$[K^+]$ Dependence of Polyamine-induced Rectification in Inward Rectifier Potassium Channels (IRK1, Kir2.1)

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ABSTRACT The effects of permeant (K^+) ions on polyamine (PA)-induced rectification of cloned strong inwardly rectifying channels (IRK1, Kir2.1) expressed in *Xenopus* oocytes were examined using patch-clamp techniques. The kinetics of PA-induced rectification depend strongly on external, but not internal, K^+ concentration. Increasing external $[K^+]$ speeds up "activation" kinetics and shifts rectification to more positive membrane potentials. The shift of rectification is directly proportional to the shift in the K^+ reversal potential (E_K) with slope factors +0.62, +0.81, and +0.91 for 1 mM putrescine (Put), 100 μM spermidine and 20 μM spermine (Spm), respectively. The time constant of current activation, resulting from unblock of Spm, also shifts directly in proportion to E_K with slope factor +1.1. Increasing internal $[K^+]$ slows down activation kinetics and has a much weaker relieving effect on block by PA: Spm-induced rectification and time constant of activation (Spm unblock) shift directly in proportion to the corresponding change in E_K with slope factors −0.15 and +0.31, respectively, for 20 μM Spm. The speed up of activation kinetics caused by increase of external $[K^+]$ cannot be reversed by equal increase of internal $[K^+]$. The data are consistent with the hypothesis that the conduction pathway of strong inward rectifiers is a long and narrow pore with multiple binding sites for PA and K^+. Key words: potassium channel • inward rectifier • spermine • spermidine • putrescine

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INTRODUCTION

Strong inward rectifier potassium channels (Katz, 1949) stabilize the resting potential of excitable cells (Hille, 1992). Several crucial properties distinguish these channels from other potassium channels. Firstly, in intact cells, rectification is so strong that currents decline to negligible levels within ~40 mV positive to the K^+ reversal potential (E_K),[1] while large inward currents are observed at voltages negative to E_K. Secondly, rectification is extremely voltage dependent with equivalent gating charge between 2 and 5. Thirdly, steady-state rectification, as well as the kinetics of channel opening and closing, depend strongly on the concentration of external K^+ (K_{OUT}), shifting in parallel with the change in E_K (Hagiwara et al., 1976; Leech and Stanfield, 1981; Saigusa and Matsuda, 1988; Cohen et al., 1989; Kelly et al., 1992; Ishihara and Hiraoka, 1994). Recent advances in the cloning of inward rectifier K channel subunits (Ho et al., 1993; Kubo et al., 1993*a*; Kubo et al., 1993*b*) have finally led to understanding of the basic mechanisms underlying the phenomenon of strong inward rectification of potassium channels (Kir). In addition to voltage-dependent block by intracellular Mg^{2+} ions (Vandenberg, 1987; Matsuda et al., 1987), very potent and extremely voltage-dependent block of Kir channels by intracellular polyamines (PA, putrescine [Put], spermidine [Spd], and spermine [Spm]) is primarily responsible for causing strong inward rectification in Kir channels (Lopatin et al., 1994; Ficker et al., 1994; Fakler et al., 1995; Lopatin et al., 1995). These studies of PA-induced rectification were carried out at fixed symmetrical potassium concentration, usually 150 mM, and the effects of external and internal potassium were not investigated. It remains to be demonstrated that PA-induced rectification is strictly dependent on K_{OUT}, as it has to be to explain classical, strong inward rectification. In this paper we present evidence that PA-induced rectification of IRK1 channels, expressed in *Xenopus* oocytes, strongly depends on K_{OUT}, mimicking classical rectification in intact cells, and thus closing the last logical gap to acceptance of PA-induced rectification as the major mechanism of strong inward rectification.

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[1] *Abbreviations used in this paper:* E_K, reversal potential for potassium ions; K_{IN}, internal $[K^+]$; Kir, inward rectifier potassium channel; K_{OUT}, external $[K^+]$; PA, polyamine; Put, putrescine; Spd, spermidine; Spm, spermine.

MATERIALS AND METHODS

Oocyte Expression of Kir Channels

cDNAs were propagated in the transcription-competent vector pBluescript SK(−) in *Escherichia coli* TG1. Capped cRNAs were transcribed in vitro from linearized cDNAs using T7 RNA polymerase. Stage V–VI oocytes were isolated by partial ovariectomy of adult female *Xenopus* under tricaine anesthesia. Oocytes were

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defolliculated by treatment with 1–2 mg/ml collagenase (Type 1A Sigma Chemical Co., St. Louis MO) in zero Ca^{2+} ND96 (below) for 1 h. Additional defolliculation was achieved by incubation of oocytes for ~10–15 min in phosphate buffer of the following composition: 100 mM K_2HPO_4, pH 6.5. 2–24 h after defolliculation, oocytes were pressure-injected with ~50 nl of 1–100 ng/μl cRNA. Oocytes were kept in ND96 + 1.8 mM Ca^{2+} (below), supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) for 1–7 d before experimentation.

