inward rectification of its i–V relationship at positive potentials (Marty, 1983; Yellen, 1984). Internal Na^+ has been shown to produce a similar inhibitory action on BK_{Ca} channels in toad gastric (Singer & Walsh, 1984) and guinea-pig taenia coli (Hu, Yamamoto, & Kao, 1989) smooth muscle cells. The possible interaction of internal Na^+ with BK_{Ca} channels in vascular smooth muscle cells has not been investigated.

The objectives of this study were to determine: (1) the properties and contribution of $i_{BK(Ca)}$ recorded in cell-attached patches from rabbit portal vein and coronary smooth muscle cells; and (2) whether block by internal Mg^{2+} and/or Na^+ is partly responsible for the reduced slope conductance of BK_{Ca} channels observed in cell-attached patch experiments. Our results indicate that in vascular myocytes, IBTX-sensitive BK_{Ca} channels recorded in cell-attached patches exhibited a much lower conductance than expected. Cytoplasmic application of Mg^{2+} reduced the slope conductance of the channel in a voltage-independent manner. On the other hand, internal Na^+ produced a flickery type of channel block at potentials positive to +20 mV resulting in marked inward rectification. The effects of Mg^{2+} and Na^+ were additive, suggesting that the two cations interact with distinct binding sites. It is concluded that combination of both ions may physiologically regulate BK_{Ca} channel activity, especially at depolarized membrane potentials.

METHODS

Cell isolation procedure

Single smooth muscle cells were isolated from rabbit portal veins and left descending coronary arteries as previously described (Miller *et al.* 1993; Leblanc *et al.* 1994, respectively). In brief, rabbits were killed with an overdose of pentobarbitone sodium (100 mg kg^{-1} in the ear artery). The heart and portal vein were quickly removed and placed in Krebs–Henseleit (KH) solution with the following composition (mM): 120 NaCl, 25 $NaHCO_3$, 4·2 KCl, 1·2 KH_2PO_4, 1·2 $MgCl_2$, 1·8 $CaCl_2$, and 5·5 dextrose (pH 7·4 after bubbling with 95% O_2–5% CO_2 at 22 °C). The adventitia was carefully removed from the vessels under a dissecting microscope and the remaining tissue cut into small pieces (2–4 mm^2). After a 30 min period in Ca^{2+}-free KH solution (no $CaCl_2$ and 0·1 mM EGTA) at 22 °C, the tissue was subsequently incubated for 15–25 min at 31–35 °C in a modified KH solution containing 10 μM Ca^{2+} and the following enzymes: collagenase (Type IA; Sigma; portal vein, 188 U ml^{-1}; coronary artery, 350 U ml^{-1}) and protease (Type XXVII, Sigma; portal vein, 0·16 U ml^{-1}; coronary artery, 0·6 U ml^{-1}). Single smooth muscle cells were mechanically dispersed by gentle trituration of the tissue pieces in 10 μM Ca^{2+} KH–enzyme solution using a Pasteur pipette. When a sufficiently high number of spindle-shaped smooth muscle cells became apparent under microscopic examination, the enzymatic incubation was stopped by several washes with enzyme-free 10 μM Ca^{2+} KH solution. The remaining pieces of tissue were triturated and the cells released in the supernatant were stored at 4 °C in 10 μM Ca^{2+} KH solution for up to 10 h after isolation.

Electrophysiological measurements

Dispersed cells were allowed to settle to the bottom of a 2 ml recording chamber on the stage of an inverted microscope (model TMS; Nikon, Tokyo) for 5–10 min. The cells were then superfused with Krebs–Hepes solution of the following composition (mM): 130 NaCl, 10 $NaHCO_3$, 4·2 KCl, 1·2 KH_2PO_4, 0·5 $MgCl_2$, 1·8 $CaCl_2$, 10 Hepes–NaOH (pH 7·35). Only relaxed elongated cells which were optically refractive and which exhibited a smooth regular surface were used in this study. All experiments were carried out at 22–24 °C.

Single-channel activity was recorded using the cell-attached and inside-out configurations of the patch-clamp technique (Hamill,

Marty, Neher, Sakmann & Sigworth, 1981). Recordings were made with an Axopatch 200A patch-clamp amplifier and Digidata 1200 A/D interface (Axon Instruments) connected to a 486 IBM clone computer. Recordings were filtered at 2 or 5 kHz by an on-board Bessel filter. In order to improve the signal/noise ratio, the tip of the patch-clamp pipette was coated with Sylgard (Dow Corning). To assure a rapid exchange of solutions across the intracellular face of excised patches, multiple perfusion lines containing various test solutions were fused to form a single outlet, thus minimizing the dead space between the tip of the patch-clamp pipette and the superfusion system. Control of the outflow through the multibarrelled outlet was accomplished by a Multiflow controller device (Norscan Instruments Ltd, Winnipeg, Canada).

Three basic voltage-clamp protocols were applied in order to record channel activity: varied holding potentials, step command pulses, and voltage ramps. With the former, the steady-state voltage dependence and conductance of unitary currents were studied by changing the holding potential knob on the patch-clamp amplifier to record 30–60 s of channel activity. Step pulses or voltage ramps were computer driven using the pCLAMP software (Clampex; Axon Instruments Inc.) and served to construct i–V relationships and generate ensemble averages of single-channel currents, respectively. For cell-attached patches from cells not exposed to depolarizing K^+ medium (Figs 1–4), voltages are expressed as relative transmembrane potentials (V_{rel}, where $V_{rel} = -$the applied voltage, $-V_{applied}$) uncorrected for the cell $V_{m,rest}$; for all other experiments (Figs 5–9), voltages are expressed as true transmembrane potentials (V_m).

Multiple channels were present in every membrane patch, consistent with the high density of BK_{Ca} channels in smooth muscle cells. As a consequence, open probability (P_o), was expressed as NP_o, where N represents the number of single channels, and calculated using the following expression (Kajioka, Kitamura & Kuriyama, 1991):

$$NP_o = (A_1 + 2A_2 + 3A_3 + \ldots nA_n)/(A_0 + A_1 + A_2 + \ldots A_n), \quad (1)$$

where A_0 is the area under the curve of an all-points amplitude histogram corresponding to current in the closed state, and $A_1 \ldots A_n$ represent the histogram areas reflecting the different open-state current levels for 1 to n channels present in the patch. Histogram parameters were obtained from multiple least-squares Gaussian fits of the data using pCLAMP software (Axon Instruments).

The permeability coefficient of K^+ through BK_{Ca} channels (P_K, in cm s^{-1}) was calculated using the simplified Goldman–Hodgkin–Katz equation (Goldman, 1943; Hodgkin & Katz, 1949) in the case of symmetrical K^+ concentrations:

$$i_K = P_K(VF^2[K^+])/(RT), \quad (2)$$

where V is the transmembrane voltage, $[K^+]$ is the concentration of K^+ on both sides of the membrane, and F, R and T have their usual thermodynamic meanings.

We employed the approach of Woodhull (1973) to obtain a value for the fractional distance within the BK_{Ca} channel at which Mg^{2+} and Na^+ block occurred using the following relationship:

$$i_O/i_B = 1 + ([B]/K_D(0)) \times \exp(zdVF/RT), \quad (3)$$

where i_O/i_B represents the ratio of the single-channel currents before and after exposure to the blocking ion, [B] is the concentration of the blocking ion, $K_D(0)$ is the dissociation constant of the blocking ion at 0 mV, z is the valence of the ion, V is the voltage drop across the membrane, and d the fraction of the voltage influencing the ion at the binding site, as measured from the intracellular surface. The values of d and $K_D(0)$, respectively, were obtained from the slope and y-intercept on a plot of $\ln(i_O/i_B - 1)$ *versus* membrane potential.

Solutions

For cell-attached patch experiments, the composition of the bathing and pipette media was identical to that described above. However in some experiments, NaCl and $NaHCO_3$ in the pipette solution were replaced by equimolar KCl to establish various transmembrane K^+ gradients ($[K^+]_o/[K^+]_i$ (mM/mM): 140/140, 75/140, 50/140, or 20/140, assuming an intracellular K^+ concentration of ~140 mM). The depolarizing medium consisted of (mM): 140 KCl, 0·5 $MgCl_2$, 10 Hepes–NaOH (pH 7·4) and 5·5 dextrose.

For inside-out patch experiments, careful attention was paid to maintain the Ca^{2+} and Mg^{2+} concentrations facing the internal side of the channel at fixed levels; chemical activities for Ca^{2+} and Mg^{2+} were fixed to values cited in the text and figures by buffering with EGTA according to dissociation constants taken from Fabiato & Fabiato (1979) and the software of C.-M. Hai (University of Virginia, Charlottesville, VA, USA). The pipette solution contained (mM): 140 NaCl, 5·4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes–NaOH (pH 7·4). The transmembrane K^+ gradient was varied using an approach identical to that described for the cell-attached experiments. The bathing medium consisted of (mM): 140 KCl, 0 or 20 NaCl, 2 or 5 EGTA (see below), 10 Hepes–KOH (pH 7·2) and various amounts of $CaCl_2$ and $MgCl_2$ to achieve $[Ca^{2+}]$ between 10 and 500 nM at a fixed level of 0 or 1 mM Mg^{2+}, or varied $[Mg^{2+}]$ between 0 and 15 mM at a fixed $[Ca^{2+}]$ of 100 nM. EGTA was 2 or 5 mM for solutions containing up to or above 100 nM Ca^{2+}, respectively.

