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A structural determinant of differential sensitivity of cloned inward rectifier K^+ channels to intracellular spermine

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Abstract Large subtype-specific differences in the sensitivity of cloned inward-rectifier K^+ channels of the IRK1, BIR10 and ROMK1 subtype to being blocked by intracellular spermine (SPM) are described. It is shown, by site-directed mutagenesis, that the four orders of magnitude larger SPM sensitivity of BIR10 channels compared to ROMK1 channels may be explained by a difference in a single amino acid in the putative transmembrane segment TMII. This residue, a negatively charged glutamate in BIR10, is homologous to the residue in IRK1 and ROMK1 which has previously been shown to change gating properties and Mg^{2+} sensitivity. Differential block by physiological SPM concentrations is suggested as a major functional difference between subtypes of inward-rectifier K^+ channels.

Key words: Inward rectifier; K^+ channel; Clone; Spermine; Site-directed mutagenesis

1. Introduction

Inward-rectifier K^+ channels mediate the resting K^+ conductance in many types of cell [1–12]. Recently several members of the family of inward-rectifier K^+ channels have been cloned and functionally expressed in *Xenopus laevis* oocytes [13–18]. K^+ channels encoded by these clones were shown to differ in their voltage-dependent gating [19,20] and their sensitivity to intracellular Mg^{2+} [21]. Mg^{2+} sensitivity in IRK1 channels could be decreased by mutating the amino acid residue at position 172, located in the putative second transmembrane segment (TMII), from aspartate (D) to glutamine (Q) or asparagine (N) [19]. Correspondingly, in ROMK1 channels, Mg^{2+} sensitivity was increased by a factor of 24 by mutating the homologous residue at position 171 from N to a negatively charged D [21]. Moreover, changing this residue in the IRK1 protein from D to Q or N could completely remove voltage-dependent kinetics in these channels [19], while the degree of inward rectification was not markedly altered [20]. SPM is a potential blocker of cation channels since it is a tetravalent cationic polyamine with an intracellular free concentration of more than 10 μM [22]. Here we show that inward-rectifier K^+ channels of the subtypes IRK1, ROMK1 and BIR10, recently cloned from rat brain [18], dramatically differ in their sensitivity to intracellular SPM, and that a glutamate (E)-to-N mutation in TMII (E158N) of BIR10 channels decreases SPM sensitivity by as much as five orders of magnitude.

2. Materials and methods

Capped cRNAs specific for IRK1 [15], BIR10 [18] and ROMK1 [13] channels were synthesized in vitro using SP6 polymerase, and injected into *Xenopus* oocytes. The BIR10-E158N mutant was prepared according to [23], subcloned into a pSP64T-derived vector and the mutation verified by sequencing. Giant patch-clamp [24] recordings under voltage-clamp conditions were made at room temperature (approximately 23°C) 3–7 days after injection. 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 (in mM) 120 KCl, 10 HEPES and 1.8 $CaCl_2$. Currents were recorded and corrected for capacitive transients with an EPC9 amplifier (HEKA Electronics, Lamprecht, Germany), and filtered at 3 kHz (−3 dB). Solutions had the following composition (in mM, pH of all solutions adjusted to 7.2 with KOH, free Mg^{2+} calculated according to [25]): K-$Int_{0\ Mg}$ (120 KCl, 10 HEPES, 10 EGTA, 1 EDTA), K-$Int_{0.1\ Mg}$ (120 KCl, 10 HEPES, 10 EGTA, 0.145 $MgCl_2$), K-$Int_{1.0\ Mg}$ (120 KCl, 10 HEPES, 10 EGTA, 1.44 $MgCl_2$). SPM was purchased from Sigma (St. Louis, MO) and added to K-$Int_{0\ Mg}$, K-$Int_{0.1\ Mg}$ or to K-$Int_{1.0\ Mg}$ to yield the final concentrations indicated. Cramming in Fig. 2 was performed by penetrating the cell membrane with the patch pipette containing the intact inside-out patch. In the second cram experiment the injury to the cell membrane caused by the first penetration was exploited to avoid a cram artifact visible in the first cramming. Block of current by SPM was quantified relative to the current measured after complete wash-out of the intracellular blocker in K-$Int_{0\ Mg}$ (0%). In Fig. 3 leakage correction of all 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 recording in 1 μM SPM.

