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Strong Voltage-Dependent Inward Rectification of Inward Rectifier K^+ Channels Is Caused by Intracellular Spermine

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Summary

Inward rectifier K^+ channels mediate the K^+ conductance at resting potential in many types of cell. Since these K^+ channels do not pass outward currents (inward rectification) when the cell membrane is depolarized beyond a trigger threshold, they play an important role in controlling excitability. Both a highly voltage-dependent block by intracellular Mg^{2+} and an endogenous gating process are presently assumed to underly inward rectification. It is shown that strong voltage dependence of rectification found under physiological conditions is predominantly due to the effect of intracellular spermine. Physiological concentrations of free spermine mediate strong rectification of IRK1 inward rectifier K^+ channels even in the absence of free Mg^{2+} and in IRK1 mutant channels that have no endogenous rectification.

Introduction

Inward rectifier potassium (K^+) channels maintain the membrane potential (E_M) near the K^+ reversal potential (E_K), in excitable and nonexcitable cells. These channels mediate a high K^+ conductance at E_K and at voltages slightly positive to E_K, which decreases when the membrane is further depolarized. Thus, inward rectifier K^+ channels have a highly stabilizing effect on E_M around E_K but allow depolarization of the cell (Hille, 1992).

The range of membrane potential over which inward rectifier K^+ channels stabilize E_M basically depends on their voltage dependence of rectification, which may be strong or mild (Hille, 1992). Strong inward rectifier K^+ channels have been described in a wide variety of cells, including skeletal muscle cells (Leech and Stanfield, 1981), cardiac muscle cells (Kurachi, 1985; Vandenberg, 1987; Matsuda, 1991), starfish egg cells (Hagiwara et al., 1976), endothelial cells (Silver and DeCoursey, 1990), and osteoclasts (Kelly et al., 1992). In the current–voltage relation (I–V) of strong inward rectifier channels, the outward current exhibits a maximum at potentials close to E_K that is followed by a region of negative slope conductance at more positive potentials. This outward current maximum is physiologically important, since it determines a trigger threshold of excitation: whenever a depolarizing current exceeds the maximal outward current, the inward rectifier K^+ channels close down, and E_M is free to change (Hille, 1992).

Both a highly voltage-dependent block by intracellular magnesium (Mg^{2+}) and an endogenous channel gating process have been supposed to underlie strong inward rectification (Vandenberg, 1987; Ishihara et al., 1989; Silver and DeCoursey, 1990; Matsuda, 1991; Elam and Lansman, 1993). Estimates for the degree of inward rectification of strong inward rectifiers were higher (Kurachi, 1985; Silver and DeCoursey, 1990) than voltage dependence found for internal Mg^{2+} block (Matsuda, 1991). This favors a gating mechanism based on a polyvalent gating charge over a simple pore block by Mg^{2+}. Recently, an unknown substance called IR (for inward rectifier substance), secreted from Xenopus oocytes, was described (Lopatin et al., 1994) that caused voltage-dependent rectification in cloned inward rectifier K^+ channels, suggesting Mg^{2+}-independent intracellular block as a third mechanism of strong inward rectification.

In a recent publication (Fakler et al., 1994b), we showed that cloned inward rectifier K^+ channels are differentially blocked by the tetravalent polyamine spermine (SPM). Sensitivity of one mild and two strong inward rectifier K^+ channels to SPM applied to their cytoplasmic side was tested in symmetrical K^+ concentrations: IRK1 (Kubo et al., 1993) was most sensitive, being half maximally blocked at an inhibitory concentration or IC_{50} of 31 nM SPM at 50 mV membrane potential. BIR10 (Bond et al., 1994) showed a slightly lower blocking affinity (IC_{50} of 40 nM at 50 mV). ROMK1 (Ho et al., 1993) was more than four orders of magnitude less sensitive to SPM (IC_{50} of 0.78 mM at 50 mV), correlating well with its mild rectification (Ho et al., 1993; Lu and MacKinnon, 1994; Taglialatela et al., 1994) and suggesting that SPM may play a role in strong inward rectification.