Chemicals and drugs

All chemicals employed in physiological salt solutions, pipette and bath solutions, as well as the enzymes used for cell dispersion were purchased from Sigma Chemical Co. Iberiotoxin, obtained from Research Biochemicals International, was dissolved in water and added to the pipette solution at a final concentration of 20 nM. Caffeine (Sigma) was directly added in powder form to the perfusate to give a concentration of 5 or 10 mM.

RESULTS

Ca^{2+}-dependent K^+ (BK_{Ca}) channels were first identified in membrane excised inside-out patches from rabbit portal vein on the basis of their large single-channel conductance, Ca^{2+} and voltage dependence, and inhibition by IBTX. Consistent with the high density of these channels in excised patches of these preparations was the presence of multiple large conductance (261 ± 4·7 pS in symmetrical K^+ solutions, 6 patches; estimated from ramp protocols) K^+ channels which were activated by membrane depolarization and physiological intracellular Ca^{2+} concentrations (100–500 nM).

Figure 1*A* and *B* illustrates the sensitivity of BK_{Ca} channels to IBTX (20 nM). Figure 1*A* shows a representative continuous recording from an inside-out patch held at +10 mV in asymmetrical K^+ conditions (pipette, 5·4 mM; bath, 140 mM). The tip of the patch pipette was filled with solution lacking the toxin and the remainder of the pipette was backfilled with solution

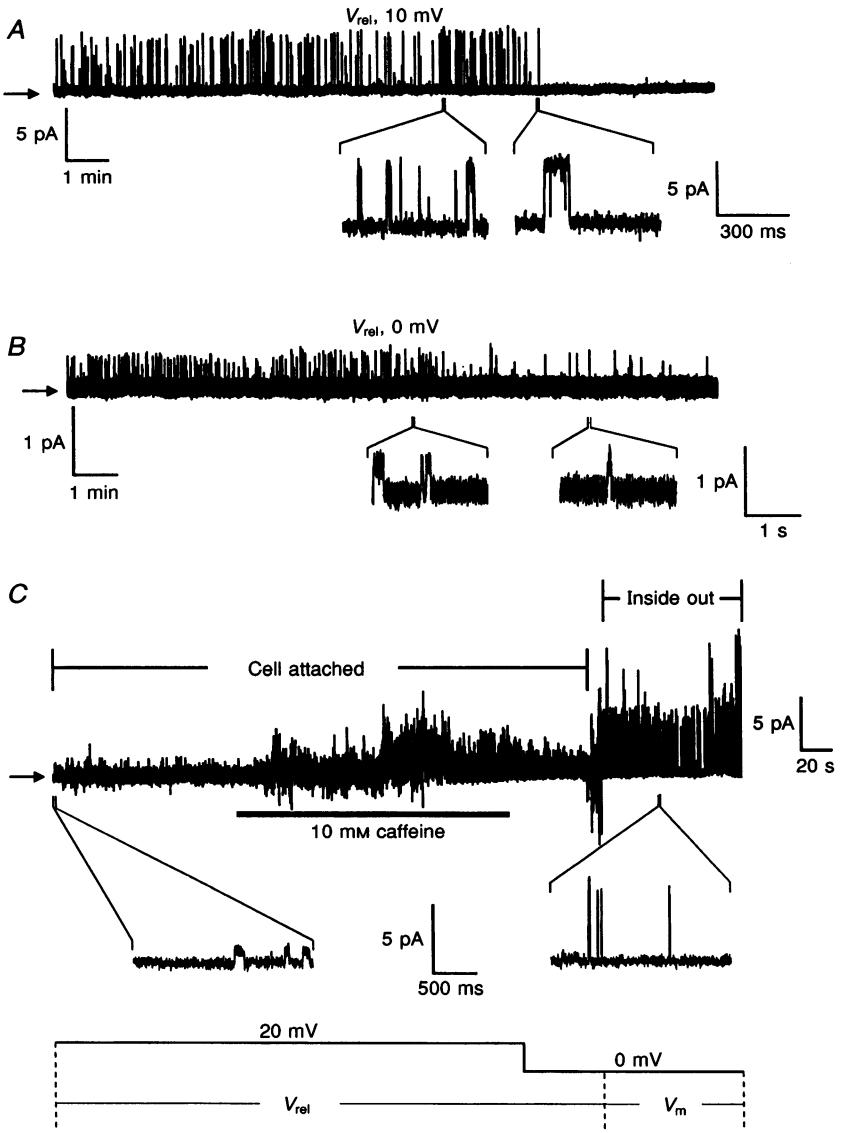

Figure 1. Delayed onset of inhibition of portal vein BK_{Ca} channels by IBTX in excised and cell-attached patch recordings

For all three panels, the pipette tip was filled with toxin-free pipette solution, and then backfilled with an identical solution containing 20 nM IBTX. Arrows indicate closed-state level. *A*, representative continuous recording started immediately after patch excision from an inside-out patch (asymmetrical K^+ solutions: pipette, 5·4 mM; bath, 140 mM) containing a single BK_{Ca} channel. Sections of the trace on an expanded time scale shown below indicate channel activity 9·5 (left) and 10·5 min (right) following patch excision. *B*, continuous recording from a cell-attached patch held at 0 mV with 5·4 mM K^+ in the pipette solution. The myocyte was bathed in standard medium and the transmembrane voltage was not corrected for the resting membrane potential (approximately −50 mV). Sections of the trace on an expanded time scale shown below were taken 7 (left), and 10·5 min (right) following the formation of the giga-ohm seal. Channel inhibition was denoted as the delayed disappearance of channel activity due to diffusion of IBTX to the cell membrane (3 patches in each condition). *C*, slow chart recording from a patch which was initially in the cell-attached mode and then excised into the inside-out configuration as indicated above. Shown below are two sections of the trace on an expanded time scale depicting a relatively small conductance channel in the cell-attached mode (left), and a large conductance channel once the patch was excised (right). Notice the enhancement of single-channel activity during exposure to 10 mM caffeine (horizontal bar). Bottom: voltage protocol; V_{rel}, $-V_{applied}$ (uncorrected for $V_{m,rest}$); V_m, true transmembrane potential. Pipette $[K^+]$, 5·4 mM; cell-attached bath $[K^+]$, 5·4 mM; inside-out bath $[K^+]$, 140 mM; $[Ca^{2+}]_i$, 100 nM. A similar observation was made in two other membrane patches.

containing IBTX in order to delay exposure of the patch to the toxin. In the example shown, 10 min of continuous recording was required before the toxin diffused to the membrane and channel activity was inhibited. Figure 1*B* illustrates a similar type of experiment except that the recording was obtained from a cell-attached patch clamped at 0 mV (V_{rel}) taken from a cell exposed to normal bathing medium (uncorrected membrane potential). Notice the small unitary amplitude of these channels when recorded with physiological K^+ concentration (pipette, 5·4 mM; bath, 140 mM) in an intact cell. The delayed inhibition of this channel by IBTX indicated that it was produced by BK_{Ca}.

Figure 1*C* shows an experiment supporting the claim that the small IBTX-sensitive channel observed in cell-attached patches results from the activity of a large conductance K^+ channel. The pipette solution contained 5·4 mM K^+. The cell was bathed in standard medium (140 mM K^+). At the beginning of the continuous recording (Fig. 1*C*, top trace), single-channel currents of ~1·5 pA (see left-hand expanded section below) were recorded for a membrane depolarization of +20 mV (larger membrane depolarizations resulted in noisy traces of which unitary currents could not be resolved with confidence). Application of 10 mM caffeine to release Ca^{2+} from internal stores enhanced the activity and recruited several channels in the patch, as expected for Ca^{2+}-dependent K^+ channels. After excision of the patch in the inside-out configuration with the inner membrane facing a solution containing 140 mM K^+ and 100 nM Ca^{2+} at 0 mV (true V_m), only currents of large unitary amplitudes (~5·6 pA, see Fig. 1*C* right-hand expanded section below) exhibiting characteristics consistent with those of BK_{Ca} channels could be recorded. Taken together, these results indicated that the permeating behaviour of the BK_{Ca} channel is different when operating in more physiological conditions.