3. Results

Fig. 1 shows currents mediated by IRK1 [15] or BIR10 [18] inward-rectifier K^+ channels measured in giant patches in response to symmetrical voltage steps from −50 to 50 mV. When the patches were excised into Mg^{2+}-free solution (K-$Int_{0\ Mg}$) an increase of inward and outward currents was observed. While inward-going currents increased very quickly, the outward currents increased slowly, within about 30 s in all experiments (n = 18). Similarly, after exposing the patch again to the cytoplasm of the oocyte by cramming through the cell membrane [26] currents were again blocked, and outward currents slowly reappeared when the patch was re-exposed to the K-$Int_{0\ Mg}$ solution (Fig. 1A; n = 5). In contrast to this slow increase of outward currents induced by wash-out of cytoplasm, the blocking effect of 1 mM Mg^{2+} on both inward and outward currents was rapidly washed-out in both types of inward-rectifier K^+ channels (Fig. 1A and C, n = 15). These results may be explained by a cytoplasmic blocker with an affinity to the channels that is higher than that of Mg^{2+} and which is therefore

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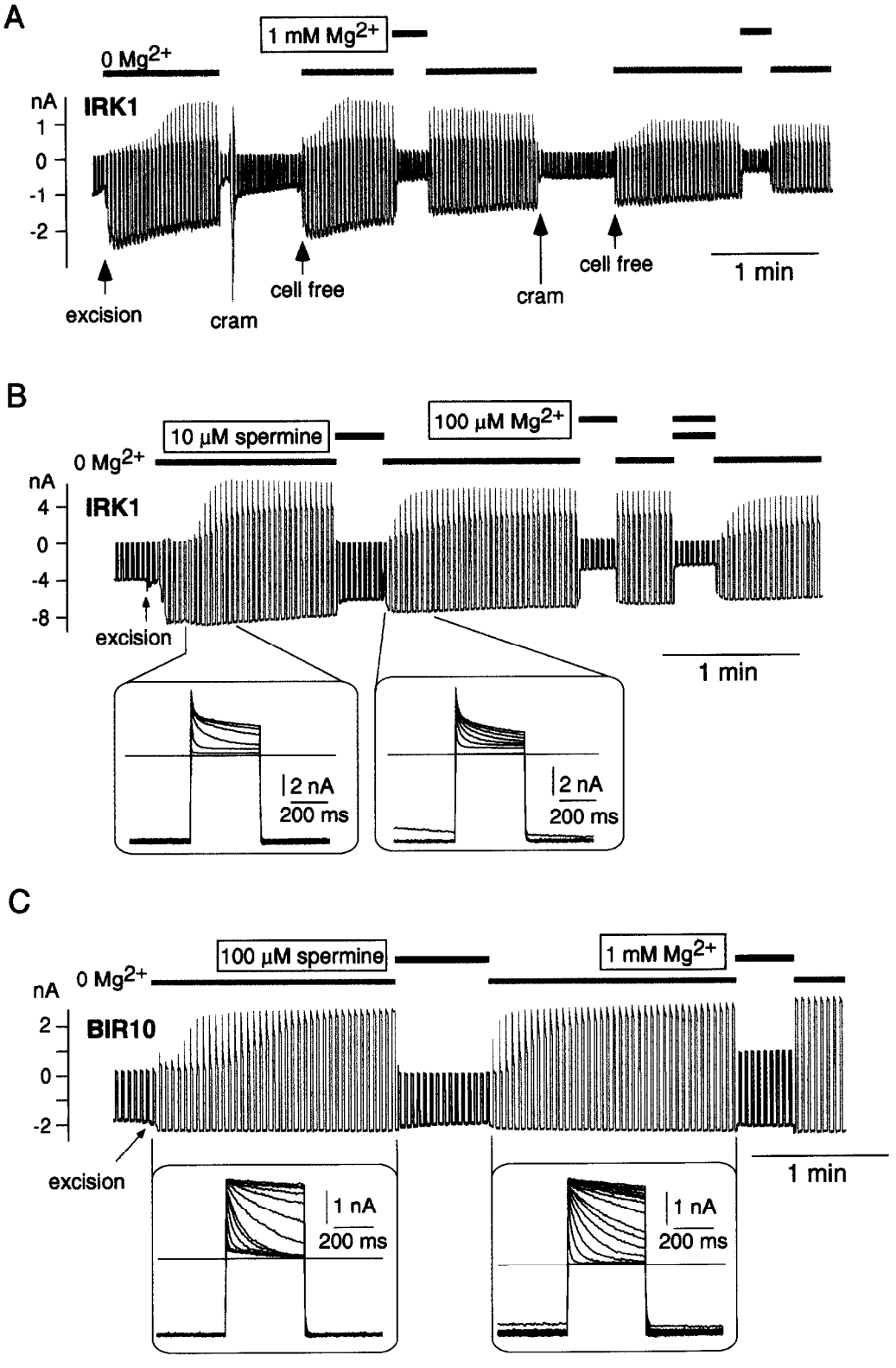

