Interaction of Permeant and Blocking Ions in Cloned Inward-Rectifier K^+ Channels

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ABSTRACT Blocking cloned inward-rectifier potassium (K_{ir}) channels from the cytoplasmic side was analyzed with a rapid application system exchanging the intracellular solution on giant inside-out patches from *Xenopus* oocytes in <2 ms. Dependence of the pore-block on interaction of the blocking molecule with permeant and impermeant ions on either side of the membrane was investigated in $K_{ir}1.1$ (ROMK1) channels blocked by ammonium derivatives and in $K_{ir}4.1$ (BIR10) channels blocked by spermine. The blocking reaction in both systems showed first-order kinetics and allowed separate determination of on- and off-rates. The off-rates of block were strongly dependent on the concentration of internal and external bulk ions, but almost independent of the ion species at the cytoplasmic side of the membrane. With K^+ as the only cation on both sides of the membrane, off-rates exhibited strong coupling to the K^+ reversal potential (E_K) and increased and decreased with reduction in intra and extracellular K^+ concentration, respectively. The on-rates showed significant dependence on concentration and species of internal bulk ions. This control of rate-constants by interaction of permeant and impermeant internal and external ions governs the steady-state current-voltage relation (*I-V*) of K_{ir} channels and determines their physiological function under various conditions.

INTRODUCTION

Inward-rectifier potassium (K_{ir}) channels play a key role in excitability by maintaining the resting potential near E_K and by determining a threshold for excitation that is dependent on both membrane voltage (E) and E_K (Hille, 1992). The functional property behind this is the channels' characteristic inward rectification, e.g., the ability of K_{ir} channels to mediate high K^+ conductance at voltages around E_K, which decreases when the membrane is further depolarized. The molecular mechanism underlying the phenomenon of inward rectification block of K_{ir} channels by intracellular Mg^{2+} and the polyamines spermine (SPM) and spermidine (SPD) has been identified (Vandenberg, 1989; Matsuda, 1991; Fakler et al., 1994a; Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1995). The pore-block by these intracellular cations is characterized by a voltage-dependence that may be strong ("strong rectifiers") or weak ("weak rectifiers") and which is determined by the quantity $E - E_K$ rather than by E alone (Hagiwara et al., 1976; Leech and Stanfield, 1981; Cohen et al., 1989; Hille, 1992). Since the latter was primarily found for changes in extracellular K^+ concentration ($[K^+]_{ex}$) inward rectification is more correctly said to depend on $[K^+]_{ex}$ and E but not on the intracellular K^+ concentration ($[K^+]_{in}$). To account for this fact a binding site for K^+ ions on the external surface of the K_{ir} channel molecule was hypothesized (Hille, 1992).

Comparison of the primary sequence of strongly and weakly rectifying K_{ir} channels identified two structural determinants involved in inward rectification. These are negatively charged residues, one in the second transmembrane segment (M2-site) (Fakler et al., 1994a; Lu and MacKinnon, 1994; Stanfield et al., 1994; Wible et al., 1994), the other in the cytoplasmic C-terminal domain (C-terminal-site) (Taglialatela et al., 1995; Yang et al., 1995). Inward-rectifiers of the $K_{ir}2.0$ subfamily (Doupnik et al., 1995; Fakler and Ruppersberg, 1996) that exhibit both the M2- and C-terminal-site display complex kinetics of SPM block (Lopatin et al., 1995; Fakler and Ruppersberg, 1996), while those carrying only the M2-site show monoexponential blocking behavior and their steady-state block is described by a single Boltzmann function (Fakler et al., 1994a; Glowatzki et al., 1995). Voltage-dependence of block is usually quantified in terms of the change in membrane voltage necessary for an e-fold increase in block. This parameter is assumed to correlate with the number of blocking charges times the percentage of the transmembrane electric field which these charges move through in the blocking reaction (electrical distance according to Woodhull, 1973). For a blocker of given charge an electrical distance (∂) of unity suggests that either one blocking molecule completely crosses the transmembrane field or that two molecules block at the same time and move through 50% of the field. For $K_{ir}2.1$ (IRK1) channels it was recently shown in voltage jump experiments that the off-rate rather than the on-rate of SPM-block is responsible for the large electrical distance found in inward-rectifier channels (Lopatin and Nichols, 1996). However, because of the permanent presence of the blocking molecule, on- and off-rates could only be measured in voltage ranges where the block is predominantly determined either by onset or release of block. Moreover, $K_{ir}2.1$ channels show complex block kinetics (Lopatin et al., 1995; Fakler

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and Ruppersberg, 1996), which made it difficult to analyze the mechanism underlying voltage-dependence of block and its control by extracellular K^+.