To investigate further the permeating properties of BK_{Ca} channels under physiological conditions, we examined the extracellular K^+ concentration dependence of $i_{BK(Ca)}$ recorded in cell-attached patches of portal vein myocytes. Figure 2 shows that reducing K^+ in the pipette from 140 to 20 mM caused a decrease in unitary conductance of channels. Since the myocytes in these cell-attached experiments were exposed to a bath solution containing normal extracellular K^+ (5·4 mM), the exact transmembrane potential is unknown. All values are given as V_{rel}; a value of about −50 mV may be assumed for $V_{m,rest}$ based on previous data (Hume & Leblanc, 1989; Miller *et al.* 1993). Indeed, Fig. 2*A* shows that the reversal potential of single-channel currents under 'presumed' symmetrical K^+ conditions is very close to that measured in current clamp (dashed line indicates +48 mV). These results support the claim that the intracellular K^+ concentration approximates 140 mM. With 20 mM K^+, the transitions are upward indicating that the currents were outwardly directed over the entire applied voltage range, i.e. the reversal potential is shifted to more negative voltages with the change in K^+

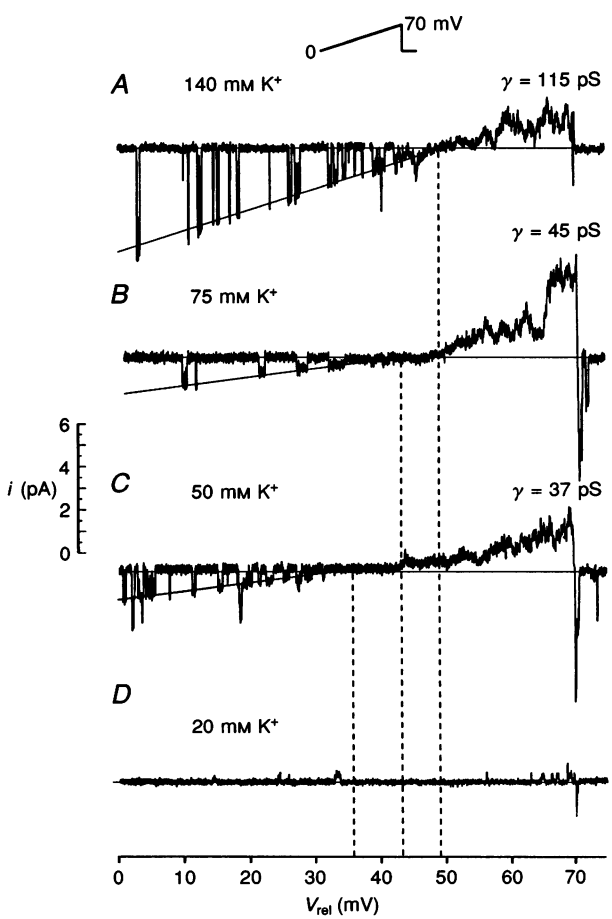

Figure 2. Conductance behaviour of BK_{Ca} channels in cell-attached patches from portal vein myocytes with varied K^+ concentrations in the pipette solution

BK_{Ca} channel activity for each condition was recorded in response to a voltage ramp ranging from 0 to 70 mV (V_{rel}). Individual experiments are shown in each panel. Cells were bathed in standard bathing solution (5·4 mM K^+, 1·8 mM Ca^{2+}). The slope of the continuous diagonal lines yielded the conductance of the channels under specified pipette [K^+]. As indicated, the estimated conductance (γ) was 115, 45 and 37 pS with 140 (*A*), 75 (*B*) and 50 mM K^+ (*C*), respectively; slope conductance could not be evaluated with 20 mM K^+ (*D*) in this particular patch. The vertical dashed lines indicate the estimated reversal potentials (obtained by extrapolation to 0 pA) corresponding to the different pipette [K^+] tested. Notice that under presumed symmetrical K^+ conditions (*A*), the extrapolated reversal potential is approximately −50 mV, which is similar to the value of $V_{m,rest}$ measured in whole-cell current-clamped portal vein cells (Hume & Leblanc, 1989; Miller *et al.* 1993). Similar observations were made in seven cells.

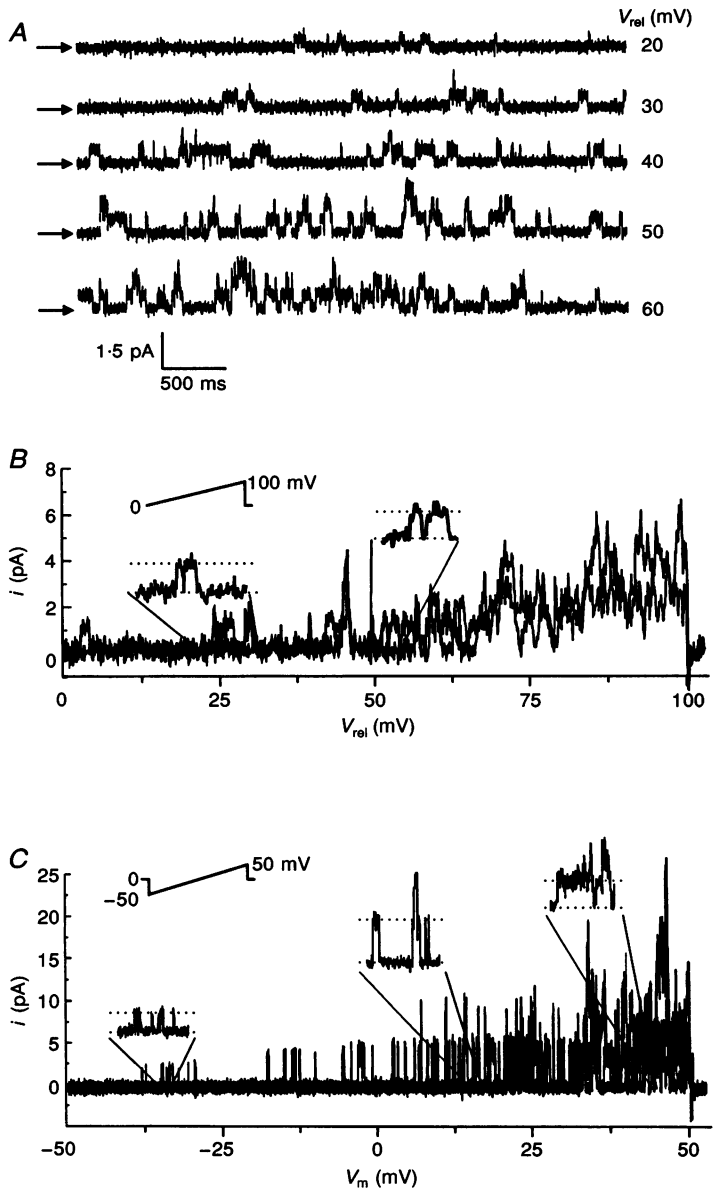

Figure 3. Reduced unitary amplitude of BK_{Ca} channels recorded in cell-attached patches from portal vein cells with 5·4 mM K^+ in the pipette solution

A, representative recordings of BK_{Ca} channel activity at relative voltages (V_{rel}) ranging between 20 and 60 mV (top to bottom) in 10 mV increments. At least three channels could be detected in the patch when applied pipette potential was −60 mV, as indicated by the multiple current levels. Arrows indicate closed-state level. *B*, three consecutive superimposed traces from a cell under similar conditions to those described in *A*. BK_{Ca} channel activity was studied in response to an applied voltage ramp protocol (V_{rel}) from 0 to 100 mV as depicted in the inset. The two enlarged time scale traces displayed above depict virtually unchanged current amplitudes at 25 (left) and 50 mV (right). *C*, three superimposed recordings from a different patch. One trace was acquired in control conditions; the following two traces were obtained following cell exposure to 5 mM caffeine to induce channel activity at more negative potentials. The cell was depolarized to ~0 mV by raising bath $[K^+]$ from 5·4 to 140 mM K^+ (Ca^{2+}-free medium, see Methods). Transmembrane potential was varied between −50 and +50 mV over 8·5 s according to the ramp protocol shown (inset). Similar results were obtained in three cells. The dotted lines in the insets of *B* and *C* indicate the amplitude of unitary currents at various potentials.

concentration (dashed lines). The single-channel conductance for each ramp in Fig. 2 is given as the slope conductance over a range of voltages where distinct transitions were apparent in the recordings (continuous lines in the figures). However, when 20 mM K^+ was employed, it was not possible to measure accurately the conductance in this patch because the transitions were very small, showed little difference in amplitude over a large range of potentials, and displayed evidence of rectification at more depolarizing voltages. Data obtained using 5·4 mM K^+ demonstrate the very small conductance and rectification of BK_{Ca} channels even more effectively.

Figure 3 shows records of single-channel recordings from cell-attached patches with 5·4 mM K^+ in the pipette. In Fig. 3*A*, the voltage across the patch (V_{rel}) was varied over the range +20 to +60 mV (from $V_{m,rest}$) in 10 mV steps with the myocyte bathed in 5·4 mM K^+ solution. Under these conditions, single-channel transitions were of a small amplitude, ranging between 0·5 and 1 pA. Similar observations were made in cell-attached patches from coronary myocytes. In four of six patch recordings, single-channel transitions could not be resolved for applied voltages in the range −50 to +50 mV. In the two remaining patches, unitary current amplitudes were between 0·4 and 0·7 pA for applied relative voltages ranging from 0 to +50 mV. It is also apparent from Fig. 3*A* that although the open probability increased as the patch was depolarized, there was little evidence of any change in the amplitude of the transitions. This point is clearly demonstrated in Fig. 3*B*, which shows superimposed recordings during applied relative voltage ramps between 0 and +100 mV under the same conditions as Fig. 3*A*. The transitions are small over the entire range of applied membrane depolarizations from rest, and at very depolarized voltages, their amplitude appears to decline. Also, notice the