washed-out much more slowly than Mg^{2+}. Although the current increase was comparably slow in both IRK1 and BIR10 channels, exposure to Mg^{2+}-free solution completely removed rectification of BIR10 channels (Fig. 1C, $n = 7$; insets show changes of the time–course of current responses to positive voltage steps), while IRK1 channels still exhibited residual rectification in $K\text{-}Int_{0\ Mg}$ due to endogenous voltage-dependent gating ($n = 24$). As shown in Fig. 1B and C a similarly slow current increase may be induced by wash-out of 10 μM (Fig. 1B) or 100 μM (Fig. 1C) of the intracellular polycation

Fig. 1. Time-dependent increase of outward currents mediated by IRK1 or BIR10 channels following patch excision, wash-out of Mg^{2+} or SPM applied to the cytoplasmic side of the inside-out patch. (A) Currents measured in response to a clamp potential of −50 mV which was stepped to 50 mV for 400 ms every 2.15 s. Outward currents were small under cell-attached conditions but slowly increased after patch excision in Mg^{2+}-free solution ($K\text{-}Int_{0\ Mg}$). This process was reproduced twice by cramming the patch into the cytoplasm and subsequent exposure to $K\text{-}Int_{0\ Mg}$. Outward current block by 1 mM Mg^{2+} ($K\text{-}Int_{1.0\ Mg}$) was rapidly washed-out. (B) Wash-out of SPM-induced outward current block (10 μM SPM) had a similar time-course as the increase of outward current following patch excision both in the presence or absence of 100 μM Mg^{2+}. Wash-out of Mg^{2+} block ($K\text{-}Int_{0.1\ Mg}$) was as fast as in A. The insets illustrate time-courses of outward currents during wash-out at an enlarged time scale, displaying every second trace of the part of the recording as indicated. (C) Similar experiment as in B with BIR10 channels. Slow wash-out of SPM (100 μM) resembles increase of outward current after patch excision. Insets illustrate time-courses of outward currents during wash-out, displaying the first 8 responses and consecutively every 8th response of the part of the recording as indicated.
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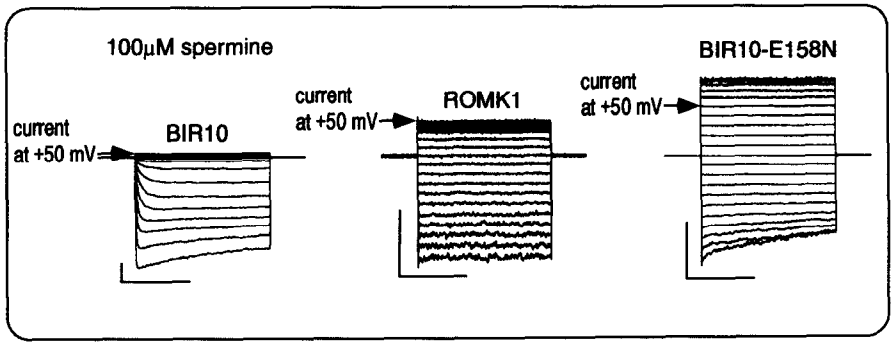