Here we report evidence for an intracellular block by SPM as the major physiological mechanism of strong inward rectification of IRK1 inward rectifier K^+ channels.

Results

Strong Inward Rectification of IRK1 Channels Is Not Due to an Intracellular Mg^{2+} Block

The physiological function of strong inward rectification of inward rectifier K^+ channels (Hagiwara et al., 1976; Silver and DeCoursey, 1990; Kelly et al., 1992) is illustrated in Figure 1A. When cell-attached patches on a Xenopus oocyte expressing IRK1 channels were examined under current–clamp conditions, voltage responses to current pulses showed a sudden increase in amplitude when the voltage reached the trigger threshold of these inward rectifier K^+ channels (Figure 1A, left trace). This effect was not present in the same patch after excision into the inside-out configuration and exposure to a solution containing 1 mM Mg^{2+} (Figure 1A, right trace).

The changes of channel properties underlying this difference were further studied under voltage–clamp conditions. Figure 1B shows steady-state I–V relations of IRK1 channels in a cell-attached patch (solid lines) that was then

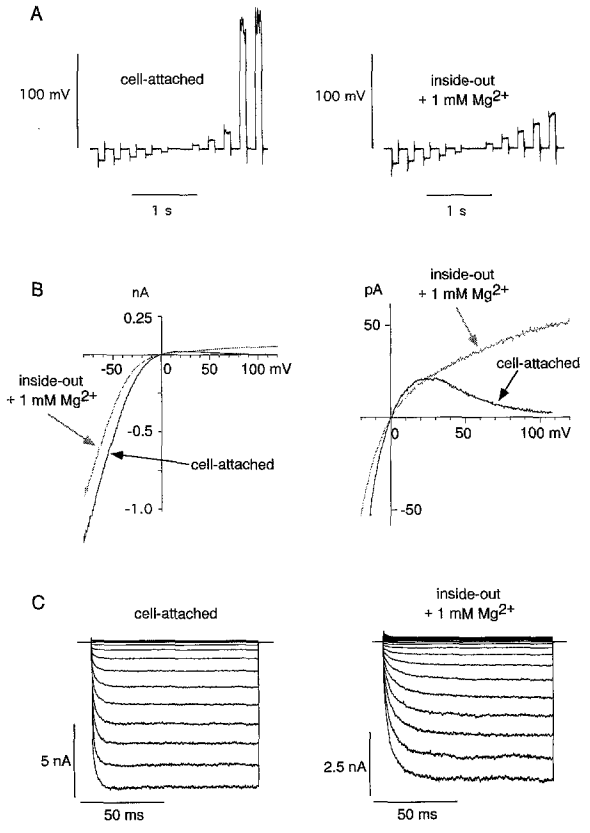

Figure 1. Properties of IRK1 Inward Rectifier K^+ Channels Are Different in Cell-Attached Patches and in Inside-Out Patches Exposed to 1 mM Mg^{2+} ($K\text{-}Int_{1.0\ Mg}$)

(A) Current–clamp recordings in a patch, cell-attached (left trace) and after excision (right trace). At zero current, the membrane potential was 0 mV. Current steps went from −50 pA to 50 pA in increments of 10 pA. Depolarizing currents resulted in a sudden increase of the voltage response when the membrane potential exceeded a threshold of about 25 mV in the cell-attached patch, while the respective voltage response in the excised patch increased almost linearly with current.

(B) Steady-state I–V relations determined under voltage–clamp conditions in response to voltage ramps of 5 s (from −80 mV to 120 mV) on two different scales. I–Vs were different in cell-attached (solid traces) and inside-out configuration (dotted traces) of the same patch.

(C) Current responses to voltage steps going from 0 mV to voltages from −100 mV to 100 mV in increments of 10 mV. Note the different rise times of the inward currents measured in cell-attached and inside-out configurations.

