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Ca^{2+}-activated K^+ channels from an insulin-secreting cell line incorporated into planar lipid bilayers

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Summary. This study evaluates the use of the planar lipid bilayer as a functional assay of Ca^{2+}-activated K^+ channel activity for use in purification of the channel protein. Ca^{2+}-activated K^+ channels from the plasma membrane of an insulin-secreting hamster Beta-cell line (HIT T15) were incorporated into planar lipid bilayers. The single channel conductance was 233 picoSiemens (pS) in symmetrical 140 mmol/l KCl and the channel was strongly K^+-selective ($P_{Cl}/P_K = 0.046$; $P_{Na}/P_K = 0.027$). Channels incorporated into the bilayer with two orientations. In 65 % of cases, the probability of the channel being open was increased by raising calcium on the cis side of the bilayer (to which the membrane vesicles were added) or by making the cis side potential more positive. At a membrane potential of + 20 mV, which is close to the peak of the Beta-cell action potential, channel activity was half-maximal at a Ca^{2+} concentration of about 15 μmol/l. Charybdotoxin greatly reduced the probability of the channel being open when added to the side opposite to that at which Ca^{2+} activated the channel. These results resemble those found for Ca^{2+}-activated K^+ channels in native Beta cell membranes and indicate that the channel properties are not significantly altered by incorporation in a planar lipid bilayer.

Key words: Ca^{2+}-activated K-channel, pancreatic Beta cell, HIT T15 cell, insulin-secreting cell line, planar lipid bilayer.

Ca^{2+}-activated K^+ channels have been found in all Beta cells and Beta-cell lines investigated [1]. Their precise functional role remains somewhat controversial, although it is now generally agreed that they probably contribute to repolarization of the Beta-cell action potential [2, 3]. Evidence also suggests that they have little influence on the glucose-dependent oscillations in Beta-cell membrane potential (slow waves) from which the action potentials arise [4] and are not essential for glucose-induced electrical activity in Beta cells [5].

The probability of Ca^{2+}-activated K^+ channels being open is increased both by intracellular Ca^{2+} and by depolarisation. The precise mechanism by which Ca^{2+} and voltage interact to increase channel activity requires knowledge of the channel structure, which is not yet available. Although a number of voltage-gated K-channels have been cloned using oligonucleotide probes based on the sequence of the Shaker K-channel, this approach has proved unsuccessful for the Ca^{2+}-activated K^+ channel [6], presumably due to lack of homology. In addition, expression cloning of the channel in oocytes is limited by low expession [7]. An alternative strategy is protein purification followed by N-terminal sequencing and construction of oligonucleotide probes for screening of cDNA libraries. For this approach, it would be useful to have a functional assay which could be used to detect the presence of the Ca^{2+}-activated K^+ channel during protein isolation. Reconstitution of solubilised channel proteins in lipid vesicles followed by fusion with planar bilayers and single channel recording is one such method [8]. In this paper we assess the usefulness of this technique as a functional assay for the Beta-cell Ca^{2+}-activated K^+ channel.

As a source of tissue we have used the clonal Beta-cell line HIT T15 (HIT). This cell line was derived by SV40 transformation of hamster islet cells [9] and has many of the properties of normal Beta cells; in particular, secretion of insulin in response to glucose [9–11]. Patch clamp studies have established that HIT cells possess Ca^{2+}-activated K^+ channels [12].

Materials and methods

Cell culture

HIT T15 cells (passage numbers 75–90) were cultured, passaged and harvested as described by Ashcroft et al. [10].

Membrane preparation

Membranes were prepared from HIT T-15 Beta cells as described by Gaines et al. [13], with slight modification. Briefly, HIT cells were collected from confluent flasks and washed twice with phosphate-

Table 1. Concentrations of EGTA and Ca^{2+} added to the KCl solution to provide the calculated free Ca^{2+} concentration. The pH was subsequently readjusted to 7.1 with KOH

Total Ca^{2+} (mmol/l)	Total EGTA (mmol/l)	Calculated free Ca^{2+}
1.0	0	1.0 mmol/l
1.0	1.0	15.6 μmol/l
1.0	2.0	0.25 μmol/l

buffered saline (Life Technologies, Paisley, UK). Cells were resuspended in ice-cold 5 mmol/l Tris base (pH 8.0) containing a cocktail of protease inhibitors (0.1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l EDTA, 1 mmol/l iodoacetamide, 10 μg/ml soybean trypsin inhibitor and 10 μmol/l leupeptin) and incubated on ice in a glass homogenizer for 40 min. The cells were then homogenized and the homogenate was centrifuged for 10 min at 900 *g*. The supernatant was collected and centrifuged at 96000 *g* for 30 min at 4°C. The pellets were resuspended at a concentration of 1–2 mg/ml protein in 0.4 mol/l sucrose, 10 mmol/l Hepes-KOH (pH 7.1), frozen immediately in liquid nitrogen and stored at −70°C.