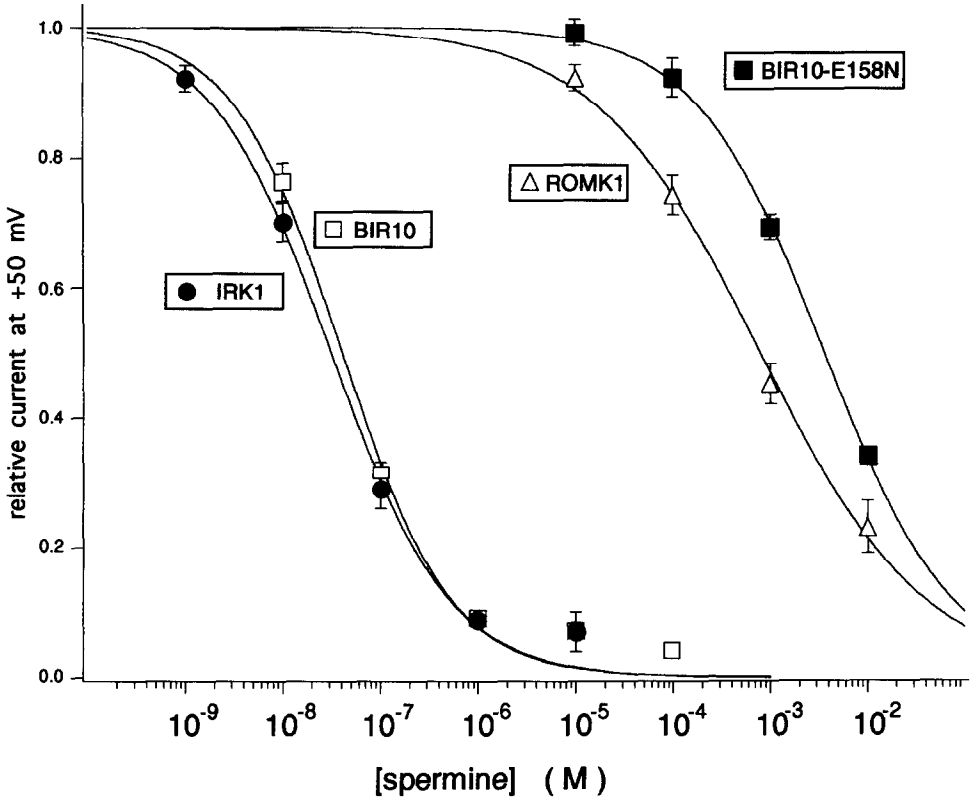

Fig. 2. Dose–response curves of the respective SPM-dependent inhibition of outward currents mediated by IRK1, BIR10, BIR10-E158N and ROMK1 channels, measured at a membrane potential of +50 mV. Each point represents the mean value of 3–8 measurements, error bars give the respective standard deviation. Fits of a logistic function to the data (solid lines) revealed estimates for half maximal inhibition of 31 nM for IRK1, 40 nM for BIR10, 3.5 mM for BIR10-E158N and 0.78 mM for ROMK1. The corresponding estimates for the Hill coefficients were 0.71, 0.78, 0.66 and 0.52, respectively. The inset illustrates differential effects of 100 μM SPM on K^+ currents in response to voltage pulses of 100 ms (going from a holding potential of 0 mM to potentials between −100 mV and 100 mV; bars represent 1 nA and 50 ms) mediated by BIR10, BIR10-E158N and ROMK1 channels. Inward currents were only a little affected by 100 μM SPM (not shown) while outward currents were inhibited differentially (in the absence of SPM, outward currents were almost rectangular and of similar amplitude as the respective inward currents in all three channel types, not shown).