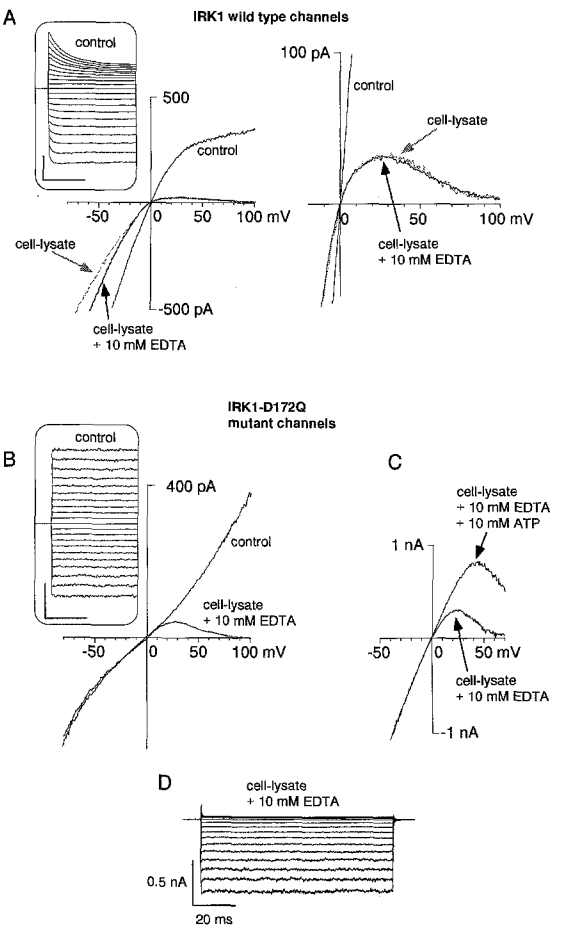

Figure 2. Effect of Cell Lysate Prepared from Xenopus Oocytes on IRK1 Wild-Type and IRK1(D172Q) Mutant Channels

(A) I–V relations determined as in Figure 1B, measured in an inside-out patch with IRK1 wild-type channels exposed to $K\text{-}Int_{0\ Mg}$ (control) and to cell lysate in the absence (dotted line) and presence (solid line) of 10 mM EDTA (right traces: enlarged scale). Inset illustrates endogenous voltage-dependent gating in $K\text{-}Int_{0\ Mg}$. Scale bars represent 5 nA and 20 ms.

(B) I–V relations measured in an excised patch with IRK1(D172Q) mutant channels exposed to $K\text{-}Int_{0\ Mg}$ (control) and to cell lysate with 10 mM EDTA. Inset illustrates lack of endogenous gating of the mutant channels in $K\text{-}Int_{0\ Mg}$. Scale bars represent 2 nA and 20 ms.

(C) I–V relations measured in an excised patch with IRK1(D172Q) mutant channels exposed to cell lysate with 10 mM EDTA and to cell lysate with 10 mM EDTA plus 10 mM ATP. Strong voltage-dependent rectification is reduced by ATP.

(D) Current responses to voltage steps as in Figure 1C, measured in a patch with IRK1(D172Q) mutant channels exposed to Mg^{2+}-free cell lysate.

excised in 1 mM Mg^{2+} (dotted lines); both I–Vs are shown on two different current scales. While under cell-attached conditions the I–V showed a current maximum at about 25 mV, the I–V measured in the inside-out configuration showed a monotonic increase of current over the whole voltage range. This experiment revealed that the voltage dependence of rectification in the cell-attached patch was 2-fold higher, visualized by a negative slope conductance, than in 1 mM Mg^{2+} (18.3 ± 0.7 mV, n = 5, and 37.5 ± 3.0 mV, n = 5 were needed for an e-fold decrease in conductance, respectively).

In addition, the time dependence of inward rectification was altered following patch excision into 1 mM Mg^{2+} (Figure 1C). Current responses to voltage steps from 0 mV to −100 mV had an instantaneous component (IC) of 55% ± 4% (mean ± SD) and an exponential component characterized by a time constant (τ) of 1.72 ± 0.34 ms (n = 10) in cell-attached patches, while in 1 mM Mg^{2+}, the respective values were 37% ± 8% and 5.23 ± 0.99 ms (n = 7).