In the present work we perform a detailed analysis of the block of $K_{ir}1.1$ channels by the monovalent cation tetrapentylammonium (T5A) applied to the cytoplasmic side. Although these channels do not carry either of the two determinants for strong rectification, they are blocked by T5A in a highly voltage-dependent manner. Blocking by T5A is studied with a rapid solution-exchange system on inside-out patches, which allows measurements of voltage and concentration dependence of on- and off-rates over a wide range. The findings obtained from T5A-block in $K_{ir}1.1$ are then compared to block of the strong rectifier $K_{ir}4.1$ by SPM. The results indicate that the mechanism of blocking is very similar in both cases and that direct interaction between permeant and blocking ions mediates strong voltage-dependence of the block and its dependence on $[K^+]_{ex}$.

MATERIALS AND METHODS

cRNA-synthesis, preparation, and injection of oocytes

Capped cRNAs were synthesized in vitro using SP6 polymerase (Promega, Heidelberg, Germany) and stored in stock solutions at −70°C. *Xenopus* oocytes were surgically removed from adult females and manually dissected. About 50 nl of a solution containing cRNA specific for $K_{ir}1.1$ and $K_{ir}4.1$ was injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (Sigma, 0.5 mg/ml) for 25 min 2 days after injection.

Electrophysiology

Giant patch recordings (Fakler et al., 1994b) in inside-out configuration were made at room temperature (~23°C) 3–7 days after injection. Pipettes used were made from thick-walled borosilicate glass, had resistances of 0.3–0.6 MΩ (tip diameter of 20–30 μm) and were filled with $[K^+]_{ex}$ solution containing either (in mM) 120 KCl and 10 HEPES or 12 KCl and 1 HEPES, pH adjusted to 7.2 with KOH. Voltage clamp recordings were performed with an EPC9 patch clamp amplifier (HEKA electronics, Lamprecht, Germany). Currents were filtered at 3 kHz (−3 dB) and sampled at 10 kHz. Step or ramp protocols were applied as indicated in the figure legends. Duration of the ramps was 5 s throughout, which was slow enough to record virtually under steady-state block conditions as deduced from currents in response to inverse ramps showing equal voltage dependence under all experimental conditions.

Excised patches were superfused with intracellular solutions composed as follows (in mM). 120 $[K^+]_{in}$: 100 KCl, 10 HEPES, 10 K_2EGTA; 12 $[K^+]_{in}$: 10 KCl, 1 HEPES, 1 K_2EGTA; 12 $[K^+]_{in}$ + 108 $[Na^+]_{in}$: 10 KCl, 108 NaCl, 1 HEPES, 1 K_2EGTA; 12 $[K^+]_{in}$ + 108 $[Rb^+]_{in}$: 10 KCl, 108 RbCl, 1 HEPES, 1 K_2EGTA; pH was adjusted to 7.2 for measurements with $K_{ir}4.1$ and to 8.0 in experiments with $K_{ir}1.1$ (Fakler et al., 1996). SPM, tetraethylammonium chloride (TEA), tetrapropylammonium chloride (T3A), and T5A were added to yield the final concentrations indicated.

Fast solution-exchange

Intracellular solutions were applied to the cytoplasmic side of excised patches via a theta-glass capillary, both barrels of which had an opening diameter of ~60 μm. Fast solution-exchange was achieved by stepping the theta capillary with a piezo-driven device, alternately placing both of the two barrels in front of the patch. The exchange speed was routinely tested by switching between solutions containing permeant (K^+) and impermeant (Rb^+ or Na^+) cations. In control experiments a complete exchange of the cytoplasmic solution was achieved within 2 ms (see Fig. 3 *A, inset*).