Planar lipid bilayers

Planar lipid bilayers were formed from a mixture of 50% phosphatidylethanolamine (bovine heart) and 50% phosphatidylserine (bovine brain) dissolved in decane at a concentration of 25 mg/ml. All lipids were obtained from Avanti Polar Lipids (Birmingham, Ala., USA). Planar lipid bilayers were formed by painting the phospholipid solution across a 300–400 μm diameter hole in a polystyrene partition separating two solution-filled chambers of 5 ml (cis) and 3 ml (trans) and allowed to thin spontaneously [14, 15]. The cis chamber (to which the vesicles were added) was voltage clamped at various potentials relative to the trans chamber: the trans chamber was grounded. All potentials refer to the potential of the cis chamber with respect to the trans chamber (i.e. cis-trans voltage).

Vesicle fusion was deemed to have occurred in those bilayers in which channel activity was observed. In this paper, we only discuss those bilayers in which vesicle fusion occurred ($n = 151$).

Data analysis

Single channel currents were recorded under voltage clamp using a standard current-to-voltage amplifier and recorded on FM tape or video tape for later analysis. The frequency response of the system was 200 Hz. The currents were later amplified (×10 or ×50) and filtered at 200 Hz using an 8-pole Bessel filter (Frequency Devices, Springfield, Mass., USA). They were then digitised at 1 kHz using an Axolab analogue-to-digital converter and analysed using an IBM AT computer and the program PCLAMP. The probability of the channel being open (the open probability) was determined from amplitude histograms constructed from all data points. The records have been redisplayed using a Gould 3200 chart recorder.

Solutions

Both the cis and trans solution initially contained 140 mmol/l KCl and 10 mmol/l HEPES (titrated to pH 7.1 with KOH: additional K^+ about 2.5 mmol/l). To the cis chamber 1 mmol/l $CaCl_2$ was added, followed by the vesicles (final protein concentration 2–10 μg/ml). Free calcium concentrations were adjusted by the subsequent addition of EGTA (titrated to pH 7.4 with KOH) and the pH was subsequently readjusted to 7.1 with KOH (Table 1). Free Ca^{2+} concentrations were calculated using the binding constants of Martell and Smith [16]. In some experiments NaCl was partially substituted for KCl, as described in the text. Experiments were done at room temperature of 20–25°C.

Statistical analysis

Results are given as mean ± the standard error of the mean (SEM).

Results

Ca^{2+}-activated K^+ channels were seen in 73 out of the 151 bilayers in which fusion occurred. The channel was able to insert into the bilayer with its intracellular side facing either the cis side or the trans side of the bilayer. The incidence of the Ca^{2+}(cis)-activated K^+ channel was 64.6% and that of the Ca^{2+}(trans)-activated K^+ channel was 35.4%. We focus our analysis principally on the properties of the Ca^{2+}(cis)-activated K^+ channel, which was distinguished by the profound decrease in its activity produced by adding EGTA to the cis side of the membrane.

Permeability

Figure 1A shows single channel currents recorded at different membrane potentials in symmetrical 140 mmol/l KCl solutions at a cis Ca^{2+} concentration of 1 mmol/l.