Electrophysiology

Oocytes were placed in hypertonic solution (HY solution, below) to shrink the oocyte membrane from the vitelline membrane. The vitelline membrane was removed from the oocyte using Dumont No. 5 forceps. Oocyte membranes were patch-clamped using an Axopatch 1D patch clamp apparatus (Axon Instruments Inc., Foster City, CA). Fire-polished micropipettes were pulled from thin-walled glass (WPI Inc., New Haven, CT) on a horizontal puller (Sutter Instrument, Co., Novato, CA). Electrode resistance was typically 0.5–1 MΩ when filled with K-INT solution (below), with tip diameters of 2–20 μm. Pipette capacitance was minimized by coating with a mixture of Parafilm (American National Can Co., Greenwich, CT) and mineral oil. Experiments were performed at room temperature in a chamber mounted on the stage of an inverted microscope (Diaphot; Nikon Inc., Garden City, NY). PClamp software and a Labmaster TL125 D/A converter were used to generate voltage pulses. Data were normally filtered at 5–20 kHz, digitized at 22 kHz (Neurocorder; Neurodata, NY) and stored on video tape. Data could then be redigitized into a microcomputer using Axotape (Axon Instruments, Inc.). Alternatively, signals were digitized on-line using PClamp, and stored on disk for off-line analysis. In most cases, especially with inside-out patches and low concentrations of PA, leak current and capacity transients were corrected off-line with a P/1 procedure (+50 mV, or higher, conditional prepulse). Currents were corrected for rundown wherever possible and necessary.

Solutions

ND96 solution for oocyte storage contained (in mM): NaCl, 96; KCl, 2; $MgCl_2$, 1; HEPES, 5; pH 7.5 (with NaOH). Hypertonic (HY) solution for shrinking oocytes contained (in mM): KCl, 60; EGTA, 10; HEPES, 40; sucrose, 250; $MgCl_2$, 8; pH 7.0. In most experiments, the control bath and pipette solutions were standard high [K^+] solution (K-INT) containing (mM): KCl, 140; HEPES, 10; K-EGTA, 1; pH 7.35 (with KOH). The bath solution additionally typically contained 1 mM K-EDTA. Concentrations of K^+ down to 20 mM were obtained by dilution of a control K-INT solution with water while keeping HEPES, EGTA, and EDTA concentrations constant. High K^+ concentrations were obtained by adding appropriate amounts of KCl. The pH of all solutions was readjusted to 7.3–7.35. No corrections for osmolarity or ionic strength were made, and no substitution for K^+ was made since we found that even relatively large cations like $NMDG^+$ (*N*-methyl-D-glucamine$^+$) cause pronounced channel block when applied intracellularly at millimolar concentrations.

Analysis

Instantaneous current-voltage (I-V) relations were obtained by extrapolation of a single exponential function, fitted to the current record, to the beginning of the test pulse, with the steady-state level taken as a free parameter. Relative currents (*R*) were calculated as a ratio between instantaneous currents measured after careful wash-out of PA and Mg^{2+} and currents measured after application of PA. Relative current-voltage relations were fit by the sum of two Boltzman equations (Eq. 1) with the sum of amplitudes *A1* and *A2* normalized to 1 ($A1 + A2 = 1$).

$$\mathbf{R}(V_M) = A_1/[1 + \mathbf{exp}\{-\lambda_1 \cdot (V_M - V_1)\}] + A_2/[1 + \mathbf{exp}\{-\lambda_2 \cdot (V_M - V_2)\}], \quad (1)$$

where, $\lambda_{1,2} = \frac{ZF}{RT}$, V_M is membrane potential and V_1 and V_2 are parameters. *Z* stands for effective valency (or steepness of rectification) of a blocking ion (PA) and *F*, *R*, and *T* have their usual meaning. After the fitting procedure, the membrane potential at which currents were half blocked, $V_{1/2}$ (no upper index), was calculated and its [K^+] dependence was determined. Microsoft Excel® was used for all analysis procedures.

RESULTS

Fig. 1 summarizes some major features of PA-induced rectification in inside-out patches excised from *Xenopus* oocytes expressing IRK1 (Kir2.1) channels. Rectification can be almost completely removed by careful wash-out of PA and Mg^{2+} (Fig. 1 *A*) and then restored by intracellular application of PA (Fig. 1, *C* and *E*). In contrast to the results obtained by Fakler et al. (1994), the IRK1 clone we used does not generally show fast rundown, and this considerably facilitated experiments. At low PA concentrations (Fig. 1 *B*), instantaneous I-V relations, rectifying weakly in the inward direction (Lopatin and Nichols, 1996), can be measured in a wide range of membrane potentials, and time- and voltage-dependent current decline can be easily resolved at positive membrane potentials (Fig. 1, *A* and *C*). Interestingly, the rate of this decline saturates at extreme positive voltages (Fig. 1 *D*), similar to that found for another strong inward rectifier channel HRK1 (Kir2.3, Lopatin et al., 1995). Again, similar to HRK1 channels, block by PA has two distinct components which are most easily resolved with spermine as the inducer of inward rectification. Instantaneous and time-dependent components of rectification are observed when the membrane potential is depolarized (Fig. 1 *E*). Relative currents for "instantaneous" and steady-state components (Fig. 1 *F*) derived from such experiments display a clear difference (as is the case for HRK1 channels, Lopatin et al., 1995). The "instantaneous" component (*dashed line*) is more shallow and can be described by the sum of two Boltzman equations with similar effective valencies ($Z_1 = 2.6$, $Z_2 = 2.9$). The steady-state component can also be fitted by the sum of two Boltzman equations, with an extremely voltage-dependent part and a shallow part ($Z_1 = 2.7$, $Z_2 = 5.6$ for this particular case). Qualitatively, this behavior is very similar to that found for HRK1 channels (Lopatin et al., 1995), although quantitative differences exist. To examine the [K^+]-dependence of PA-induced inward rectification in a manageable way, the membrane potential at which channels were half blocked ($V_{1/2}$) was calculated from