Volume calculation

Calculation of volumes for TEA, T3A, and T5A were performed with a homemade software (program in C), which integrates volume voxels within a molecular body whose boundaries are given by the superposition of the van der Waals spheres of all atoms in the molecule. The radii used for the van der Waals spheres were 1.25 $Å^3$ for H-, 1.55 $Å^3$ for O-, 1.69 $Å^3$ for N-, and 1.87 $Å^3$ for C-atoms; the steps used for integration yield an accuracy better than 1%.

Data evaluation

I-V values were recorded in the absence and presence of the blocker by 5-s voltage ramps. *g-V* plots were obtained by normalizing the current measured in the presence of the blocker to that recorded in its absence. Groups of 100 adjacent data points recorded were averaged for the final *g-V*. For the conductance at E_K, which is not defined as chord conductance, a slope-conductance value was calculated from a monoexponential fit to the neighboring data points.

Sets of *g-V* data were fitted with a first-order Boltzmann function (Eq. 1)

$$g = g_0/\{1 + \exp[(V - V_{1/2})\partial zF/\mathrm{RT}]\}$$

In Eq. 1, g/g_0 represents the normalized conductance change due to block; V is membrane voltage; $V_{1/2}$ is membrane voltage for half-maximal inhibition; ∂ is electrical distance of block; z is charge of the blocking ion (4 in the case of SPM); F, R, and T have their usual meaning.

Time constants for onset and release of SPM and T5A were obtained from single exponentials fitted to the currents in response to voltage steps and to blocker application or washout. Time constants for release of SPM in 12 mM $[K^+]_{in}$ were only determined for potentials < -20 mV because of nonspecific binding of SPM to the cytoplasmic surface of the patches which affected washout of the blocker. All fits were performed with commercial software (Igor, Wave Metrics, Portland, OR).

RESULTS

Block of $K_{ir}1.1$ channels by TEA, T3A, and T5A

Intracellular block of $K_{ir}1.1$ channels by TEA, T3A, and T5A exhibits high voltage-dependence (Fig. 1). This is in contrast to voltage-dependent K^+ channels which are blocked by these ammonium derivatives almost independently of membrane voltage (Hille, 1992). As shown in Fig. 1, *A* and *B*, both blocking affinity and voltage-dependence of block increased with the size of the blocking molecule. The voltage for half-maximal block ($V_{1/2}$) and the electrical distance (∂) were obtained by fitting a first-order Boltzmann function (Eq. 1) to the conductance voltage relations as in Fig. 1 *B*. For 1 mM TEA (calculated volume of 181 $Å^3$), $V_{1/2}$ was 68.8 ± 5.1 mV ($n = 6$); for 1 mM T3A (volume: 256 $Å^3$) and 1 mM T5A (volume: 406 $Å^3$), $V_{1/2}$ was 34.8 ± 1.8 mV ($n = 5$) and 7.2 ± 4.2 mV ($n = 10$), respectively. The electrical distances obtained from the same fits were 0.94 ± 0.02, 1.00 ± 0.02, and 1.35 ± 0.04 for TEA, T3A, and T5A, respectively. Thus T5A proved to be the most potent blocker of the ammonium derivatives tested.