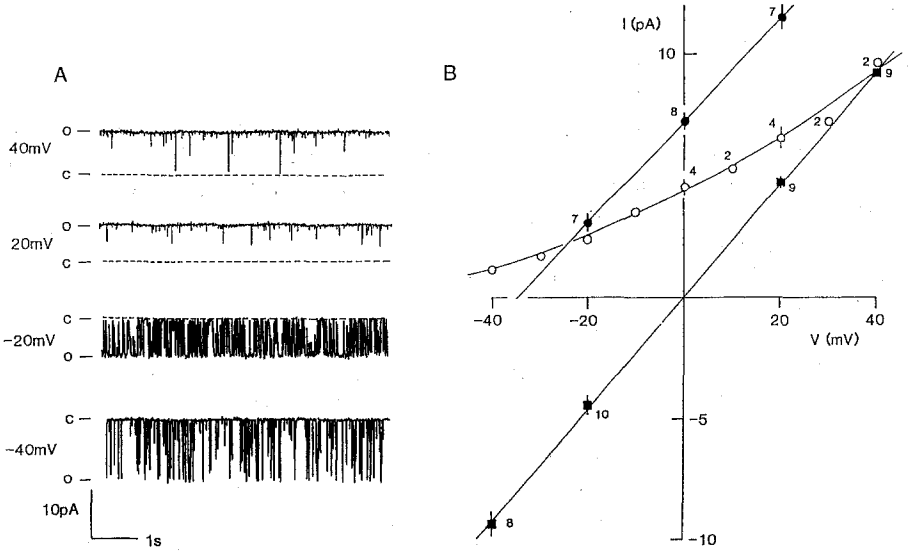

Fig. 1 A, B. Single channel currents and current-voltage (I–V) relations of the Ca^{2+}(cis)-activated K^+ channel.
A Ca^{2+}(cis)-activated K^+ channel currents recorded at different membrane voltages (indicated to the left of each trace). C and O indicate the closed and open levels respectively. cis: 140 mmol/l KCl, 1 mmol/l $CaCl_2$; trans: 140 mmol/l KCl.
B Mean single channel I–V relations. (■) cis: 140 mmol/l KCl, 1 mmol/l $CaCl_2$; trans: 140 mmol/l KCl. (●) cis: 250 mmol/l KCl, 1 mmol/l $CaCl_2$; trans: 50 mmol/l KCl. (○) cis: 140 mmol/l KCl, 1 mmol/l $CaCl_2$; trans: 135 mmol/l NaCl, 5 mmol/l KCl. The numbers adjacent to each point indicate the number of bilayers. The vertical lines indicate ± SEM. The solid lines have slopes of 213 pS (●) and 233 pS (■). The continuous line through the open circles (○) was fit to equation (1) in the Results

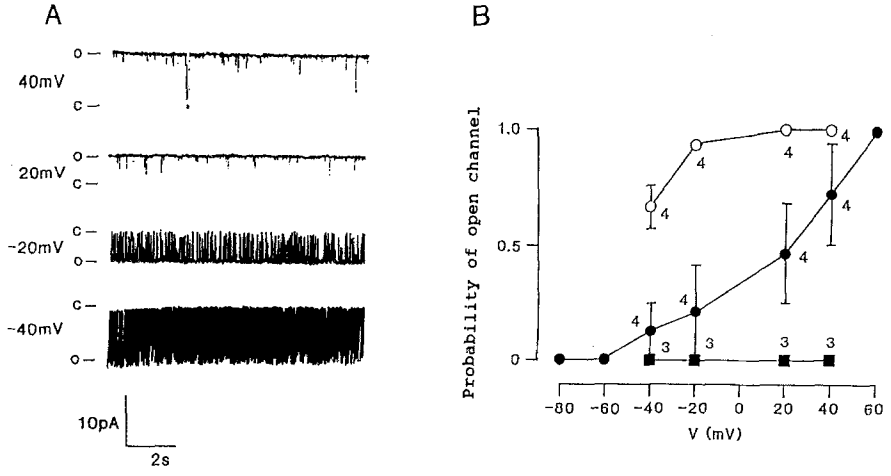

Fig. 2 A, B. Ca^{2+} dependence and voltage dependence of the Ca^{2+}(cis)-activated K^+ channel.
A Ca^{2+}(cis)-activated K^+ channel currents recorded at different membrane voltages (indicated to the left of each trace). cis: 140 mmol/l KCl, 1 mmol/l Ca^{2+}; trans: 140 mmol/l KCl. C and O indicate the closed and open levels respectively.
B Relationship between the channel open probability and membrane potential at different cis calcium concentrations: 1 mmol/l (○), 15.6 μmol/l (●), 0.25 μmol/l (■). cis and trans: 140 mmol/l KCl. The numbers adjacent to each point indicate the number of bilayers