These results indicate that for IRK1 channels, Mg^{2+} block is not sufficient to explain the inward rectification seen in cell-attached patches.

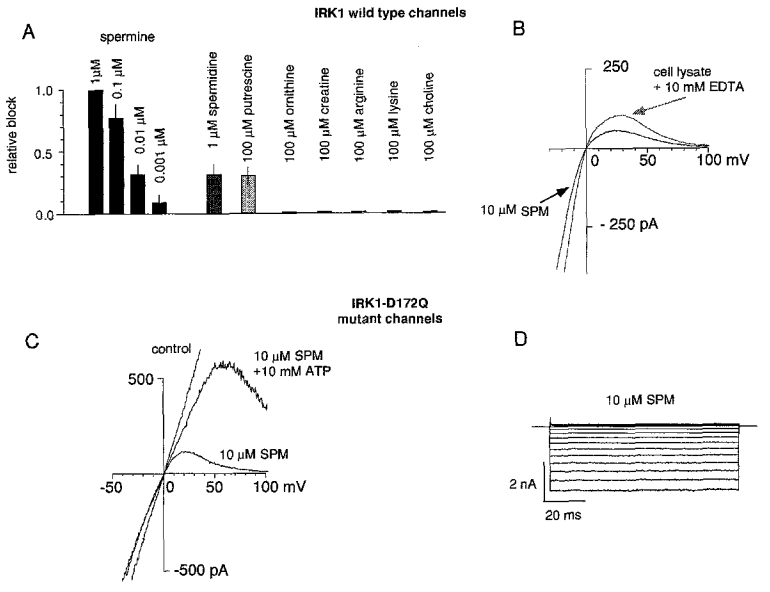

Figure 3. Polyamines Induce Strong Voltage-Dependent Block of IRK1 Wild-Type and IRK1(D172Q) Mutant Channels

(A) In IRK1 wild-type channels, the polyamines SPM, SPD, and putrescine induced a significant block of steady-state current (given as mean ± SD) at a membrane potential of 50 mV (1 μM SPM, 100% block; 0.1 μM SPM, 77% ± 10% block; 0.01 μM SPM, 31% ± 8% block; 0.001 μM SPM, 9% ± 6% block, n = 3; 1 μM SPD, 31% ± 8% block, n = 6; 100 μM putrescine, 30% ± 7% block, n = 5), while other organic cations had no blocking effect (n = 3).

(B) Steady-state I–V relations measured in an excised patch with IRK1 wild-type channels exposed to cell lysate with EDTA (dotted line) or 10 μM SPM (solid line).

(C) Steady-state I–V relations measured in an excised patch with IRK1(D172Q) mutant channels exposed to K-Int$_{0\ Mg}$, 10 μM SPM, or 10 μM SPM plus 10 mM ATP.

(D) Current responses of IRK1(D172Q) channels exposed to 10 μM SPM in voltage steps as in Figure 1C. The voltage-dependent block is virtually instantaneous.

Mg^{2+}-Free Cell Lysate Mediates Strong Inward Rectification of IRK1 Channels

Exposure of an excised patch to Mg^{2+}-free solution revealed a weak endogenous voltage dependence of conductance (about 100 mV for an e-fold decrease in conductance; Figure 2A, control and inset). When a cell lysate prepared from Xenopus oocytes (see Experimental Procedures) was applied to the cytoplasmic side of the excised patch, the strong voltage dependence of rectification was completely restored (17.3 ± 0.3 mV for an e-fold decrease in conductance, n = 8; Figure 2A, dotted line). Similar results as for the lysate were obtained when an excised patch was crammed back into the oocyte (data not shown). Strong voltage dependence of rectification was also restored by cell lysate to which the Mg^{2+}-chelator EDTA (10 mM) was added (17.9 ± 1.1 mV, n = 11; Figure 2A, solid line). This indicates that the cytoplasm contains a factor that is able to induce strong voltage dependence of rectification also in the absence of free Mg^{2+}. This factor might either potentiate voltage dependence of endogenous rectification due to channel gating or represent a positively charged blocking particle mediating a highly voltage-dependent channel block.