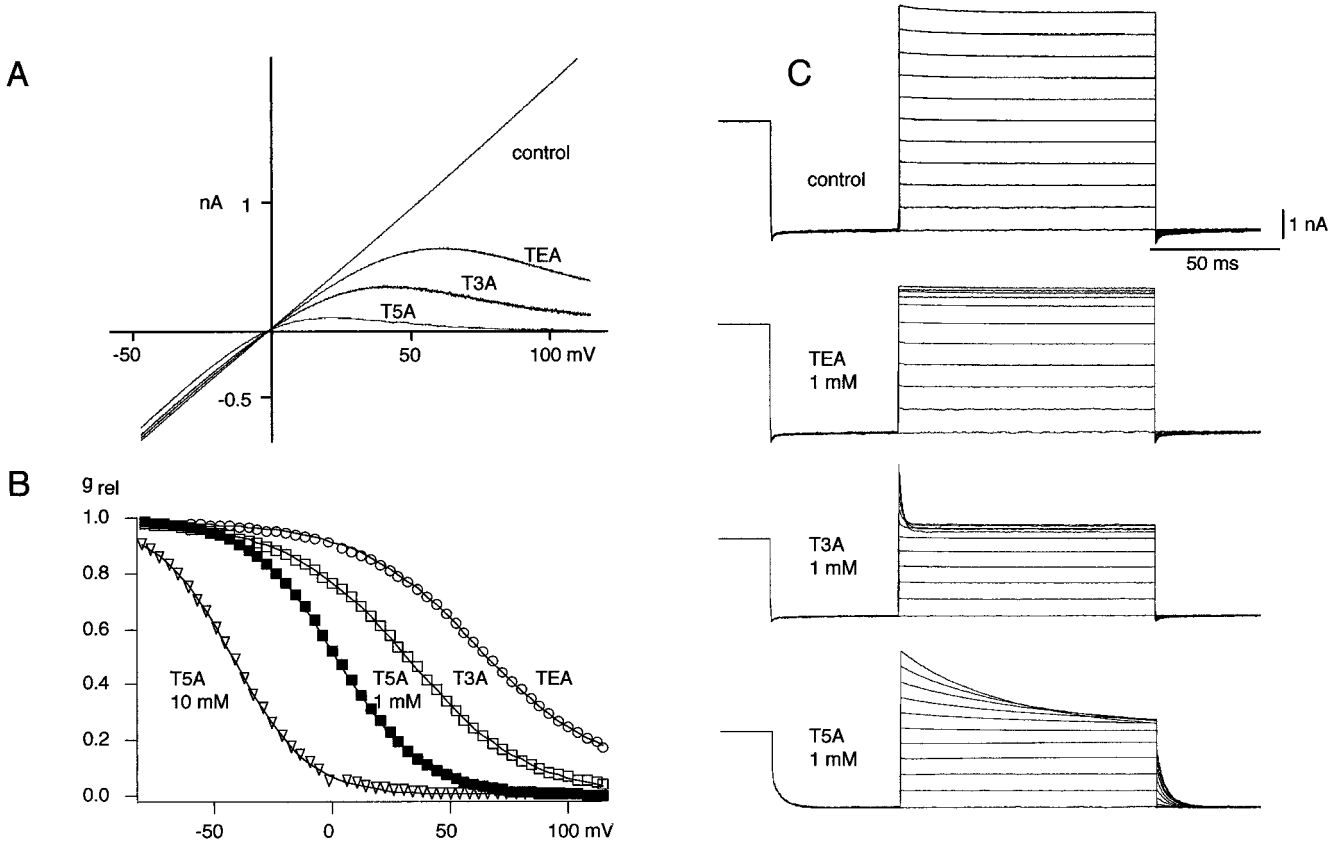

FIGURE 1 Block of $K_{ir}1.1$ channels by tetraalkylammonium derivatives. (*A*) Current-voltage relations (*I-V*) measured in response to voltage ramps (−80 to 120 mV, 5 s) in symmetrical 120 mM K^+ in the absence (control) and presence of 1 mM TEA, T3A and T5A at the cytoplasmic side of a giant inside-out patch. (*B*) Conductance (g_{rel})-voltage relation obtained from an experiment as in (*A*), fitted with a single Boltzmann function. (*C*) Current responses to 100 ms voltage steps from −100 mV to +100 mV in 20-mV increments in the presence of 1 mM TEA, T3A, or T5A. Voltage protocol starts with a step from 0 to −100 mV to completely remove the block. Scale bars as indicated. Note the difference in onset of block for the three ammonium derivatives.

In addition to voltage-dependence, the time constant for onset of block (τ_{on}) was also dependent on the size of the blocking molecule (Fig. 1 *C*). While the block by TEA measured in response to a voltage jump from −80 to 100 mV could not be resolved ($\tau_{on} < 0.3$ ms), τ_{on} for T3A was 1.3 ± 0.2 ms ($n = 4$) and that obtained for T5A was 39.0 ± 4.7 ms ($n = 7$).