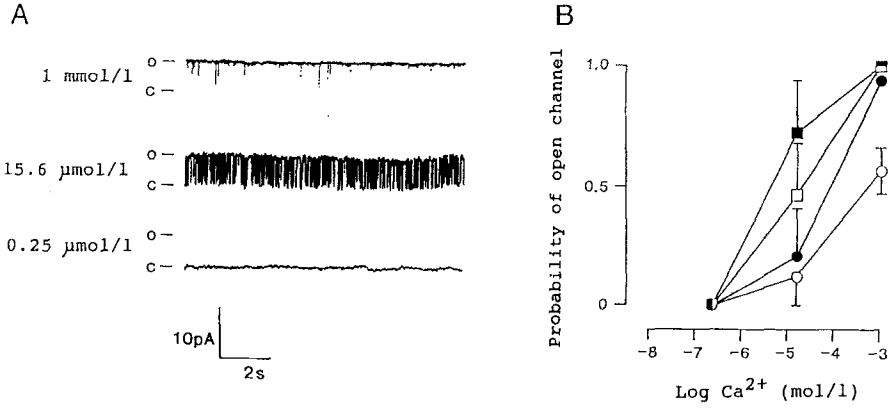

Fig. 3.(A, B)
A Ca^{2+}(cis)-activated K^+ channel currents recorded at different cis calcium concentrations at +20 mV. Free calcium concentrations are indicated to the left of each trace. cis and trans: 140 mmol/l KCl. C and O indicate the closed and open levels respectively.
B Relationship between the channel open state probability and the cis side calcium concentration, at four different membrane potentials: +40 mV (■) +20 mV (□), −20 mV (○), −40 mV (○)

The mean single channel current-voltage (I–V) relation measured under these conditions (Fig. 1 B) is linear, with a slope conductance of 233 picoSiemens (pS) and a reversal potential close to 0 mV. The mean single channel I–V relation measured with 250 mmol/l KCl cis and 50 mmol/l KCl trans is also shown in Figure 1 B. The reversal potential of −34.5 mV was close to the calculated K^+-equilibrium potential (−40 mV) and indicates that the channel strongly selects K^+ over Cl^-: the permeability ratio, P_{Cl}/P_K, was 0.046.

Under quasi-physiological ionic conditions (cis: 140 mmol/l KCl and trans: 135 mmol/l NaCl, 5 mmol/l KCl), the slope conductance between −20 mV and +20 mV was reduced to 149 pS (Fig. 1 B). However, the single-channel I–V relation is no longer linear and was better fit by the Goldman-Hodgkin-Katz equation:

$$I = \frac{P_K VF^2}{RT}\left\{\frac{([K]_{cis} - [K]_{trans}\exp(VF/RT))}{(1-\exp(VF/RT))}\right\} + \frac{P_{Na} VF^2}{RT}\left\{\frac{([Na]_{cis} - [Na]_{trans}\exp(VF/RT))}{(1-\exp(VF/RT))}\right\} \quad (1)$$

where F is the Faraday, R is the gas constant and T is the absolute temperature, V is the membrane potential and I is the current. P_K and P_{Na} are the permeability to K^+ and Na^+, respectively. $[K]_{trans}$ and $[K]_{cis}$ are the trans and cis K^+ concentrations, respectively and $[Na]_{trans}$ and $[Na]_{cis}$ are the trans and cis Na^+ concentrations, respectively. The continuous line drawn through the open circles in Figure 1 B was fit to equation (1) using a least squares method. The best fit was obtained with a K permeability of 3.42×10^{-13} cm³/s and a P_{Na}/P_K ratio of 0.027. This permeability ratio indicates that the channel is considerably less permeable to Na^+ than K^+.

In symmetrical 140 mmol/l KCl solutions the K permeability was 4.25×10^{-13} cm³/s and with 250 mmol/l KCl cis and 50 mmol/l KCl trans, the K permeability was 3.66×10^{-13} cm³/s.

Effects of calcium and voltage

Channel activity was dependent on both Ca^{2+} and voltage. The effect of membrane potential on single channel currents and the probability of the channel being open (open probability) is shown in Figure 2. Making the cis potential more positive increased the channel open probability: at a cis Ca^{2+} concentration of 16 μmol/l, some channel activity was evident at −40 mV and the open probability was half maximal at about +20 mV.

As the cis Ca^{2+} concentration was raised, the relationship between the open probability of the channel and membrane potential shifted to more negative membrane potentials, so that at any given potential channel activity was greater. The opposite effect was found when the cis Ca^{2+} concentration was lowered and no channel activity was observed with 0.25 μmol/l Ca^{2+} even at +40 mV.