To discriminate between these hypotheses, Mg^{2+}-free cell lysate was applied to IRK1(D172Q) mutant channels (Stanfield et al., 1994), known to lack voltage-dependent channel gating (Stanfield et al., 1994) (Figure 2B, inset). Similarly to wild-type channels, IRK1(D172Q) mutant channels exhibited strong voltage dependence of inward rectification in Mg^{2+}-free lysate (17.6 ± 1.3 mV, n = 5; Figure 2B). This strongly suggests that endogenous gating is not necessary for strong rectification caused by the cytoplasmic factor. Rectification induced by the lysate in mutant channels was virtually instantaneous (Figure 2D), suggesting that the cytoplasmic factor responsible for strong voltage dependence of rectification is a pore blocker of IRK1 channels rather than a modulator.

Since voltage dependence of the block by lysate is about 2-fold higher than for divalent Mg^{2+}, the cytoplasmic blocker may have a higher valence or a different binding site located deeper in the channel pore than for Mg^{2+}. The suggestion of a higher valence was supported by the result that the tetravalent ATP (10 mM) reduced the activity of the cytoplasmic blocker (Figure 2C, n = 3), while the divalent chelators EGTA and EDTA (10 mM), which form complexes with di- and trivalent metal ions (Gutteridge et al., 1990; Cho and Geum-Yi, 1991), failed to have an effect. Thus, the cytoplasmic blocker is most likely a polyvalent organic cation rather than a di- or trivalent metal ion.

Strong Rectification Induced by Cell Lysate Is Predominately Due to Spermine

Since strong binding to ATP is well known for the tetra- and trivalent polyamines SPM and spermidine (SPD) (Watanabe et al., 1991), and block of IRK1 channels by SPM has already been reported (Fakler et al., 1994b), we compared SPM sensitivity of IRK1 wild-type channels at 50 mV membrane potential with their sensitivity to other intracellular organic cations (Figure 3A). While SPM has a half-maximal block at a concentration of 31 nM (Fakler et al., 1994b), which is much lower than the intracellular concentration of free SPM (8.2–75.7 μM free, 0.88–1.57 mM total concentration, values according to Watanabe et al., 1991), the affinity for SPD was about 100-fold lower. Putrescine, a precursor of SPD and SPM, was again 100-fold less effective than SPD and ornithine, a precursor of putrescine, and all other intracellular cations tested had negligible effects.

SPM in a concentration of 10 μM, which is close to the lower limit of the physiological range of free SPM, reason-

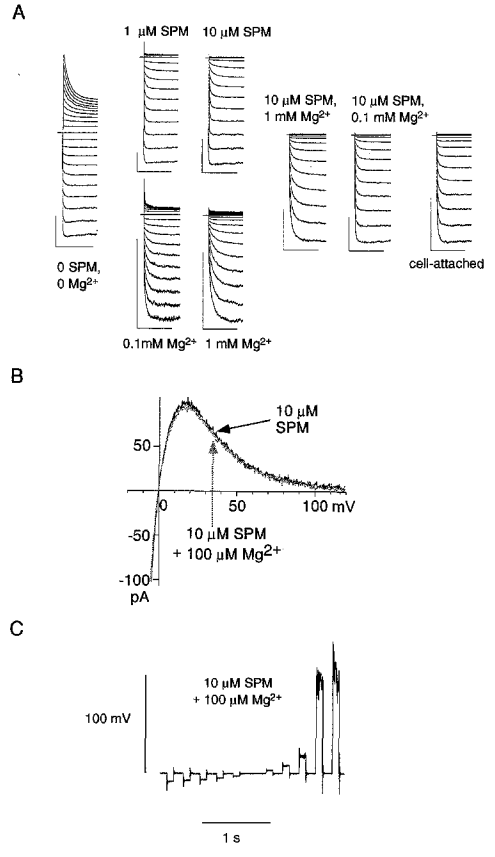

Figure 4. Properties of IRK1 Wild-Type Channels as Determined in Cell-Attached Patches Can Be Mimicked in Inside-Out Patches Exposed to 10 μM SPM, Together with 0.1 mM Mg^{2+} ($K\text{-}Int_{0.1\ Mg}$)

(A) Onset of current in response to voltage steps going from 0 mV to potentials between −100 mV and 100 mV in increments of 10 mV under various conditions as indicated. Scale bars represent 5 nA and 30 ms.