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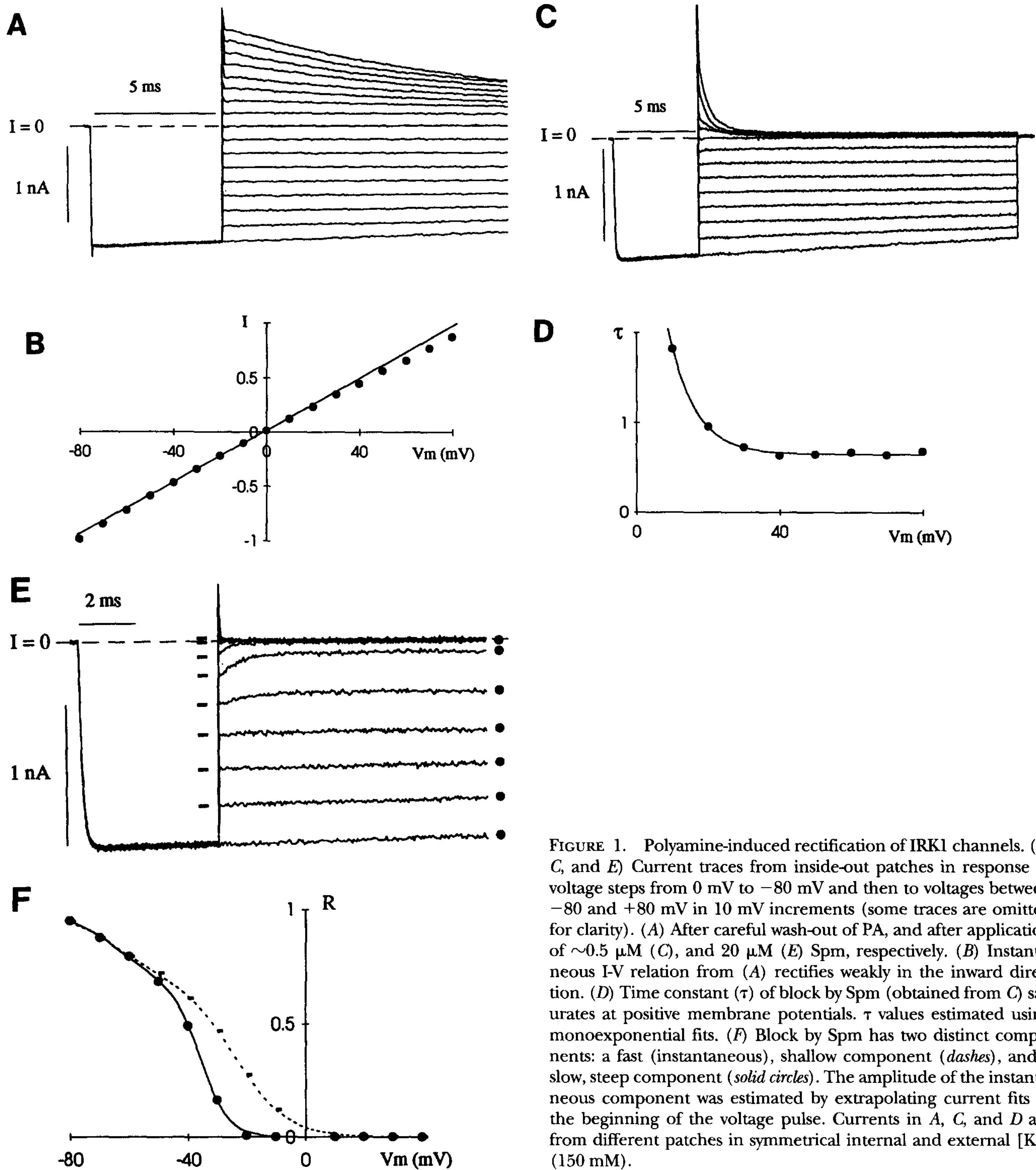

FIGURE 1. Polyamine-induced rectification of IRK1 channels. (*A*, *C*, and *E*) Current traces from inside-out patches in response to voltage steps from 0 mV to −80 mV and then to voltages between −80 and +80 mV in 10 mV increments (some traces are omitted for clarity). (*A*) After careful wash-out of PA, and after application of ~0.5 μM (*C*), and 20 μM (*E*) Spm, respectively. (*B*) Instantaneous I-V relation from (*A*) rectifies weakly in the inward direction. (*D*) Time constant (τ) of block by Spm (obtained from *C*) saturates at positive membrane potentials. τ values estimated using monoexponential fits. (*F*) Block by Spm has two distinct components: a fast (instantaneous), shallow component (*dashes*), and a slow, steep component (*solid circles*). The amplitude of the instantaneous component was estimated by extrapolating current fits to the beginning of the voltage pulse. Currents in *A*, *C*, and *D* are from different patches in symmetrical internal and external [K^+] (150 mM).

these double Boltzman fits to steady-state (20 ms) relative currents as described above.