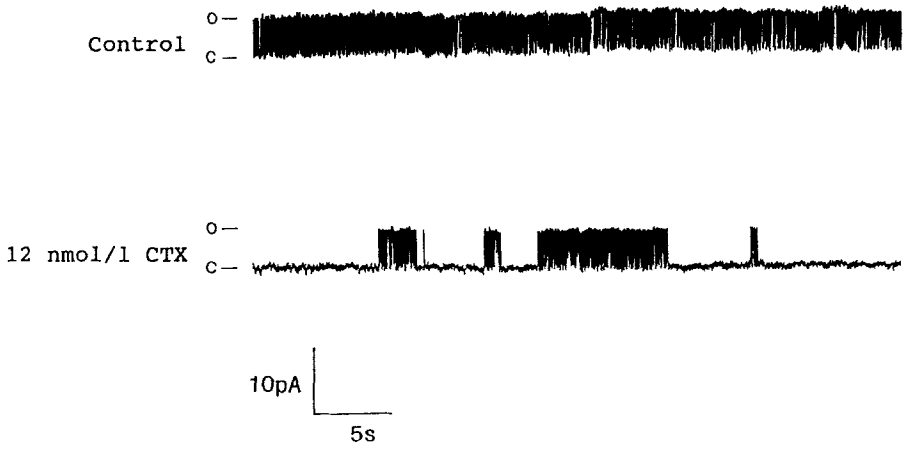

Fig. 4. Effect of charybodotoxin (CTX) on the Ca^{2+}(cis)-activated K^+ channel. 12 nmol/l CTX was added to the trans side. Membrane voltage, +20 mV. C and O indicate the closed and open levels respectively. cis: 140 mmol/l KCl, 15.6 μmol/l Ca^{2+}. trans: 140 mmol/l KCl

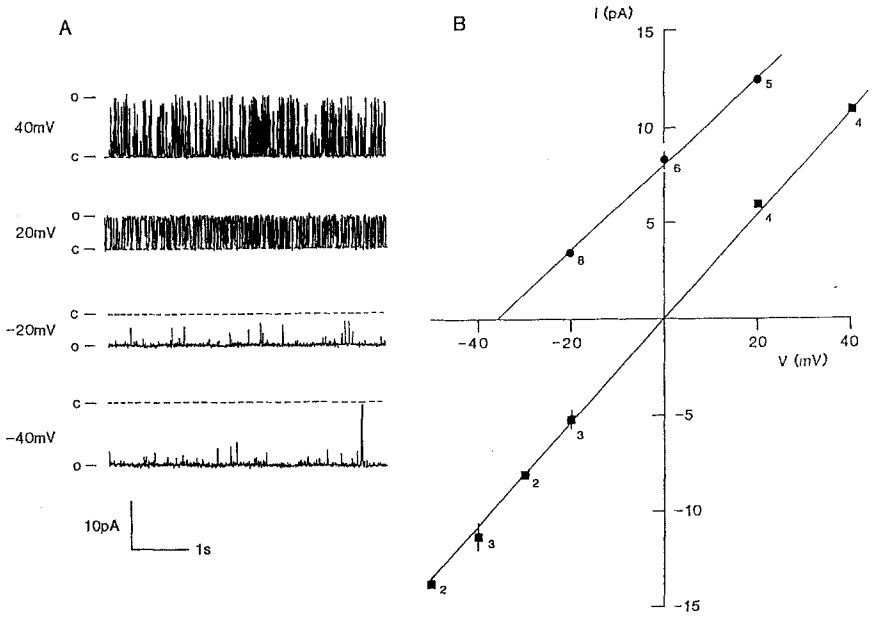

Fig. 5 A, B. Single channel currents and current-voltage (I–V) relations of the Ca^{2+}(trans)-activated K^+ channel. **A** Ca^{2+}(trans)-activated K^+ channel currents recorded at different membrane voltages (indicated to the left of each trace). cis: 140 mmol/l KCl, 1 mmol/l $CaCl_2$; trans: 140 mmol/l KCl, 1 mmol/l $CaCl_2$. **B** Mean single channel I–V relation. (■) cis: 140 mmol/l KCl, 1 mmol/l $CaCl_2$; trans: 140 mmol/l KCl. (●) cis: 250 mmol/l KCl, 1 mmol/l $CaCl_2$; trans: 50 mmol/l KCl. The numbers adjacent to each point indicate the number of bilayers. The vertical lines indicate ± SEM. The lines have slopes of 225 pS (●), and 269 pS (■)