(B) I–V relations determined as in Figure 1B, measured in a giant patch exposed to 10 μM SPM, showed identical outward currents in the presence (dotted line) and absence (solid line) of 100 μM Mg^{2+} ($K\text{-}Int_{0.1\ Mg}$).

(C) Voltage response to current steps as in Figure 1A showed a sudden increase when the membrane was depolarized beyond a trigger threshold of about 25 mV.

ably mimicked the high voltage dependence of rectification (wild-type channels, 18.0 ± 0.6 mV, n = 7, Figure 3B; IRK1(D172Q) mutant channels, 17.4 ± 1.4 mV, n = 7, Figure 3C) as induced by Mg^{2+}-free cell lysate (Figure 2). As also shown for the cell lysate, addition of 10 mM ATP to the 10 μM SPM solution reduced the blocking effect of SPM in IRK1(D172Q) channels (Figure 3C). Moreover, voltage-dependent rectification induced by 10 μM SPM in IRK1(D172Q) was virtually instantaneous (Figure 3D), similar to that shown for the Mg^{2+}-free lysate in Figure 2D. This strongly suggests that SPM might indeed be the cytoplasmic blocker in the Mg^{2+}-free cell lysate.

Since in the physiological situation the cytoplasmic blocker acts in the presence of Mg^{2+}, we tested the time dependence of inward rectification of IRK1 wild-type channels under various combinations of SPM and Mg^{2+}. The series of experiments shown in Figure 4A revealed that

Table 1. Time Constants and Values for the Instantaneous Current Component Determined by a Monoexponential Fit to the Onset of Inward Current at a Voltage Step from 0 mV to −100 mV

Condition	τ (ms)	IC (%)	n
0 mM Mg^{2+}, 0 mM SPM	1.23 ± 0.27	81 ± 2	7
1 μM SPM	1.03 ± 0.54	73 ± 7	3
10 μM SPM	0.87 ± 0.25	65 ± 4	3
0.1 mM Mg^{2+}	5.56 ± 2.05	58 ± 6	6
1.0 mM Mg^{2+}	5.23 ± 0.99	37 ± 8	7
1.0 mM Mg^{2+}, 10 μM SPM	4.76 ± 1.78	47 ± 1	3
0.1 mM Mg^{2+}, 10 μM SPM	1.30 ± 0.55	64 ± 6	6
Cell-attached	1.72 ± 0.34	55 ± 4	10

Time constants, τ, given in milliseconds. Values for the instantaneous current component, IC, given in percentage of the steady-state current.
[a] τ and IC are mean ± SD of n experiments.

the rising phase of inward currents and the outward current transients measured under 10 μM SPM together with 0.1 mM Mg^{2+} were most similar to the results obtained from cell-attached patches (fit results are given in Table 1). At a lower SPM concentration of 1 μM, outward currents became clearly more pronounced than in the cell-attached measurement, while at a higher Mg^{2+} concentration of 1 mM, the inward current increase became significantly slower (Figure 4A; Table 1). Therefore, 10 μM SPM with 0.1 mM Mg^{2+} represents a possible estimate of the intracellular free concentrations, although we cannot exclude higher free SPM in combination with higher Mg^{2+} concentrations. Figure 4B shows that 10 μM SPM applied to excised patches in the presence of 0.1 mM Mg^{2+} induced a strong voltage dependence of rectification (Figure 4B, dotted line, 16.8 ± 0.3 mV, n = 4), which was again as high as in cell-attached patches or in cell lysate and significantly higher than for 10 μM SPD with 0.1 mM Mg^{2+} (21.1 ± 0.3 mV, n = 4). Moreover, rectification appeared virtually unchanged when Mg^{2+} was completely removed from the same patch (Figure 4B, solid line), similar to that shown for the cell lysate with 10 mM EDTA (see Figure 2A, solid line).