A reason for this strikingly slow τ_{on} seen for T5A might be competition with other ions entering the internal part of the channel pore more rapidly than T5A. To test this hypothesis $[K^+]_{in}$ was reduced from 120 to 12 mM. This reduction in $[K^+]_{in}$ led to a decrease of τ_{on} to 12.7 ± 1.1 ms ($n = 6$; Fig. 2 *A*) while the same reduction in $[K^+]_{ex}$ (to 12 mM) did not affect τ_{on} (38.3 ± 3.6 ms; $n = 6$; data not shown) compared to the symmetrical 120 mM K^+ situation. This acceleration of τ_{on} upon decrease in $[K^+]_{in}$ is in accordance with the competition hypothesis. Alternatively, the reduced ionic strength of the cytoplasmic solution might have led to an accumulation of the blocking molecules due to a reduced compensation of negative surface charges.

The time constant for onset of block, τ_{on}, was not only sensitive to changes in concentration but also to changes of the internal ion species. When 108 mM of the intracellular K^+ was replaced by rubidium (Rb^+; Fig. 2 *B*) τ_{on} was slowed down to 78.5 ± 2.7 ms ($n = 7$) and the steady-state block was shifted to more positive potentials (Fig. 2 *C*; $V_{1/2}$: 13.1 ± 2.6 mV; δ: 1.43 ± 0.05; $n = 8$). Surprisingly, a similar shift in steady-state block was obtained in the experiment with reduced $[K^+]_{in}$, in which the omitted 108 mM K^+ had not been substituted for by other monovalents ($V_{1/2}$: 13.8 ± 2.3 mV; δ: 1.40 ± 0.03; $n = 4$). Since the on-rate in this case was more than six times faster than in the experiment with Rb^+ substituted for K^+ (see above, Fig. 2, *A* and *B*), a straightforward explanation for the identical steady-state blocks in Fig. 2 *C* might be a compensating change in the off-rate. An increase in off-rate might also explain why the steady-state block in 12 mM $[K^+]_{in}$ is shifted to the right compared to the symmetrical 120 mM K^+ (Fig. 2 *C*), although the on-rate in 12 mM $[K^+]_{in}$ was approximately three times faster than for the symmetrical K^+ condition. These considerations hold only in systems, however, which show a blocking reaction of simple first-order kinetics.

To test for this presumption, we carried out experiments in which T5A was applied to $K_{ir}1.1$ channels with a fast piezo-driven application-system allowing exchange of the internal solution at giant inside-out patches in <2 ms (Fig. 3 *A, inset*). With this setup on- and off-rates could be