Polyamine-induced Rectification Is Strongly Relieved by External K^+ Ions

To study the dependence of PA-induced rectification on external K^+ concentration, inside-out patches with different pipette K^+ concentrations (K_{OUT}, 25–350 mM) were isolated into a constant intracellular [K^+] (K_{IN}, 150 mM) bath solution. Patches were first carefully washed-out of endogenous PA and then exogenous PAs were applied and relative currents constructed. Fig. 2 *A* shows an example of a family of relative currents (fit with the sum of two Boltzmann functions) measured at

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four different K_{OUT} when 100 μM Spd was used to induce rectification. Reducing K_{OUT} causes a dramatic shift of relative currents to a more negative membrane potential. Increasing K_{OUT} causes a dramatic shift to a more positive potential. Hence, at any given membrane potential, block by spermidine is increased by reducing K_{OUT}, or decreased by elevation of K_{OUT}. Qualitatively the same phenomenon was observed with Put and Spm-induced rectification. For further quantitative analysis, graphs like those in Fig. 2 *A* were transformed to show the dependence of the voltage at which channels are half-maximally blocked ($V_{1/2}$), on the corresponding reversal potential for potassium ions (E_K). For all three species of PA—Put, Spd and Spm—inward rectification shifts directly in proportion to the shift in E_K (Fig. 2 *B*). At the PA concentrations examined (0.5 mM Put, 100 μM Spd, and 20 μM Spm), the fitted coefficients of proportionality were 0.62, 0.81, and 0.91, respectively.

Intracellular K⁺ Ions Also Relieve Polyamine Block

In parallel to the effect of K_{OUT}, K_{IN} also has a relieving effect on PA-induced rectification. Fig. 3, *A* and *B* shows that voltage-dependent block by 20 μM Spm (rectification) can be dramatically relieved by increasing K_{IN} while keeping K_{OUT} constant at 150 mM. With 20 μM Spm, virtually no outward currents are seen at positive membrane potentials in symmetrical potassium solutions (Fig. 3 *A*). However, when K_{IN} is increased to 625 mM, large outward currents with relatively slow decay are observed (Fig. 3 *B*). As in the previous section, relative currents can be obtained for each concentration of K^+, and the midpoint of rectification ($V_{1/2}$) plotted against E_K. Fig. 3 *C* shows averaged data from such experiments. The dependence of $V_{1/2}$ on E_K has a slope of only −0.15, compared to +0.91 for changes of K_{OUT}. Clearly, the shift in rectification is not a dependence on E_K per se, as earlier suggested by Saigusa and Matsuda (1988), it is also clear that the relief afforded by intra- and extracellular K^+ ions is not the same; extracellular ions are more effective.

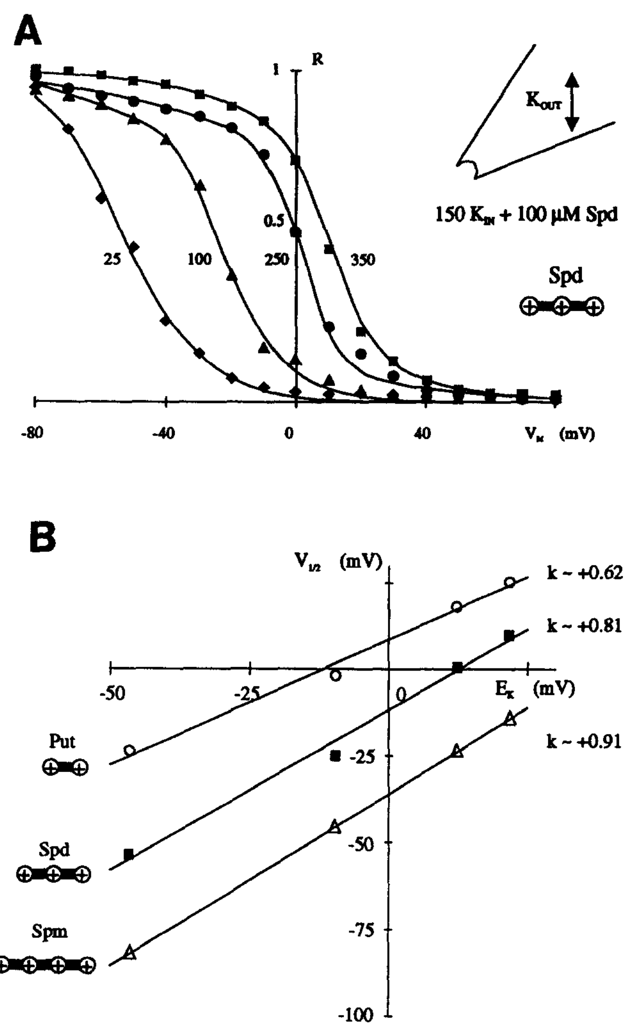

FIGURE 2. Polyamine-induced rectification depends strongly on external [K⁺]. (*A*) Steady-state relative currents (*R*) were measured at different K_{OUT} (pipette), 350 mM (■), 250 mM (●), 100 mM (▲), and 25 mM (◆) and at fixed 150 mM K_{IN} with 100 μM Spd as the inducer of inward rectification. Solid lines are fits with the sum of two Boltzman equations (see METHODS). (*B*) The midpoint of steady-state rectification ($V_{1/2}$), measured for 1 mM Put (○), 100 μM Spd (■), and 20 μM Spm (△), is plotted against E_K for experiments like that presented in (*A*). Solid lines are linear approximations with slope factors 0.62, 0.81, and 0.91 for Put, Spd, and Spm, respectively.