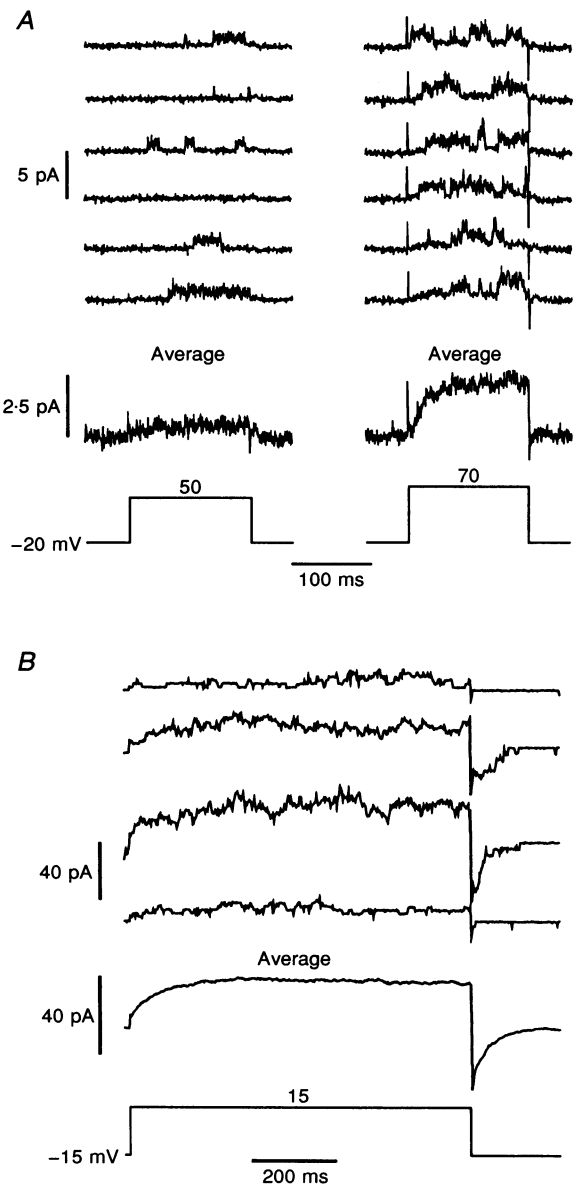

Figure 4. Ensemble averages of BK_{Ca} channels in cell-attached and excised-membrane patches from portal vein myocytes

A, non-stationary analysis of BK_{Ca} channel activity in a typical cell-attached patch with 5·4 mM K^+ in the pipette solution. The patch was repetitively depolarized (150 ms steps) to either +50 (left traces) or +70 mV (right traces) from a holding potential of −20 mV (protocols illustrated below each family of traces) at a frequency of 0·5 Hz. Six consecutive sweeps (top six traces) and the resulting ensemble average of sixty-four steps (lower trace) are shown for the two voltages studied. Leak and capacitive current transients were compensated for by adding to each individual current sweep a scaled current elicited by a hyperpolarizing step to −40 mV, a voltage that did not elicit channel openings. Slope conductance was 31 pS between +20 and +50 mV. *B*, ensemble average of BK_{Ca} channel activity (213 pS) from an inside-out patch exposed to symmetrical K^+ solutions (140 mM K^+; leak and capacitative currents uncorrected). This patch was repetitively depolarized to +15 mV (750 ms steps) from holding potential of −15 mV according to the protocol shown at the bottom. Four representative current recordings are shown at the top, and the average of sixty-four sweeps is displayed below. The time constants of activation and deactivation were 67 and 41 ms, respectively.

increased noise in the open state compared with excised patch recordings (Fig. 1*A* and *C*).

To circumvent the problem of transmembrane voltage in the cell-attached patches with 5·4 mM K^+ in the bath solution, we switched the bath solution to a depolarizing medium with 140 mM K^+ so that membrane potential would be shifted to ~0 mV. Figure 3*C* shows records obtained during voltage ramps over transmembrane potentials of −50 to +50 mV; three superimposed traces are shown. Since the bathing medium was low in Ca^{2+}, very little channel activity could be observed at potentials negative to about +25 mV. For this reason, we acquired one trace in Fig. 3*C* before and two records after the myocyte was exposed to 5 mM caffeine. The release of Ca^{2+} from internal stores due to caffeine made it possible to detect transitions negative to 0 mV and permitted a complete record of single-channel amplitudes over the entire range of voltages during the ramp. The voltage dependence of the channels is clear from these recordings. However, the amplitude of the transitions was reduced compared with excised patches in asymmetrical conditions; positive to +25 mV, the transitions exhibited distinct evidence of rectification and a region of negative slope conductance.

We were concerned that the very small transitions recorded with 5·4 mM K^+ in the pipette may have reflected the activity of another channel type. This appeared unlikely in view of the high sensitivity to IBTX of these small unitary K^+ currents in inside-out and cell-attached patch experiments under physiological K^+ gradient (Fig. 1*A* and *B*). To rule out this possibility further we also examined ensemble average recordings in cell-attached patches from myocytes in 140 mM K^+ bath solution with 5·4 mM K^+ pipette solution for evidence of time and voltage dependence. Figure 4 shows ensemble averages of single-channel transitions in cell-attached and inside-out patches. Ensemble currents obtained in a cell-attached patch with 5·4 mM K^+ in the pipette solution showed no evidence of inactivation during test pulses to +50 and +70 mV but were clearly voltage and time dependent (Fig. 4*A*). Activation time constants of the ensemble currents were 26 and 20 ms at +50 and +70 mV, respectively. The mean time constant at +50 mV (holding potential, −20 mV) was 29·2 ± 1·7 ms (4 patches). These values are similar to those reported for BK_{Ca} channels in colonic smooth muscle cells (Carl & Sanders, 1989). Figure 4*B* shows that with symmetrical K^+ solutions (140 mM), qualitatively similar results were obtained with an inside-out patch, but the transitions were of greater amplitude, as were the ensemble currents. Although not pooled because different step potentials were used, similar data were obtained in two additional inside-out patches. These data indicate that the small rectifying transitions seen in cell-attached and excised patches with 5·4 mM K^+ in the pipette were due to BK_{Ca} channels.

We next sought to determine the extent to which unitary current amplitudes in a physiological K^+ gradient departed from that expected based on theoretical predictions. To estimate an idealized i–V for BK_{Ca} channel amplitude with asymmetrical K^+ solutions (pipette, 5·4 mM; bath, 140 mM), the permeability coefficient for K^+ was first calculated based on the mean conductance (262 pS) estimated from the i–V relation of BK_{Ca} channels recorded in inside-out patches exposed to symmetrical 140 mM K^+ as depicted in Fig. 5*A*. Equation (2) was employed to calculate P_K; a value of $4{\cdot}87 \times 10^{-13}$ cm s^{-1} was obtained and used to generate a theoretical i–V plot from −100 to +100 mV for a

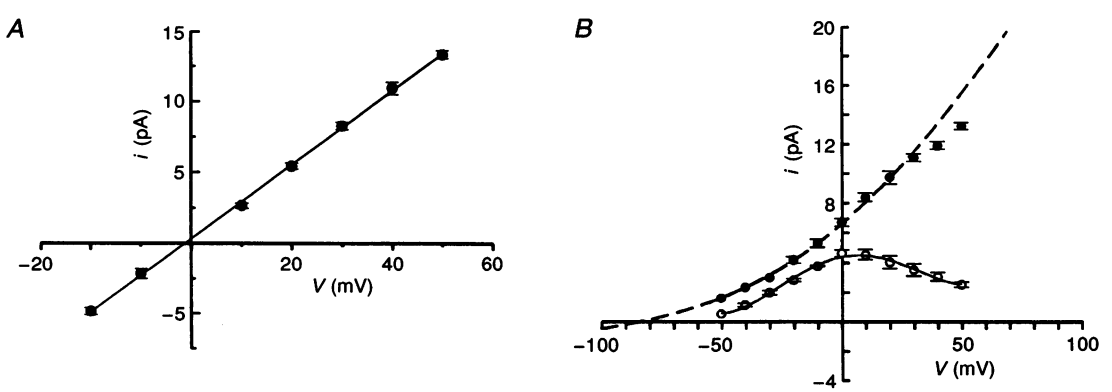

Figure 5. Conductance properties of BK_{Ca} channels in excised and cell-attached patches from rabbit portal vein myocytes

A, mean i–V relation pooled from four inside-out patches exposed to symmetrical 140 mM K^+ solutions. Each data point is a mean ± S.E.M. The mean slope conductance (γ) was 262 ± 6·6 pS in the potential range between −20 and +50 mV. *B*, voltage dependence of unitary currents through BK_{Ca} channels recorded with 5·4 mM K^+ in the pipette solution from inside-out (●) and cell-attached (○; cells bathed in depolarizing medium) patches. Each data point is the mean ± S.E.M. of 6 and 5 cells for the inside-out and cell-attached data, respectively. The dashed line represents the theoretical i–V relation for asymmetrical K^+ solutions (pipette, 5·4 mM; bath, 140 mM) and was generated using the Goldman–Hodgkin–Katz relation (eqn (2)) and the slope conductance determined under symmetrical K^+ conditions (*A*).

physiological K^+ gradient (Fig. 5*B*). Figure 5*B* shows the idealized *i–V* (dashed line) superimposed on experimental data from inside-out patch experiments with a K^+ gradient (pipette, 5·4 mM; bath, 140 mM; filled circles). Experimental data derived from excised patches (in the absence of Na^+ and Mg^{2+} and only 100 nM Ca^{2+} in the bath) closely approximated the predicted *i–V* relation between −50 and +30 mV and slightly deviated from the theoretical curve beyond +30 mV. In contrast, data derived from cell-attached patches of myocytes bathed in K^+ depolarizing solution (Fig. 5*B*, open circles; Fig. 4*C*) departed from the theoretical Goldman–Hodgkin–Katz *i–V* relation at all potentials and displayed inward rectification at potentials more positive than 0 mV. One possibility to account for this discrepancy could be that one or several intracellular factors may inhibit K^+ permeation through BK_{Ca} channels when recorded in cell-attached mode with 5·4 mM K^+ in the pipette solution. In subsequent experiments, we tested the hypothesis that block by intracellular Mg^{2+} and Na^+ caused the reduced unitary currents and inward rectifying properties of BK_{Ca} channels observed in cell-attached patch experiments.