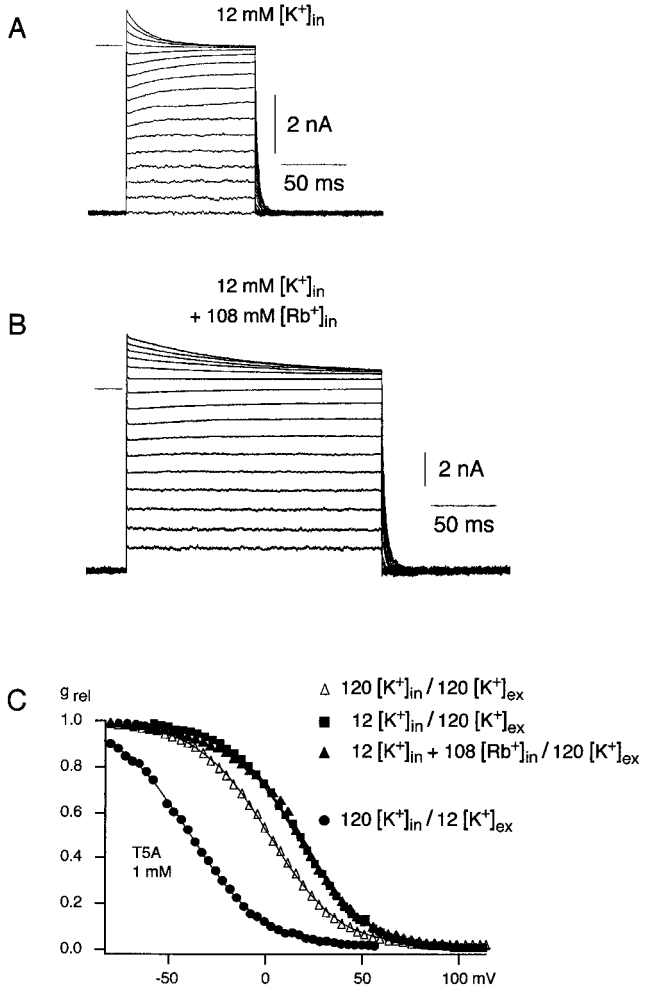

FIGURE 2 Concentration and species of intracellular monovalent cations affect time course and steady state of block. (*A* and *B*) Experiments as in Fig. 1 *C* with 1 mM T5A on $K_{ir}1.1$ channels in 12 mM $[K]_{in}$ (*A*) and 12 mM $[K]_{in}$ + 108 mM $[Rb]_{in}$ (*B*). Onset of block is accelerated in (*A*) and slowed down in (*B*) with respect to symmetrical 120 mM $[K^+]$ (Fig. 1 *C*). (*C*) Steady-state block determined as in Fig. 1 for the conditions indicated.

measured separately and over a wide voltage range. Fig. 3 *A* shows the change in conductance evoked by application and washout of 1 mM T5A at 100, 70, 30, 10, −10, and −20 mV ($n = 7$). When onset and release of block (*dots*) were fitted with monoexponentials (*lines*), deviations between data and fit were found to be only ~1% of signal. These results do not indicate a significant contribution of more than one monoexponential function and strongly suggest a blocking reaction of first-order kinetics. Accordingly, it should be possible to derive the steady-state conductance from the on- and off-rates calculated from the time constants of the monoexponential fits (τ_{on}/τ_{off}). In Fig. 3 *B*, the steady-state conductances obtained from the asymptotic values of the fits to the time course of block are plotted versus the steady-state conductance calculated from the on- and off-rates. As expected for a first-order process, the resulting points were nicely fitted by a straight line that is almost a bisector (slope: 1.09, offset: 0.005). Moreover, the voltage-