Activation Kinetics Strongly Depend on External But Not Internal [K⁺]

The kinetics of activation, or voltage-dependent unblock, were examined using spermine as the inducer of inward rectification. Unblock of spermine at negative membrane potentials is the slowest of the three PA species and can be reliably measured over a wide range of potentials. As found for HRK1 (Lopatin et al., 1995), PA unblock of IRK1 channels is highly voltage dependent, and estimated sensitivities were 39.7 mV (n = 1) and 18.5 ± 4 mV (n = 3) per e-fold change in activation tau for 100 μM Spd and 20 μM Spm, respectively (compare 33.3 and 15.0 mV, respectively, for activation taus in HRK1).[2]

Fig. 4 *B* illustrates the major finding, that Spm-induced activation tau depends very strongly on K_{OUT}. The volt-

[2]Change in Spm-induced activation tau for HRK1 channels was estimated to be ~25% for 20-fold change in Spm concentration (Lopatin et al., 1995). In the present study we have not systematically examined the dependence of activation tau on PA concentration. However, the similarity of rectification properties between IRK1 and HRK1 suggests that the PA concentration dependence of IRK1 activation kinetics should also be negligible.

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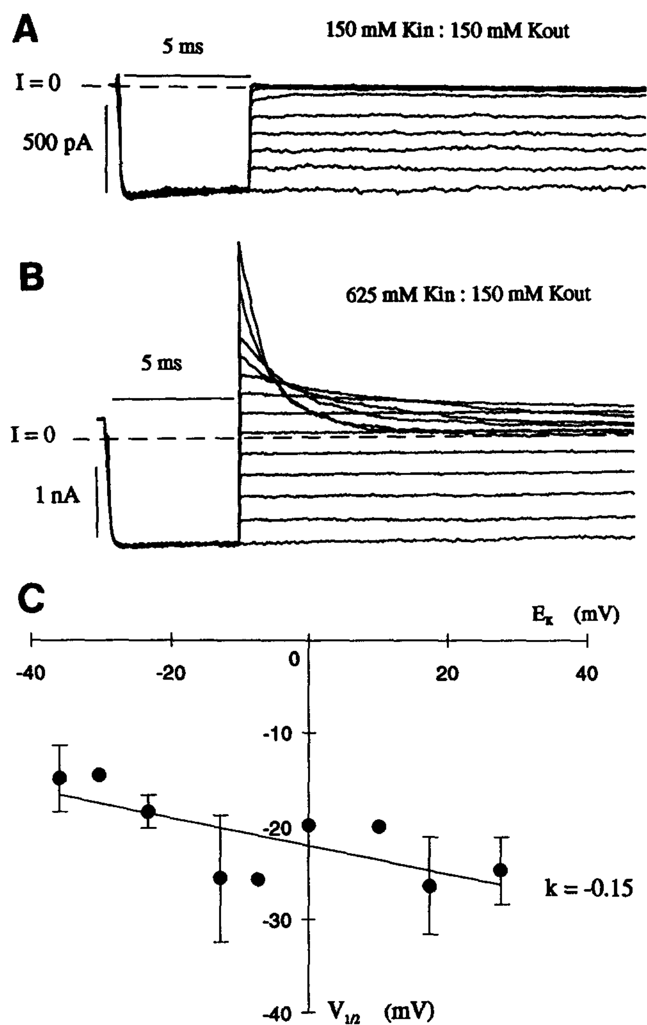

FIGURE 3. Block by spermine can be relieved by increase of intracellular K^+. (*A*) Currents from inside-out patches in response to voltage steps from 0 to −80 mV and then to voltages between −80 and +80 mV after application of 20 μM Spm in symmetrical 150 mM K^+. (*B*) In the same patch as in *A*, K_{IN} was increased to 625 mM while keeping Spm concentration constant at 20 μM. The same voltage protocol was applied. (*C*) The midpoint of rectification ($V_{1/2}$) measured in the range of K_{IN} between 50 and 625 mM (constant 150 mM K_{OUT}) is plotted against E_K (for n = 1–3 measurements at each K_{IN}). The solid line is a linear fit with slope factor $k = -0.15$.

age dependence of Spm-induced activation was measured at K_{OUT} ranging from 25 to 350 mM, with K_{IN} kept constant at 150 mM. Decrease of K_{OUT} caused a considerable leftward shift of activation tau, essentially without change in the voltage sensitivity (the slope of linear approximations, Fig. 4 *B*). Given the decrease in open channel conductance at very low K_{OUT} (Lopatin and Nichols, 1996), the small apparent decrease in voltage sensitivity at the lowest K_{OUT} (25 mM) may not be statistically reliable, and further studies are necessary to clarify this observation. Graphs like that in Fig. 4 *B* can be interpreted in two ways. Firstly, at any fixed membrane potential (e.g., −50 mV, *dashed vertical line*), activation slows down (higher values of *Log(τ)*) with decreasing K_{OUT}. Secondly, the membrane potential at which activation tau adopts a specific value (e.g., $V_{\log(\tau)\,=\,-0.5}$ at Log(τ) = −0.5, *dashed horizontal line*) shifts to negative membrane potentials with decreasing K_{OUT}. In Fig. 4 *C*, $V_{\log(\tau)\,=\,-0.5}$ is plotted against E_K for separate data sets obtained either by changing K_{OUT} (separate patches in each case) or by changing K_{IN} (measurements obtained with the same patch). Filled symbols represent experiments where K_{OUT} was varied at constant 150 mM K_{IN}; open symbols represent experiments where K_{IN} was varied at constant 150 mM K_{OUT}. Linear approximations give the following slopes of straight lines: 1.08 and 0.31 ± 0.04 (n = 3) for K_{OUT} and K_{IN} dependence, respectively.