Inside-out patches containing IBTX-sensitive BK_{Ca} channel activity were exposed to varied Mg^{2+} concentrations in the bath solution to assess the ability of this divalent cation to block outward K^+ current through BK_{Ca} channels under asymmetrical K^+ conditions. Figure 6*A* shows typical single-channel recordings from a representative patch under control conditions (0 Mg^{2+}, 100 nM Ca^{2+}), and after increasing the concentration of free Mg^{2+} to 2 and 5 mM (pipette, 5·4 mM K^+) at constant holding potential of +30 mV. Figure 6*B* displays the corresponding all-point amplitude histograms. Magnesium applied to the

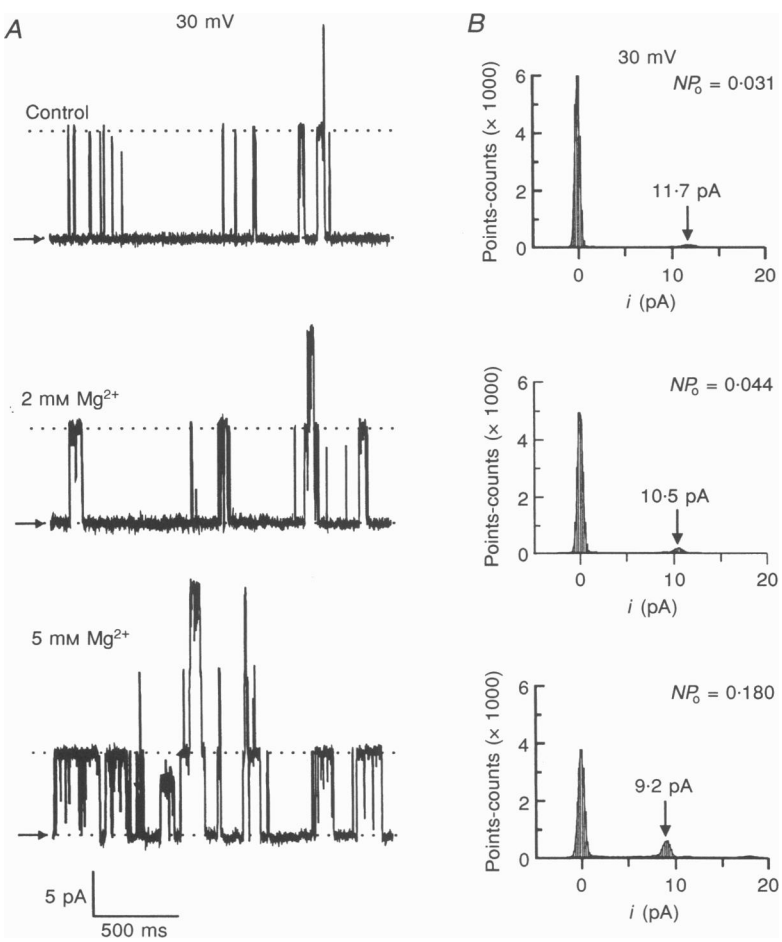

Figure 6. Effects of internal Mg^{2+} on BK_{Ca} channel unitary current amplitude and open probability from a typical inside-out patch experiment in a portal vein cell

The inside-out patch shown here was exposed to asymmetrical K^+ solutions (pipette, 5·4 mM; bath, 140 mM) and held at +30 mV. *A*, sample recordings are depicted in control (0 Mg^{2+}; top trace), 2 mM Mg^{2+} (middle trace) and 5 mM Mg^{2+} (bottom trace) with the internal face of the channels exposed to 100 nM Ca^{2+}. The dotted lines indicate the unitary amplitude of the first open level. Notice the decrease in current amplitude and increase in open probability as bath [Mg^{2+}] is increased. *B*, amplitude histograms derived from the corresponding data in *A*. NP_o values were calculated according to eqn (1) described in Methods. The amplitude of the initial current level is indicated for each condition.

cytoplasmic face of the channels dose-dependently decreased their unitary current amplitude and increased their open probability. Figure 6*B* shows that peak current of the first open level was reduced by 10 and 21% in 2 and 5 mM Mg^{2+}, respectively. This effect on single-channel conductance was concomitant to an increase in channel activity; in this patch, NP_o increased 1·4- and 6-fold in 2 and 5 mM Mg^{2+}, respectively. The effect of Mg^{2+} on unitary conductance and NP_o for BK_{Ca} channels was also apparent during voltage ramps.

Figure 7*A* depicts current traces recorded from an excised patch over the range of −20 to +60 mV with 5·4 mM K^+ in the pipette and either 0, 2 or 5 mM Mg^{2+} in the bath (100 nM Ca^{2+}). Decreased unitary current amplitude and increased channel activity were apparent with high Mg^{2+}. *i–V* relations of BK_{Ca} channels from pooled data with increasing Mg^{2+} were determined from ramps between +10 and +70 mV for 0, 2 and 5 mM Mg^{2+} (Fig. 7*B*, top trace). Unitary current amplitude decreased across the entire voltage range studied, but there was no evidence of rectification similar to that observed in cell-attached recordings. On average, single-channel conductance (chord conductance at +70 mV) decreased by 20 and 33% in 2 and 5 mM Mg^{2+} compared with that in the absence of Mg^{2+}. The bottom graph of Fig. 7*B* shows plots of the voltage dependence of block for the two concentrations of Mg^{2+}. For both concentrations, the percentage block of BK_{Ca} channels increased linearly with membrane depolarization, indicating that Mg^{2+} inhibits the channel in a voltage-dependent manner.

Similar experiments were carried out in coronary myocytes. Inside-out patches from these cells exposed to either 2 or 5 mM Mg^{2+} reduced single-channel amplitude and increased NP_o in a fashion comparable to that observed with patches from portal vein myocytes (data not shown). In the absence of Mg^{2+} (symmetrical K^+ solutions), the slope conductance was 201 ± 13 pS (4 patches). It decreased by an average of 29 and 39% with 2 and 5 mM Mg^{2+}, respectively. These

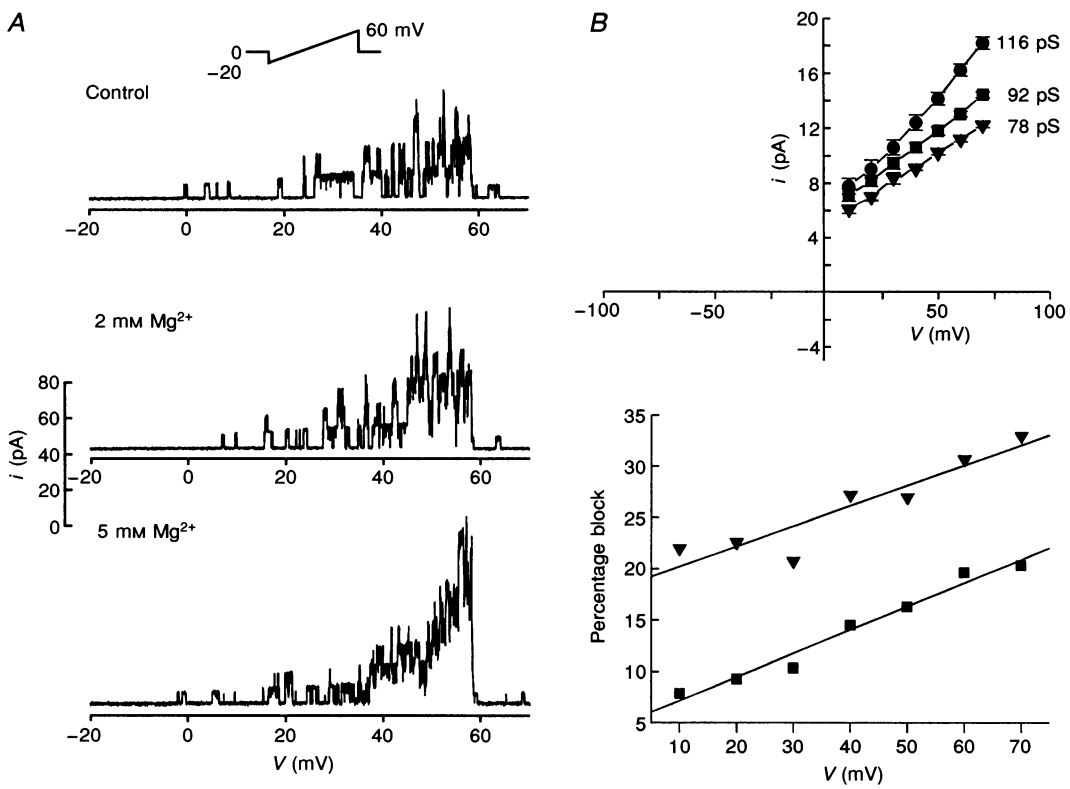

Figure 7. Voltage dependence of the effects of internal Mg^{2+} on $i_{BK(Ca)}$ recorded in inside-out patches from portal vein cells