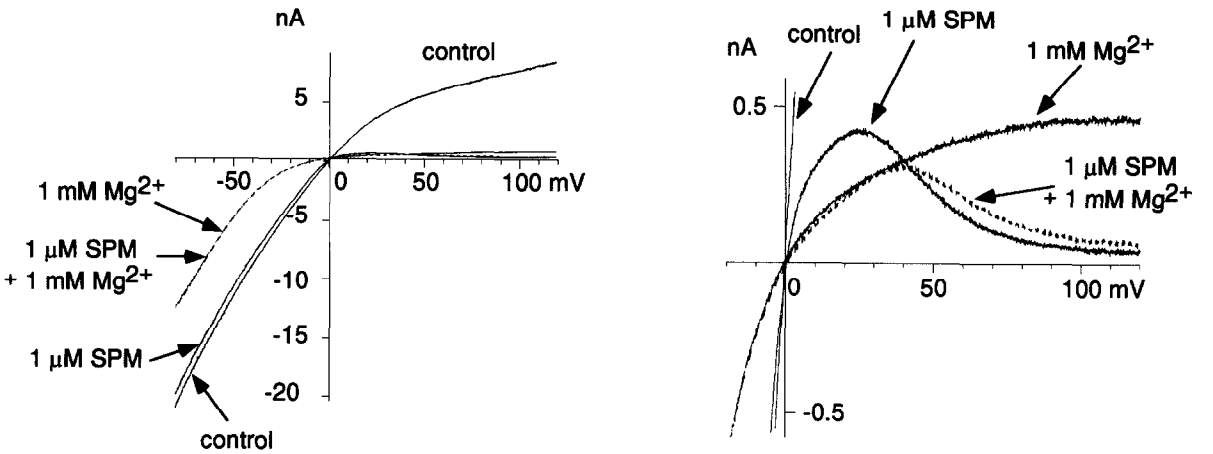

Fig. 3 Interaction of Mg^{2+} and SPM block. *I–V* relations in response to voltage ramps of 5 s (going from −80 to 120 mV) on two different current scales measured in a giant inside-out patch with IRK1 channels. The same patch was either exposed to Mg^{2+}-free ($K\text{-}Int_{0\ Mg}$) solution (control), to 1 mM Mg^{2+} ($K\text{-}Int_{1.0\ Mg}$), 1 μM SPM or to 1 μM SPM plus 1 mM Mg^{2+} (dashed lines). For the *I–V* relation in 1 μM SPM plus 1 mM Mg^{2+}, positive to +40 mV SPM determined the outward current block while negative to +40 mV 1 mM Mg^{2+} determined the outward current amplitude (right traces: enlarged scale); inward currents were only dependent on Mg^{2+} concentration (left traces).

SPM (physiological intracellular concentration: 8.2–75.7 μM free, 0.88–1.57 mM total, values according to [22]), both in the presence and in the absence of Mg^{2+} (Fig. 1B; $n = 9$). We therefore investigated the affinities of three subtypes of inward-rectifier K^+ channels (IRK1, BIR10 and ROMK1) to being blocked by SPM applied to the cytoplasmic side of the excised patches.

Fig. 2 shows dose–response curves for the three channel subtypes and for mutated BIR10 channels (BIR10-E158N) measured at a membrane potential of +50 mV in symmetrical K^+ concentration (original traces at different voltages in the presence of 100 μM SPM are shown in the inset). While the values for half-maximal block (IC_{50}) in IRK1 and BIR10 channels were in the sub-micromolar range (31 nM for IRK1 channels and 40 nM for BIR10 channels) ROMK1 channels were largely insensitive to SPM (IC_{50} of 0.78 mM). Recently Mg^{2+}-sensitivity of ROMK1 channels has been increased 24-fold by a point mutation in TMII introducing negatively charged residues at position 171 [21], where the IRK1 and BIR10 sequences exhibit an aspartate or glutamate residue, respectively. The inverse mutation in the IRK1 (D-to-N) did, however, not substantially alter inward rectification of the resulting mutant channels [19,20]. We now mutated the homologous glutamate residue (amino acid 158) in the BIR10 channel to a neutral asparagine (BIR10-E158N). To our surprise, as shown in Fig. 2, this mutation (BIR10-E158N) caused a loss by as much as five orders of magnitude of sensitivity to SPM (IC_{50} of 3.5 mM).

Since in IRK1 channels affinity to SPM was highest, even much higher than sensitivity for Mg^{2+} (IC_{50} of 2.5 μM at +50 mV, data not shown) we were interested in the interaction between SPM and Mg^{2+} at the blocking site. Fig. 3 shows the steady-state current–voltage (*I–V*) relation of a patch with IRK1 channels between −80 and +120 mV. 1 mM Mg^{2+} and 1 μM SPM were either applied separately or in combination. The curve measured for the combination was similar to that with 1 mM Mg^{2+} at potentials below 40 mV, while at potentials positive to 40 mV, block was predominately due to the thousand-fold lower concentration of SPM. Interestingly, in the voltage range where SPM dominated the block, the current was even larger when Mg^{2+} was added. This suggests a two-step reaction where the competition between SPM and Mg^{2+} occurs one step before either of the cations blocks the channel.

4. Discussion

Polyamines such as SPM comprise an ubiquitous class of intracellular cation, usually present at micro- to millimolar concentrations in cells [22]. High affinity block of inward-rectifier K^+ channels by SPM might therefore represent a potent mechanism of regulating outward currents through channels of this K^+ channel family. Differential sensitivity to SPM found between IRK1, BIR10 and ROMK1 correlates well with the degree of inward rectification observed for the respective channels in cell-attached patches; for example, inward rectification is weak in ROMK1 channels [21]. This difference in rectification, however, may also be due to differences in sensitivity to intracellular Mg^{2+} or to differences in channel kinetics. Nevertheless the mutation in BIR10 which caused almost complete insensitivity to SPM also changed the rectification behaviour of the resulting BIR10-E158N channels which could hardly be distinguished from ROMK1 channels in cell-attached patches (not shown). The interaction between SPM and Mg^{2+} as shown in Fig. 3 suggests that, at least under certain conditions, SPM may dominate the cytoplasmic block of inward-rectifier K^+ channels. Differential block by physiological SPM concentrations may therefore represent an important functional difference between the various inward-rectifier K^+ channel subtypes.

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