Fig. 4 *D* adds one more dimension to this subject: the speed up of activation that is observed when K_{OUT} is increased from 150 to 500 mM cannot be reversed by equivalent increase of K_{IN} to bring E_K back to 0 mV. In other words, not the value of E_K, but the absolute values of K_{OUT} and K_{IN}, determine activation kinetics, and as shown above, the steady-state rectification, of IRK1 channels.

DISCUSSION

K_{OUT} Dependence of Polyamine-induced Rectification Explains K_{OUT} Dependence of Classical Inward Rectification

It has long been recognized (see Hille, 1992) that both steady-state and kinetic properties of classical inward rectifier channels depend strongly on external $[K^+]$ (K_{OUT}). One peculiarity of this behavior is the coincidence between the voltage-dependence of rectification and the potassium reversal potential (E_K). In intact cells any shift in E_K caused by change in K_{OUT} leads to an almost equivalent shift of rectification. This has been taken as implying that rectification depends not on the absolute value of the membrane potential (V_M), but rather on the permeant ion driving force ($V_M - E_K$), and has become accepted as one of the hallmarks of strong inward rectification. This phenomenon has been observed in a variety of cell types: starfish eggs (Hagiwara et al., 1976), guinea-pig ventricular cells (Saigusa and Matsuda, 1988), and Purkinje fibres (Cohen et al., 1989), rabbit osteoclasts (Kelly et al., 1992), frog skeletal muscle (Leech and Stanfield, 1981), and also with cloned IRK1 channels (Ishihara and Hiraoka, 1994) from mouse heart. In this paper we show that PA-induced rectification of IRK1 channels expressed in *Xenopus* oocytes also depends strongly on K_{OUT}. The voltage dependence of block by single PA species is rather complex (Fig. 1 *E*), and relative currents (*R*, Fig. 1 *F*) in most cases cannot be described by a single Boltzmann

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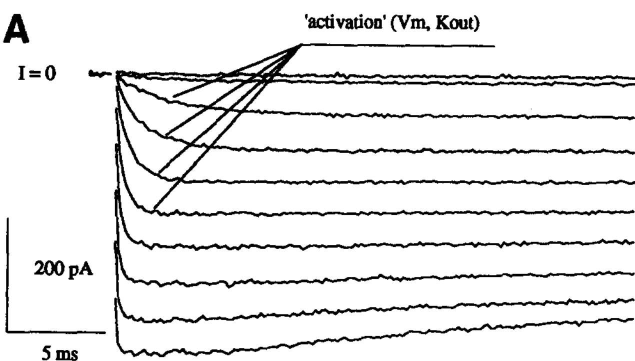