As a consequence of the complete restoration of strong voltage dependence of inward rectification induced by 10 μM SPM and 0.1 mM Mg^{2+}, current-clamp experiments revealed a similar trigger threshold for depolarizing currents (Figure 4C) as in cell-attached patches (see Figure 1A).

Discussion

Our results establish that strong voltage-dependent inward rectification of IRK1 channels is largely based on intracellular SPM. SPM is effectively bound to ATP (Watanabe et al., 1991) but not to EDTA, similar to the intracellular blocker present in the cell lysate. It is 100 times more potent than the nearest related polyamine, SPD, and only SPM induces voltage dependence of rectification that is as strong as that measured in cell-attached patches.

The higher voltage dependence of rectification induced by SPM compared with SPD is most likely due to the higher valence of the tetravalent SPM compared with the trivalent

SPD. The mechanism by which SPM induces strong voltage-dependent inward rectification is presumably a simple pore block of the channel, since the block was very fast and had an unchanged voltage dependence in channels of IRK1(D172Q), a mutant that lacks voltage-dependent gating (Stanfield et al., 1994). Correspondingly, the electrical distances (δ) calculated from valence and voltage-dependent decrease of conductance were similar for 1 mM Mg^{2+} (δ = 0.33), SPD (δ = 0.39), and SPM (δ = 0.37). However, these electrical distances are difficult to compare with data from the literature, since their absolute values depend on the method of determination (Silver and DeCoursey, 1990).

The high sensitivity of IRK1(D172Q) channels to SPM block (IC_{50} of about 500 nM at 50 mV; data not shown) is remarkable, because a corresponding mutation in BIR10 channels (Fakler et al., 1994b) caused an almost complete loss of SPM sensitivity. This suggests that SPM sensitivity of IRK1 and BIR10 channels may be based on different structural determinants. Nevertheless, BIR10 wild-type channels are similarly sensitive to SPM (Fakler et al., 1994b) as IRK1 channels and also exhibit a strong voltage dependence of SPM block (data not shown).

The time constants of inward current increase measured in the presence of various concentrations and combinations of SPM and Mg^{2+} were used as a test for the possible blocker concentrations in cell-attached patches. The results of fitting presented in Table 1 suggested 10 μM free SPM plus 100 μM free Mg^{2+} as reasonable estimates for the intracellular concentrations in Xenopus oocytes. In contrast with Mg^{2+}, which markedly slowed down the inward current increase, SPM did not significantly change the corresponding time constant. In the experiment with 10 μM SPM plus 100 μM Mg^{2+}, SPM could even mask this effect of Mg^{2+} on the time constant. A higher intracellular concentration of 1 mM free Mg^{2+} induced a slow time constant of about 5 ms, also in the presence of 10 μM SPM, which was never observed in cell-attached patches. The mechanism by which this time constant is slowed down by Mg^{2+} was not further investigated. It might either reflect an effect of Mg^{2+} on endogenous gating of the channels or a particular slow unbinding of Mg^{2+} in IRK1 wild-type channels. Such a slow time constant was absent in IRK1(D172Q) channels, which favors the first hypothesis.

Inside cells, most of the intracellular SPM is bound to nucleotides, but nevertheless, free SPM is present in micromolar concentrations (Watanabe et al., 1991). Therefore, changes in nucleotide concentrations, as for example in ATP concentration, will most likely induce changes in free SPM. This adds another potential mechanism to the regulatory pathways already shown for IRK1 channels (Fakler et al., 1994a). Thus, SPM block determines the trigger threshold for depolarizing currents, similar to the external Mg^{2+} block in N-methyl-D-aspartic acid (NMDA) glutamate receptors (Ruppersberg et al., 1994). Polyamines are already known to inhibit cell proliferation (Heby, 1981) and to regulate protein synthesis (Giannakouros et al., 1990). Voltage-dependent block of K^+ channels represents a further regulatory function for polyamines that probably contributes to control of cellular excitability.