The channels were exposed to asymmetrical K^+ solutions (pipette, 5·4 mM; bath, 140 mM). *A*, three original tracings elicited by voltage ramps ranging from −20 to +60 mV. The acquired currents were plotted as a function of ramp voltage and recorded under identical conditions to those of Fig. 6, i.e. control (0 Mg^{2+}; top trace), 2 mM Mg^{2+} (middle trace), and 5 mM Mg^{2+} (bottom trace); internal Ca^{2+} was buffered at 100 nM. *B*, top panel, corresponding *i–V* plots reporting the effects of internal Mg^{2+} on BK_{Ca} channel conductance. Each data point is a mean ± S.E.M. of 6 cells. Plotted are the *i–V* curves for control (●), 2 mM Mg^{2+} (■), and 5 mM Mg^{2+} (▼), respectively; chord conductance (γ) values are indicated to the right of each curve. Bottom graph, plots of percentage block of unitary current amplitude as a function of voltage for 2 mM Mg^{2+} (■) and 5 mM Mg^{2+} (▼). Calculated data derived from those of the top graph.

results indicate that the effects of Mg^{2+} on the permeating and gating behaviour of BK_{Ca} channels are not unique to the portal vein but seem to represent general properties of these channels.

To quantitate accurately the effect of Mg^{2+} on conductance, we examined the effect of a range of concentrations between 0 and 15 mM Mg^{2+} on unitary current amplitude at +40 mV. Mean values for unitary current amplitudes normalized to the value in the absence of Mg^{2+} are shown in Fig. 8*A*. The K_d for the Mg^{2+} block at +40 mV was 9·7 mM (Fig. 8*A*, inset). The Mg^{2+} block was weakly voltage dependent. Using the Woodhull (1973) model, a value of 0·23 for the fractional distance (eqn (3)) across the channel as measured from the inside was calculated for 2 mM Mg^{2+} with a $K_D(0)$ of 37·6 mM. Similar results were obtained in coronary myocytes; the estimated fractional distance with 2 mM Mg^{2+} was 0·22 with a $K_D(0)$ of 28·5 mM.

We next examined the effects Mg^{2+} on open probability of BK_{Ca} channels over a range of Ca^{2+} concentrations between 10 and 500 nM. The concentrations of Mg^{2+} and Ca^{2+} were chosen to reproduce the physiological concentrations of these divalent cations as determined in several studies (Jelicks & Gupta, 1990; Okada, Ishikawa & Saito, 1992; Sturek, Kunda & Hu, 1992; Quamme, Dai & Rabkin, 1993). If Mg^{2+} and Ca^{2+} stimulate BK_{Ca} channels by interacting with the same binding site, Mg^{2+} should be more potent at enhancing its activity at low $[Ca^{2+}]$. Figure 8*B* indicates that 1 mM Mg^{2+} enhanced the open probability of BK_{Ca} channels at +40 mV over the range of Ca^{2+} concentrations studied; the Mg^{2+}-induced increase in NP_o ranged from 3·5- to 7-fold (Fig. 8*B*, inset); these increases were not significantly different from each other ($P < 0·05$). The fact that the Mg^{2+}-induced increase in NP_o was similar at all Ca^{2+} concentrations tested supports the notion that Mg^{2+} stimulates BK_{Ca} channel activity through an allosteric mechanism. Whether or not co-operativity (Hill coefficient >1 for Ca^{2+} activation) is involved in the enhancement produced by Mg^{2+} cannot be assessed from the present experiments as previously suggested (Golowasch *et al.* 1986; Trieschmann & Isenberg, 1989; Zhang *et al.* 1995).

The preceding data (Fig. 8*A*) clearly indicated that Mg^{2+} reduces BK_{Ca} channel conductance; although the effect is slightly voltage dependent, it cannot account for the rectification and negative slope conductance at positive potentials observed in the cell-attached patches. Na^+ was

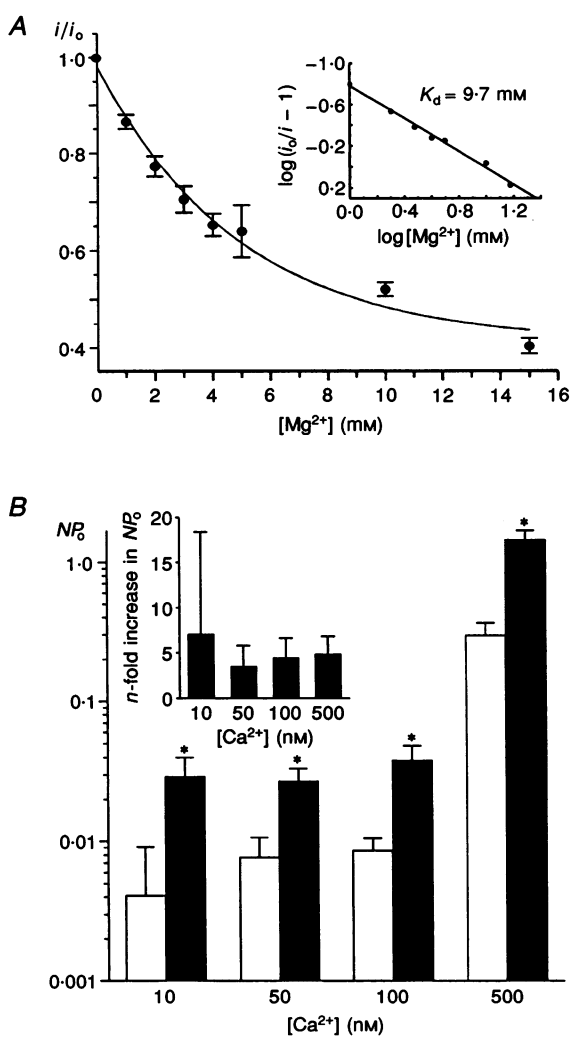

Figure 8. Biophysical properties of Mg^{2+}-induced effects on conductance and gating of BK_{Ca} channels in portal vein myocytes

A, the internal face of inside-out patches containing BK_{Ca} channels was exposed to free $[Mg^{2+}]$ ranging from 0 to 15 mM while membrane patches were held at +40 mV; free $[Ca^{2+}]_i$, 100 nM. Each data point indicates mean ± S.E.M. unitary current (i) normalized relative to current amplitude in the absence of Mg^{2+} (i_o) and expressed as i/i_o (5 patches). Superimposed fit is a single exponential decaying function. Inset, the same data are plotted on a double logarithmic scale to provide a better estimate of the $[Mg^{2+}]$ required to produce half-maximal inhibition of the current (K_d); the indicated K_d value of 9·7 mM was derived from the slope of the linear regression fit superimposed on the data points. *B*, effects of internal Ca^{2+} on the Mg^{2+}-induced enhancement of NP_o of BK_{Ca} channels. Bath $[Ca^{2+}]$ was varied between 10 and 500 nM in the presence (■) and absence (□) of 1 mM Mg^{2+}. *Significantly different ($P < 0·05$) from control data obtained in the absence of Mg^{2+} (6 patches). Inset, bar graph reporting the n-fold increase in NP_o for the Ca^{2+} concentration range tested. An analysis of variance revealed no significant differences ($P < 0·05$) among the four groups.

previously shown to interfere with K^+ permeation in BK_{Ca} channels of smooth muscle (Singer & Walsh, 1984; Hu *et al.* 1989) and other cell types (Marty, 1983; Yellen, 1984). For this reason, we considered the possibility that the combination of Mg^{2+} and Na^+ might account for the cell-attached data. To characterize further the effects of Na^+ on BK_{Ca} channels, excised patches were exposed to Na^+ alone, or in combination with Mg^{2+}. Figure 9*A* shows representative data for ramps applied in the absence of Na^+ and Mg^{2+}, in the presence of 2 mM Mg^{2+} or 20 mM Na^+, and in the presence of both ions. Na^+ increased the open-state noise and caused rectification at potentials positive to +20 mV; however, Na^+ produced little effect on unitary current amplitude at potentials negative to −20 mV. In contrast to Mg^{2+}, application of 20 mM Na^+ to the internal side of the BK_{Ca} channel produced strong voltage-dependent block at potentials more positive than +20 mV resulting in the appearance of a negative slope region on the *i–V* relationship. Although not analysed in detail, internal sodium ions induced a flickery type of block that resembled that described in chromaffin cells (Marty, 1983; Yellen, 1984) and intestinal myocytes (Hu *et al.* 1989). From eqn (3), we also estimated an electrical distance value of 1·8 for Na^+ permeation-induced block. Our estimated value was slightly higher than the value reported for taenia coli smooth muscle cells (Hu *et al.* 1989; *d*, 1·2) and similarly indicates that Na^+ can penetrate deeply inside the conduction pathway. A value higher than the upper limit (*d*, 1·0) predicted by the Woodhull (1973) formulation has generally been explained on the basis that BK_{Ca} channels may be occupied by more than one cation at a time (Yellen, 1984; Hu *et al.* 1989).