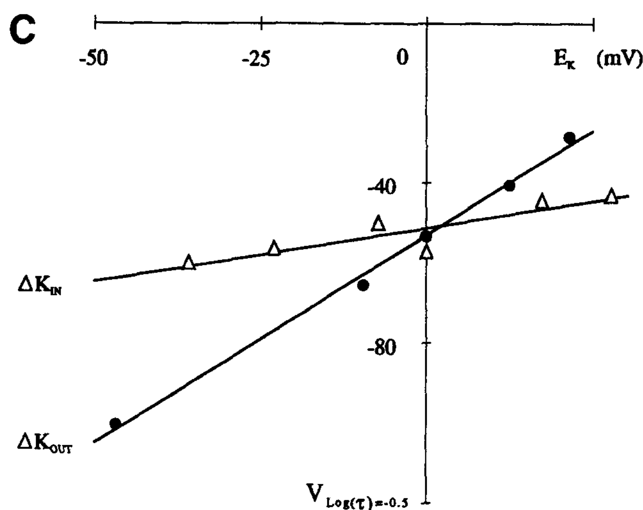

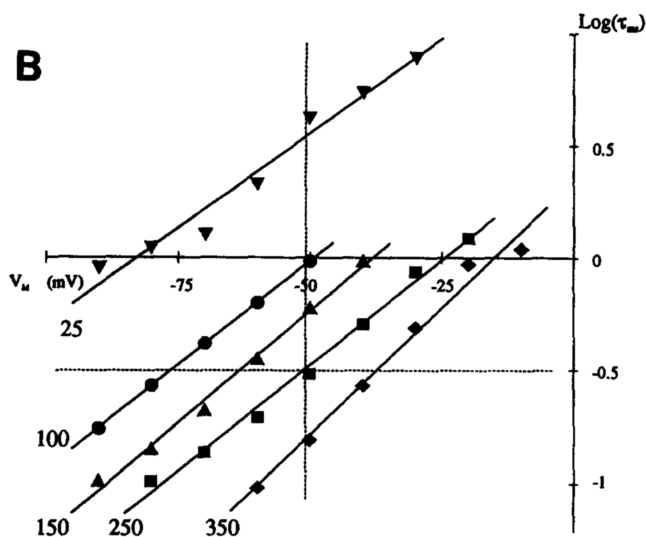

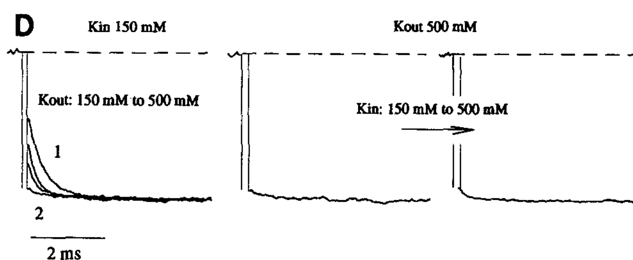

FIGURE 4. Activation kinetics depend strongly on external, but not internal, $[K^+]$. (*A*) Family of current traces in response to voltage steps from +50 mV holding potential to voltages between 0 and −90 mV in 10 mV increment. Symmetrical 150:150 mM potassium with 20 μM Spd as inducer of rectification. The 200-μs interval at the beginning of each voltage pulse is omitted. (*B*) Activation tau (τ) obtained by monoexponential fits to records such as those in *A* is plotted against membrane potential for 25 mM (▼), 100 mM (●), 150 mM (▲), 250 mM (■), and 350 mM (◆) K_{OUT} (at constant 150 mM K_{IN}). Tau is plotted on a logarithmic scale. Solid lines are linear fits to the data. The dashed line shows the level of $Log(\tau) = -0.5$, the most reliable data lie in this range. (*C*) Sensitivity to K_{OUT} (●) and K_{IN} (△) (while keeping $[K^+]$ constant at 150 mM on the other side of the membrane) is plotted against the corresponding E_K for a representative experiment in each case. The solid lines are linear fits with slopes of 1.08, and 0.26, for K_{OUT} and K_{IN}, respectively. (*D*) An inside-out patch was first isolated into symmetrical 150:150 mM K^+ solutions (*left panel, 1*). The patch pipette was then perfused with 500 mM K_{OUT} (*2*), and K_{IN} was subsequently changed to 500 mM (*right panels*). 20 μM Spm was always present at the intracellular side of the membrane. The 200-μs interval at the beginning of the voltage pulse is omitted. Currents were normalized to the same amplitude.

equation. Steady-state relative currents have at least two components with steepness (effective valency, *Z*) ranging between 1 and 6. Complexity of relative currents is also observed with classical rectification in intact cells. One of the best examples is seen with inward rectifiers in frog skeletal muscle (Leech and Stanfield, 1981) where both shallow and steep parts of the relative current-voltage relationship are observed. DeCoursey et al. (1984) considered this to be an artifact resulting from K^+ accumulation, but the observation of similar behavior in excised membrane patches suggests that it is a real phenomenon. In other cell types, inward rectification is more simple and apparently well described by a single Boltzmann equation (Silver et al., 1994; Saigusa and Matsuda, 1988). Although PA-induced rectification of IRK1 channels is very similar to that of HRK1 channels (Lopatin et al., 1995), there are some differences: the instantaneous part of Spm-induced rectification of IRK1 channels is not well described by a single Boltzmann equation as it is for HRK1 channels. For the sake of simplicity in this paper we did not consider the $[K^+]$ dependence of each component of relative currents. Instead, after the fitting procedure, which assumes no specific model, we calculated the voltage at which chan-

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nels are half blocked ($V_{1/2}$), and then examined the [K^+]-dependence of this empirical parameter. [K^+]-dependence of rectification defined in this way is of limited use for biophysical interpretation, but retains physiological meaning.

As shown in Fig. 2, PA-induced rectification depends strongly on K_{OUT}, with apparent sensitivity increasing from putrescine to spermine. Phenomenologically, these results provide the last significant piece of evidence required to explain classical inward rectification as resulting from PA-induced channel block; the [K^+] dependence of PA-induced rectification can be as strong as that of classical rectification. From the biophysical point of view, these results resolve another question, raised nearly 20 years ago by Hille and Schwarz (1978): is it possible to explain the virtually perfect shift of rectification due to change of extracellular [K^+] by simple voltage-dependent block by multivalent cations? Theoretical analysis with a single file pore model (Hille and Schwarz, 1978) revealed at least one problem: blocking particles with valency greater than 1, for example Mg^{2+}, could not produce an appropriate shift of rectification (because of increased electrical repulsion within the pore). With polyvalent PA this problem would be even worse. The above results demonstrate that the experimental answer is yes, it is possible. Moreover, block by the tetravalent Spm molecule may be even more sensitive to K_{OUT} than block by Spd (valency +3) and Put (+2), although, without further extensive experiments, we cannot formally discount the possibility that these differences result from a dependence on PA concentration. Preliminary theoretical considerations using multisite-multibarrier models indicate that blocking by multivalent particles is not an intrinsic problem of the barrier model to explain perfect K_{OUT} dependence of rectification (Lopatin et al., 1996).

K_{IN} Dependence of Polyamine-induced Rectification Demonstrates Competition Effects

Previous studies with intact cells have demonstrated contradictory effects of intracellular [K^+] (K_{IN}) on rectification, and although there has been wide acceptance of the notion that classical rectification depends on the K^+ driving force ($V_M - E_K$), this dependence only holds universally for changes in K_{OUT}. When E_K was changed by varying K_{IN} in starfish eggs (Hagiwara and Yoshii, 1979), frog muscle (Hestrin, 1981; Leech and Stanfield, 1981) or Purkinje myocytes (Cohen et al., 1989), the mid-point of rectification did not follow E_K (although in some cases there was still a weak positive correlation with E_K). In guinea-pig ventricular cells (Saigusa and Matsuda, 1988), the measured K_{IN} dependence of rectification was as strong as the K_{OUT} dependence. This is in contrast to the present results, wherein K_{IN} dependence of rectification has a negative correlation (k ~ -0.15, Fig. 3 *C*). This apparent contradiction might be resolved after consideration of the control of PA concentration in intact cells. The total intracellular PA concentration is enormous, reaching tens of millimolar in some cells (Bachrach, 1973), while micromolar amounts are necessary to induce sufficient rectification to explain classical rectification (Lopatin et al., 1996). Because of the huge cellular buffering capacity, most PAs are bound to low and high affinity binding sites (DNA, RNA, proteins) (Watanabe et al., 1991). It has also been shown that intracellular PA levels can be modulated by increasing concentration of monovalent (K^+) or divalent (Mg^{2+}) cations, and ATP (Watanabe et al., 1991). It is therefore possible that in intact cells, changes in K_{IN} may affect the free, or active, intracellular PA concentrations, thus complicating the experiments. In inside-out patch experiments, with presumably almost complete wash-out of PAs, this complication should be avoided. In the present experiments, channel block by PA is relieved, and outward currents are observed as K_{IN} is increased (Fig. 3). We observe a weak negative correlation between $V_{1/2}$ and K_{IN} (Fig. 3 *C*), qualitatively explainable by a competition between intracellular K^+ ions and PAs.