Experimental Procedures

Mutagenesis and cRNA Synthesis

The IRK1(D172Q) mutant was prepared according to the protocols of Herlitze and Koenen (1990) and subcloned into a pSP64T-derived vector; the mutation was verified by sequencing.

Capped cRNAs specific for IRK1 (Kubo et al., 1993) and IRK1(D172Q) (Stanfield et al., 1994) mutant channels were synthesized in vitro by use of SP6 polymerase (Promega, Heidelberg, Federal Republic of Germany) and stored in stock solutions at −70°C.

Preparation and Injection of Oocytes

Xenopus oocytes were surgically removed from adult females and manually dissected. About 50 nl of a solution containing cRNA specific for IRK1 wild-type or mutant channels was injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (Sigma, 0.5 mg/ml) and incubated at 19°C for 1–3 days before use.

Electrophysiology

Expression of IRK1 wild-type and mutant channels was tested by recordings using two microelectrodes, and oocytes providing inward rectifying currents of more than 10 μA at a membrane potential of −100 mV were used for patch-clamp experiments. Giant patch recordings (Hilgemann et al., 1991) under voltage- and current-clamp conditions were made at room temperature (approximately 23°C) 3–7 days after injection. Current-clamp experiments in patches were possible when the leakage conductance was less than 1% of the conductance mediated by the expressed channels (Ruppersberg et al., 1991a). Pipettes used were made from thick-wall borosilicate glass, had resistances of 0.3–0.6 MΩ (tip diameter of 20–30 μm), and were filled with 120 mM KCl, 10 mM HEPES, and 1.8 mM $CaCl_2$. Currents were sampled at 10 kHz and corrected for capacitive transients with an EPC9 amplifier (Heka Electronics, Lamprecht, Federal Republic of Germany), with analog filter set to 3 kHz (−3 dB). In Figure 1B, leakage correction of both traces was made by subtracting the asymptotic conductance value obtained from a monoexponential fit to the final 10% of the decay of conductance calculated from the cell-attached recording. The fit provided the voltage (given as mean ± SD) for e-fold decrease in conductance cited in the text.

Block of current by organic cations (given as mean ± SD) was quantified relative to the current recorded cell-attached (100%) and the current measured after complete washout of the intracellular blocker in $K\text{-}Int_{0\ Mg}$ (0%). Solutions were applied to the cytoplasmic side of the excised patches as described (Ruppersberg et al., 1991b) and had the following composition (pH adjusted to 7.2 with KOH, free Mg^{2+} calculated according to Fabiato, 1988): $K\text{-}Int_{1.0\ Mg}$ (120 mM KCl, 10 mM HEPES, 10 mM EGTA, 1.44 mM $MgCl_2$), $K\text{-}Int_{0\ Mg}$ (120 mM KCl, 10 mM HEPES, 10 mM EGTA, 1 mM EDTA), $K\text{-}Int_{0.1\ Mg}$ (120 mM KCl, 10 mM HEPES, 10 mM EGTA, 0.145 mM $MgCl_2$).

K_2ATP, polyamines, amino acids, choline, and creatine were purchased from Sigma (Sigma, St. Louis, MO) and added to $K\text{-}Int_{0\ Mg}$, $K\text{-}Int_{0.1\ Mg}$, or $K\text{-}Int_{1.0\ Mg}$ to yield the final concentrations indicated.

Preparation of Cell Lysate

Lysate from Xenopus oocytes was prepared as follows: the surgically removed ovary was rinsed four times with $K\text{-}Int_{0\ Mg}$, frozen at −70°C overnight, thawed, diluted 1:10 (v/v) in $K\text{-}Int_{0\ Mg}$, and centrifuged for 1 hr at 20,000 U/min. The supernatant was carefully recovered, filtered at 0.2 μm, and transferred to the application system. In some experiments, K_2ATP and EDTA were added to yield the final concentrations indicated.

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