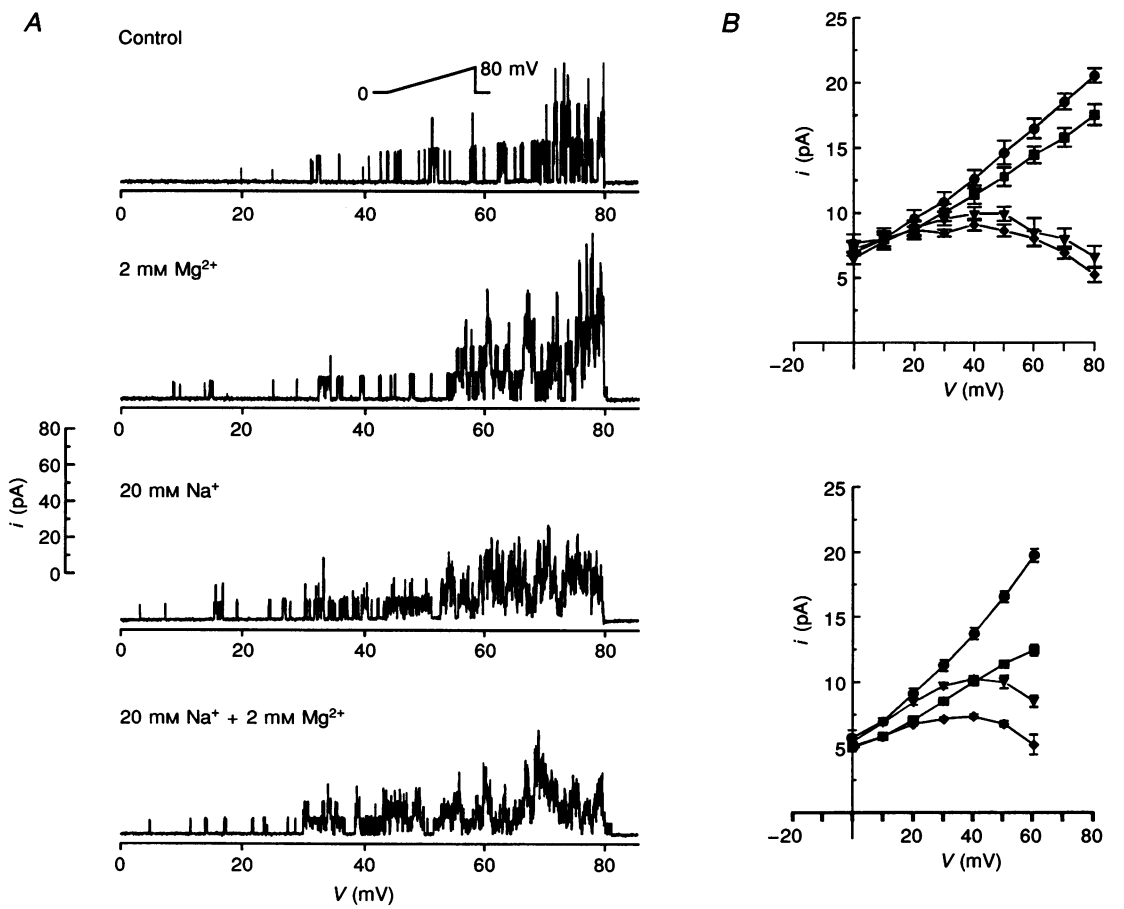

Figure 9. Effects of internal Mg^{2+} and Na^+ on current amplitude and open probability of BK_{Ca} channels recorded in inside-out patches from portal vein cells

A, excised-membrane patches were subjected to a ramp protocol (inset, top panel) where transmembrane potential was varied between 0 and +80 mV. Representative recordings are shown in four experimental conditions (from top to bottom): control, 2 mM Mg^{2+}, 20 mM Na^+, and in the combined presence of 20 mM Na^+ and 2 mM Mg^{2+}. Free $[Ca^{2+}]_i$, 50 nM. As in Fig. 6, membrane current was plotted as a function of ramp voltage. *B*, corresponding *i–V* plots for data obtained in experimental conditions identical to those described in *A*. Each data point indicates mean ± S.E.M. (6 patches). The four curves refer to data acquired in control (●), 5 mM Mg^{2+} (■), 20 mM Na^+ (▼), and 20 mM Na^+ plus 2 (top plot) or 5 mM (bottom plot) Mg^{2+} (◆).

In the presence of both Mg^{2+} and Na^+, decreased unitary conductance was observed over the entire voltage range tested, rectification occurred positive to +20 mV, and increased open-channel noise was apparent. The depression of unitary conductance by Na^+ and Mg^{2+} positive to +20 mV was additive. Mean data for unitary current amplitude *versus* voltage in the presence of 20 mM Na^+ and 2 (top) or 5 mM Mg^{2+} (bottom) are shown in Fig. 9*B*. The ability of Na^+ to cause rectification positive to +20 mV is apparent; however, it is evident that only Mg^{2+} decreases unitary conductance at potentials negative to +20 mV in a dose-dependent manner. Thus, depressed unitary current amplitude above and below +20 mV, rectification positive to +20 mV, and increased open channel noise were observed only in the combined presence of Mg^{2+} and Na^+ at the concentrations tested.

DISCUSSION

This study was designed to investigate the conductance and gating properties of large conductance Ca^{2+}-activated K^+ channels of vascular smooth muscle cells under conditions which least perturb the physiological ionic environment, that is with a preserved transmembrane K^+ gradient. Our data indicate that the unitary conductance of BK_{Ca} channels recorded in cell-attached patches is significantly lower than expected in the potential range of −50 to +50 mV. The *i–V* relationship of BK_{Ca} channels displayed marked inward rectification at potentials more positive than 0 mV. Experiments conducted with excised inside-out patches suggest that with free Ca^{2+} buffered to within the physiological range (100–500 nM), block by both intracellular Mg^{2+} and Na^+ may participate in the reduction of unitary current amplitude, inward rectification at positive potentials, and noisy behaviour of single channels in on-cell patches (this study) and whole-cell $I_{BK(Ca)}$ (Hume & Leblanc, 1989; Beech & Bolton, 1989; Miller *et al.* 1993; Leblanc *et al.* 1994). Whereas physiological concentrations of Mg^{2+} depressed $i_{BK(Ca)}$ in a weakly voltage-dependent fashion, Na^+ produced little effect negative to +20 mV but exhibited strong voltage-dependent inhibitory effects and induced flickery block at potentials positive to this voltage.

Identification of BK_{Ca} channels in cell-attached patches

A novel finding of the present study was the surprisingly small amplitude of BK_{Ca} channels in cell-attached patches of portal vein and coronary myocytes when exposed to normal extracellular $[K^+]$ (5·4 mM). Such a pattern of channel behaviour was misleading as the magnitude of these single-channel currents approached that measured for delayed rectifier (K_V; Beech & Bolton, 1989; Gelband & Hume, 1992; Volk & Shibata, 1993), ATP-dependent (K_{ATP}; Kajioka *et al.* 1991), and inward rectifier (K_{IR}; Quayle, McCarron, Brayden & Nelson, 1993) K^+ channels under both symmetrical and asymmetrical K^+ conditions. However, it is unlikely that the K^+ channel most consistently observed in cell-attached patches resulted from the activity of one of the latter channels for the following reasons. (1) The channel under investigation was clearly activated by membrane depolarization; in contrast, single and whole-cell currents through K_{ATP} channels are voltage independent (Kajioka *et al.* 1991), whereas K_{IR} channels produce little, if any, current at depolarized membrane potentials (Quayle *et al.* 1993; Nelson & Quayle, 1995). (2) Accurate determination of the *i–V* relationship of the channel in cell-attached patches from myocytes bathed in depolarizing medium to minimize the influence of the cell's own $V_{m,rest}$ required the presence of caffeine, which is known to release Ca^{2+} from internal stores, thus supporting the view that the K^+ channel was Ca^{2+} dependent. (3) Ensemble averages of single-channel currents in cell-attached patches displayed activation kinetics that were similar to those generated by BK_{Ca} channels in excised patches exposed to symmetrical K^+ solutions, and whole-cell BK_{Ca} channels, and did not inactivate as reported for K_V channels (Beech & Bolton, 1989; Hume & Leblanc, 1989; Volk & Shibata, 1993; Leblanc *et al.* 1994). (4) The channel was blocked by a low concentration of iberiotoxin, a scorpion venom toxin considered to be a highly specific blocker of BK_{Ca} channels in smooth muscle cells (Galvez *et al.* 1990; Nelson & Quayle, 1995). These results support the notion that under physiological conditions, the conductance of BK_{Ca} channels is much lower than that previously assumed and, in light of these findings, its contribution to whole-cell K^+ current must be re-evaluated.