[K^+] Dependence of Activation Kinetics Reflect Lock-in by K_{IN} and Competition by K_{OUT}

The kinetics of PA-induced rectification depend strongly on K_{OUT} (Fig. 4), as observed with rectification in intact cells (Leech and Stanfield, 1981; Hagiwara et al., 1976; Harvey and Ten Eick, 1988; Ishihara and Hiraoka, 1994), but only weakly on K_{IN} (Fig. 4 *C*). There is great contrast in published dependencies of activation kinetics on K_{IN}. In Purkinje myocytes, the kinetics of activation at any given membrane potential are reportedly slowed by reduction of K_{IN} (Cohen et al., 1989), while in our experiments reduction of K_{IN} causes a moderate speeding up of activation. In guinea-pig ventricular myocytes, the speeding up by decrease in K_{IN} is even more marked (Saigusa and Matsuda, 1988). The proportionality to E_K has coefficients of proportionality of -1, $+0.3$, and $+1$ for these three studies, respectively. This variability of results might be explained by differences in perfusion techniques. In experiments with Purkinje myocytes, Cohen et al. (1989), for example, used 100 mM choline$^+$ chloride$^-$ (2-hydroxyethyl trimethylammonium chloride) for K^+ substitution; without direct estimation of the effects of choline$^+$ ions on channel kinetics it would be inappropriate to put much emphasis on these results. We did not use any cation substitution since we found that at least one popular replacement, NMDG$^+$ (*N*-methyl-D-glucamine$^+$), causes chan-

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nel block when applied intracellularly in the millimolar range.

Biophysical Implications of K^+ Interactions with PA Block

Experiments presented in this paper were carried out on inside-out patches with full control of K_{OUT}, K_{IN}, Mg^{2+}, and free PA concentrations. Consequently, this data is valuable from the biophysical perspective of obtaining further insights into the pore structure of inward rectifiers. Based on previous (Lopatin et al., 1995; Yang et al., 1995) and current findings (see also Lopatin et al., 1996). At least two PA molecules may be involved in blocking of Kir channels, sequentially sliding under the membrane electrical field into the long and relatively narrow pore region and then binding at deep, high affinity (slow) and shallow, low affinity, sites (fast). This is, at least in part, consistent with current understanding of the molecular structure of Kir channels. It has been shown that at least two negatively charged amino acid residues in IRK1 channels, namely D172 (Stanfield et al., 1994; Wible et al., 1994; Ficker et al., 1994) and E224 (Yang et al., 1995) in each of the four probable subunits that make up a functional Kir channel (Glowatzki et al., 1995), greatly affect rectification properties and Mg^{2+} and PA binding. The presence of two PA binding sites gives enough complexity to the model to explain both steady-state concentration and voltage dependence, as well as kinetics properties of Kir channels at fixed symmetrical concentration of K^+ ions (Lopatin et al., 1995). The validity of such a model is further strengthened by the fact that only minor additions have to be made to the previous model to accommodate new data on K^+ dependence of conductance (Lopatin and Nichols, 1996) and block by PA (Lopatin et al., 1996). We postulate the presence of a relatively high affinity, externally located binding site for K^+ ions (putatively at the selectivity filter of the channel) which is not accessible from the intracellular side of the membrane for the larger PA molecules. Secondly, we postulate an electrostatic interaction between K^+ ions bound to this site and to other cations (K^+, Mg^{2+}, or PA^{n+}) in the pore.

According to these postulates, the apparently perfect dependence of steady-state PA block on K_{OUT} arises because of strong electrostatic interaction between K^+ ions bound with high affinity to the selectivity filter (which is easily accessible from the outside) and PA in the deep, high affinity binding site. Occupancy of this deep binding site, and hence the degree of channel block, depends strongly on K^+ occupancy of the selectivity filter, and rectification therefore follows E_K (when K_{OUT} is changed). In the same way, increased occupancy will speed up activation (PA unblock) due to electrostatic destabilization of the PA binding, and this again will be proportional to the K^+ occupancy of selectivity filter and, hence, E_K.

The predicted effect of K_{IN} on polyamine block is somewhat different from that of K_{OUT}. Increasing K_{IN} will increase K^+ occupancy of the innermost low affinity binding site(s) and lock (Neyton and Miller, 1988) the PA in the deep one, thus causing a slowing down of activation at any given membrane potential, or shifting the voltage at which tau has a given value to more negative voltages, as observed (Fig. 4 *C*). However, even though the PA off rate is slowed down by increased K_{IN}, occupancy of the deep binding site by PA will be reduced because of competition by K^+ ions for binding, the net effect being relief of steady-state PA block (Fig. 3 *B*).

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