Figure 3A shows single channel currents at +20 mV with different cis Ca^{2+} concentrations. The channel open probability is plotted as a function of cis Ca^{2+} concentration in Figure 3B, for four different membrane potentials. At a membrane potential of 20 mV, the open probability is half-maximal in ~16 μmol/l Ca^{2+}. Depolarisation shifts the relationship between open probability and Ca^{2+} to lower Ca^{2+} concentrations. Conversely, hyperpolarization reduces the Ca^{2+}-sensitivity.

The sensitivity of the channel to cis calcium and to cis positive potentials suggests that the intracellular end of the protein faces the cis solution and extracellular end faces the trans solution.

Blocking by charybdotoxin

Charybdotoxin (CTX) is a potent blocker of high conductance Ca^{2+}-activated K^+ channels [5, 17, 18]. Figure 4 shows that 12 nmol/l CTX greatly reduced the channel open probability when added to the side opposite to that at which Ca^{2+} activated the channel. CTX induced a long-lived closed state, which separates the channel openings into bursts.

Ca^{2+}(trans)-activated K^+ channel

As discussed, the Ca^{2+}-activated K^+ channel was also able to incorporate into the bilayer with its intracellular side facing the trans side of the bilayer. In this case, channel activity was not influenced by the addition of EGTA to the cis side but was sensitive to variation of the trans Ca^{2+} concentration.

Figure 5A shows single channel currents recorded at different membrane potentials in symmetrical 140 mmol/l KCl solutions in the presence of a trans free calcium concentration of 1 mmol/l. The mean I–V relation, given in Figure 5B, reverses at 0 mV and has a slope conductance of 269 pS.

The mean reversal potential shifted to −36.0 mV when the cis solution contained 250 mmol/l KCl, 1 mmol/l $CaCl_2$ and the trans solution contained 50 mmol/l KCl (Fig. 5B) giving a permeability ratio, P_{Cl}/P_K, of 0.031. The mean single channel conductance with these solutions was 225 pS.

The Ca^{2+} sensitivity of the channel resembled that of the Ca^{2+}(cis)-activated K^+ channel. Likewise, positive potentials applied to the Ca^{2+}-activated side of the bilayer

increased the channel open probability (i.e., cis hyperpolarization). Together with the similarity of the single channel conductance and the K^+-selectivity, these results confirm our idea that this channel is the Ca^{2+}(cis)-activated K^+ channel with a different orientation in the bilayer.

Discussion

Ca^{2+}-activated K^+ channels have already been reported in patch clamp experiments on the insulin-secreting cell lines HIT T15 [12] and RINm5F [19] and in mouse [3] and rat pancreatic Beta cells [20–22]. The single channel conductance of ~230 pS found in our studies falls within the range of 211–250 pS reported in these previous studies (measured in symmetrical 140–150 mmol/l KCl solutions).

The reported Ca^{2+}-sensitivity of Ca^{2+}-activated K^+ channels in Beta cells, measured in patch clamp experiments, varies both between species and also from patch to patch. When measured in inside-out patches excised from HIT cell membranes, channel activity at 0 mV was half-maximal at about 1 μmol/l Ca^{2+} [12]. In our observations the calcium sensitivity was somewhat lower: $K_{0.5}$ = ~16 μmol/l at 20 mV. One possible reason may be that our solutions did not contain Mg^{2+}, whereas in the patch clamp experiments, the intracellular solution contained 1.1 mmol/l Mg^{2+} [12]. Mg^{2+} is known to increase the Ca^{2+}-sensitivity of Ca^{2+}-activated K^+ channels [23]. The Ca^{2+}-sensitivity we measure is still greater than that found in mouse Beta cells [20–22], however, which may explain why the Ca^{2+}-activated K^+ current contributes a larger fraction of the delayed outward current in HIT cells [2].

In summary, we have shown that the properties of Ca^{2+}-activated K^+ channels from HIT cell membranes, incorporated into planar lipid bilayers, do not differ significantly from those reported for these channels in native membranes. This suggests that reconstitution of Ca^{2+}-activated K^+ channels in planar lipid bilayers may be used as a functional assay to follow the channel during protein purification.

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