Possible mechanisms implicated in the reduction of BK_{Ca} channel conductance

Reduced conductance at negative potentials is expected from the non-linear *i–V* relationship predicted by the Goldman–Hodgkin–Katz constant field equation (Goldman, 1943; Hodgkin & Katz, 1949). Inside-out patch experiments carried out using a physiological K^+ gradient (pipette, 5·4 mM; bath, 140 mM) revealed a good correlation between original data and the outward rectifying theoretical curve based on the Goldman–Hodgkin–Katz equation and the value of P_K estimated from the conductance of BK_{Ca} channels measured in symmetrical conditions (140 mM K^+). However, the *I–V* relation of iberiotoxin-sensitive BK_{Ca} channels in cell-attached patches markedly deviated from the excised patch data and theoretical relationship at all potentials studied, displaying inward rectification at potentials positive to 0 mV and a prominent negative slope region.

One possible explanation for this observation is that the intracellular K^+ concentration ($[K^+]_i$) in the intact cell was lower than that expected (~140 mM), as a result of the cell isolation procedure, the method of cell storage, or reduced activity of the Na^+–K^+ pump at room temperature. This appears unlikely since extrapolation of the *i–V* curve of

BK_{Ca} channels recorded in cell-attached patches with 140 mM K^+ in the pipette solution in cells bathed in standard perfusate yielded reversal potential values which were near the reported values of $V_{m,rest}$ measured in current-clamped portal vein (Hume & Leblanc, 1989; Miller *et al.* 1993) and coronary myocytes (Leblanc *et al.* 1994). The latter findings support the notion that a low $[K^+]_i$ is probably not involved in altering the conductance of the BK_{Ca} channel when operating in its native environment. One alternative explanation may be that physiological levels of intracellular particles or ions inhibit outwardly directed movement of K^+ through BK_{Ca} channels.

Effects of internal Mg^{2+} and Na^+

Our data showed that both internal Mg^{2+} and Na^+ have the ability to interact with BK_{Ca} channels although their mechanisms of action differ. At physiological concentrations, Mg^{2+} exerted a dual effect on portal vein and coronary smooth muscle BK_{Ca} channels: it depressed their conductance and enhanced their open probability. Intracellular magnesium induces voltage-dependent block and inward rectification, or modulates the gating properties of several types of K^+ channels including cardiac K_{ATP} (Horie *et al.* 1987), cardiac (Matsuda *et al.* 1987) and endothelial (Elam & Lansman, 1995) K_{IR}, single A-type K^+ channels in cultured rat locus ceruleus neurons (Forsythe *et al.* 1992), renal smooth muscle delayed rectifier K^+ channels (Gelband *et al.* 1994), and K^+ channel clones of the inward rectifier super family comprising the ROMK and IRK subclasses (Jan & Jan, 1994). Mg^{2+} has been reported to reduce the conductance of Ca^{2+}-activated K^+ channels in a dose- and voltage-dependent manner with enhanced potency of block at depolarized membrane potentials in rat skeletal muscle (Ferguson, 1991) and cerebral artery smooth muscle cells (Zhang *et al.* 1995). Our reported values for the fractional distance of block with 2 mM Mg^{2+} (*d*, 0·21–0·23) are similar to those obtained by Ferguson (1991; *d*, ~0·2) and Zhang *et al.* (1995; *d*, 0·08–0·3). Our data are consistent with the idea that Mg^{2+} interacts with a site near the inner mouth of the channel pore. Although internal Mg^{2+} may participate in the reduction of BK_{Ca} channel conductance observed in cell-attached patches, it is probably not involved in the negative slope region of the i–V relationship of BK_{Ca} channels recorded under similar conditions. No evidence of a region of negative slope conductance was obtained with excised patches exposed to varied Mg^{2+} concentrations.

One novel observation of the present study was the demonstration that the application of 20 mM Na^+ in the presence of 2 or 5 mM Mg^{2+} further decreased BK_{Ca} channel conductance. However, the mechanisms by which the two ions inhibit K_{Ca} channels seemed to differ for the following reasons. (1) Except for high concentrations of Mg^{2+} (5 mM or higher, see Fig. 7), flickery activity was not observed in the presence of Mg^{2+} alone, whereas it was typical of Na^+-induced block. (2) The i–V relationship of BK_{Ca} channels in the presence of the two ions nearly represented the algebraic sum of the individual i–V curves obtained in the presence of either ion (Fig. 9*B*). These results support the view that Mg^{2+} and Na^+ interact with two independent binding sites closely associated with the permeation pathway. The general shape of the i–V relationship in the presence of the two cations was similar to that for BK_{Ca} channels recorded from cell-attached patches (compare Figs 5*B* and 9*B*). The appearance of the negative slope region was apparent at approximately +10 mV for the cell-attached patches, but was evident only above +40 mV for inside-out patches, as similarly observed in other smooth muscle cells (Singer & Walsh, 1984; Hu *et al.* 1989). The reason for this discrepancy remains unclear. One assumption in this comparison is that the ionic conditions used in our excised patch experiments mimic those of unperturbed cells. Future experiments will be carried out to determine the effects of other combinations of $[K^+]_i$, $[Na^+]_i$ and $[Mg^{2+}]_i$ on the voltage dependence of unitary currents through BK_{Ca} channels. On the other hand, our study cannot rule out the possibility that other inorganic or even organic (Fakler, Brandle, Glowatzki, Weidemann, Zenner & Ruppersberg, 1995) blocking particles might be implicated in the phenomenon.

Ca^{2+}-dependent K^+ channels have been viewed as large conductance channels under physiological conditions in vascular smooth muscle cells. In asymmetrical K^+ solutions (pipette, ~5·4 mM; bath, 140 mM), the conductance was estimated to lie between 100 and 130 pS at 0 mV (Beech & Bolton, 1989; Miller *et al.* 1993). In light of our findings, this concept may need revision; our cell-attached data clearly indicated that BK_{Ca} channels exhibit complex voltage-dependent conducting properties which would bring them closer to the category of 'medium' conductance K^+ channels with unitary characteristics resembling those of certain delayed rectifier K^+ channels (Volk & Shibata, 1993; Gelband & Hume, 1992). Although internal Mg^{2+} and Na^+ are probably not the sole participants in depressing the conductance of BK_{Ca} channels, they probably play a major role in this process. It is possible that Na^+-induced flickery block of BK_{Ca} channels at positive potentials may also partially explain the noisy and oscillatory nature of TEA- or charybdotoxin-sensitive whole-cell $I_{BK(Ca)}$ current recorded at positive potentials in rabbit portal vein (Hume & Leblanc, 1989; Beech & Bolton, 1989; Miller *et al.* 1993) and coronary myocytes (Leblanc *et al.* 1994).

Can internal Mg^{2+} and Na^+ regulate BK_{Ca} channel activity under physiological conditions?

Studies using the magnesium indicator Mg-fura-2 or Mg^{2+}-induced ^{31}P-NMR spectral shifts of ATP have indicated that basal $[Mg^{2+}]_i$ lies in the range 0·25–0·52 mM in vascular smooth muscle cells (Jelicks & Gupta, 1990; Okada *et al.*

1992; Quamme *et al.* 1993), but can transiently reach ~1–3 mM after stimulation with arginine vasopressin or endothelin (Okada *et al.* 1992), or following interventions which mobilize internal Ca^{2+} stores (Okada *et al.* 1992; Quamme *et al.* 1993). The above studies provided evidence in favour of the existence of active mechanisms regulating the activity of Mg^{2+} within tight limits. Resting $[Na^+]_i$ determined with the fluorescent Na^+ dye SBFI or ^{23}Na-NMR has been reported to lie in the range 10·1–24·1 mM in various types of normotensive vascular smooth muscle cells (Jelicks & Gupta, 1990; Johnson, Theler, Capponi & Vallotton, 1991; Tepel *et al.* 1994). Intracellular Na^+ is a highly regulated cation in smooth muscle and can change in response to a variety of stimuli including the inhibition of the Na^+–K^+ pump and indirect stimulation of Na^+–Ca^{2+} exchange (Ashida & Blaustein, 1987), stimulation of Na^+–H^+ exchange (Tepel *et al.* 1994), and alterations in $[Ca^{2+}]_i$ (Tepel *et al.* 1994). Rat aortic myocytes from spontaneously hypertensive rats exhibited increased (Jelicks & Gupta, 1990) or decreased (Tepel *et al.* 1994) levels of $[Na^+]_i$. It is not yet known whether the subsarcolemmal concentrations of Mg^{2+} and Na^+ are significantly different than that measured in 'bulk' cytoplasm, as has been proposed for Ca^{2+} in vascular smooth muscle cells (Sturek *et al.* 1992). The above studies justify the concentrations of Mg^{2+} and Na^+ used in our experiments. Although the gating and conductance of BK_{Ca} channels are significantly influenced by Mg^{2+} and Na^+ within their physiological concentration ranges, it remains to be established whether a modulation of BK_{Ca} channels through dynamic alterations of the two ions represents a meaningful physiological mechanism because the biophysical effects of both ions predominantly occur at depolarized membrane potentials. It is possible that the enhancing effect of Mg^{2+} on gating may play a more important role than its inhibitory effects on conductance in the physiological range of membrane potentials as recently suggested by Zhang *et al.* (1995) in cerebral arterial smooth muscle cells. These authors proposed that Mg^{2+} would exert a 'tonic' facilitation of BK_{Ca} channel activity.

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