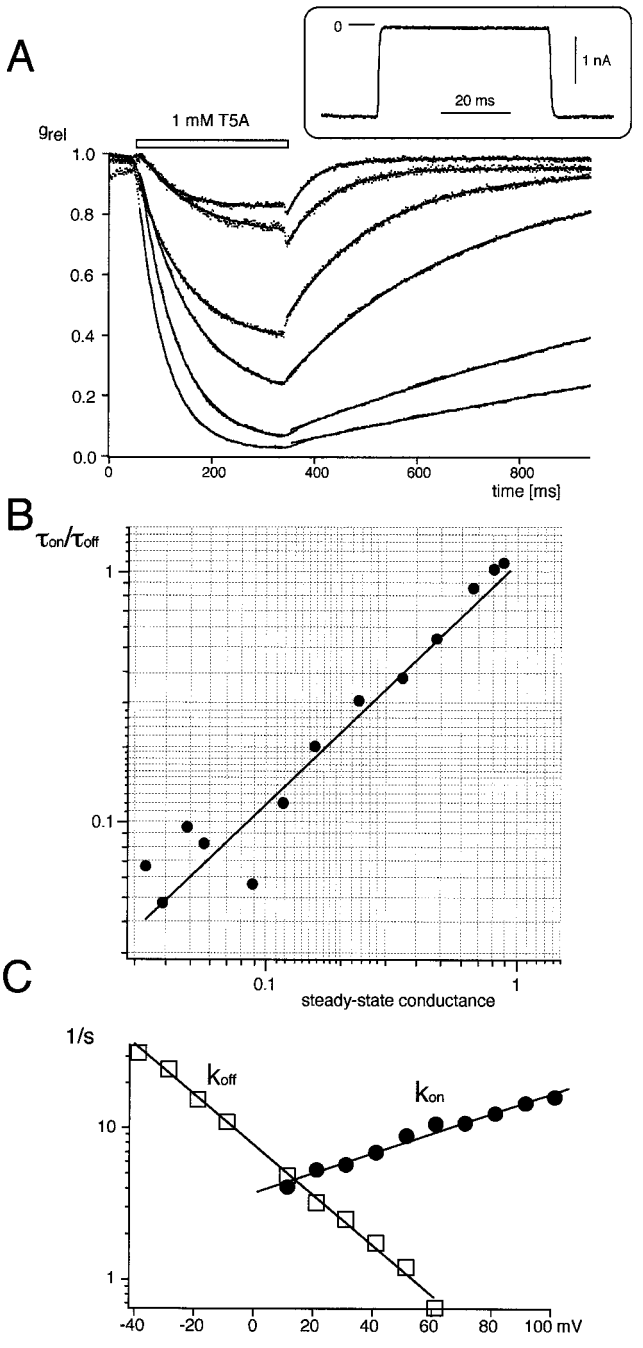

FIGURE 3 Onset and release of T5A-block in $K_{ir}1.1$ channels measured with a rapid application-system. (*A*) Rapid application of 1 mM T5A for 300 ms at various voltages (−20, −10, 10, 30, 70, and 100 mV) in symmetrical 120 mM $[K^+]$. Currents are normalized to the amplitude preceding application of T5A and plotted as relative conductance (g_{rel}). Onset and release of block are fitted with single exponentials. *Inset:* Exchange of intracellular Rb^+ versus K^+ at 0 mV (120 mM $[K^+]_{ex}$). Solution exchange is complete in <2 ms. (*B*) Deviation between data points and fit from the experiment in (*A*) at 10 mV; time axis as in (*A*). (*C*) Ratio τ_{on}/τ_{off} obtained from the fits in (*A*) plotted on a logarithmic scale versus steady-state block obtained from the asymptotic value of the exponentials fitted to the time course of block. Resulting data points were fitted by a straight line with a logarithmic weight. (*D*) Voltage-dependence of on- (k_{on}) and off-rates (k_{off}) determined from the fits to the time course of onset and release of block as $k_{off} = 1/\tau_{off}$ and $k_{on} = (1/\tau_{on}) - k_{off}$ according to first-order kinetics.

dependence of steady-state conductance in terms of electrical distance is approximately explained by the sum of electrical distances calculated from the voltage-dependence of on- and off-rates (Fig. 3 *C*) which were 0.38 and 0.95, respectively.

Taken together, blocking of $K_{ir}1.1$ channels by T5A is characterized by simple first-order kinetics, i.e., one blocking molecule per channel, and by time constants that are far above the exchange time of the fast application device. Thus the $K_{ir}1.1$-T5A system should be an appropriate tool for studying on- and off-rates of block over a wide voltage range and under various ionic conditions on both sides of the membrane.

Dependence of block by T5A on ion-ion interaction

Based on these results it seems interesting to return to the questions on whether the off-rates are dependent on $[K^+]_{in}$ and on how much they contribute to the changes in steady-state block seen upon changes in $[K^+]_{in}$ (Fig. 2 *C*). Fig. 4 shows that the off-rate of T5A is indeed considerably changed by changes in $[K^+]_{in}$ or $[K^+]_{ex}$, while it is virtually independent of the blocker concentration. Almost identical values for τ_{off} are found for 1 and 10 mM T5A, as shown in Fig. 4 *A* for a membrane potential of −30 mV (36.1 ± 2.9 ms, $n = 6$; and 36.2 ± 2.3 ms, $n = 4$), and exhibits almost equal voltage-dependence (Fig. 4 *B*; electrical distance of 0.97 and 0.95, respectively). In contrast, τ_{off} obtained for 10 mM T5A was largely dependent on $[K^+]_{in}$ and $[K^+]_{ex}$. While τ_{off} was 7.2 ± 1.2 ms ($n = 3$) in 12 mM $[K^+]_{in}$ at a membrane potential of −30 mV, the respective value increased ~23-fold at 12 mM $[K^+]_{ex}$, resulting in a τ_{off} of 164 ± 19 ms ($n = 4$). Thus the voltage for a given τ_{off}, e.g., 20 ms, is shifted by as much as 48 mV ($[K^+]_{in}$ 12 mM) and −42 mV ($[K^+]_{ex}$ 12 mM) compared to symmetrical 120 mM K^+. The shift in τ_{off} is thus close to the shift in E_K of ~55 mV calculated for a 10-fold change in concentration at

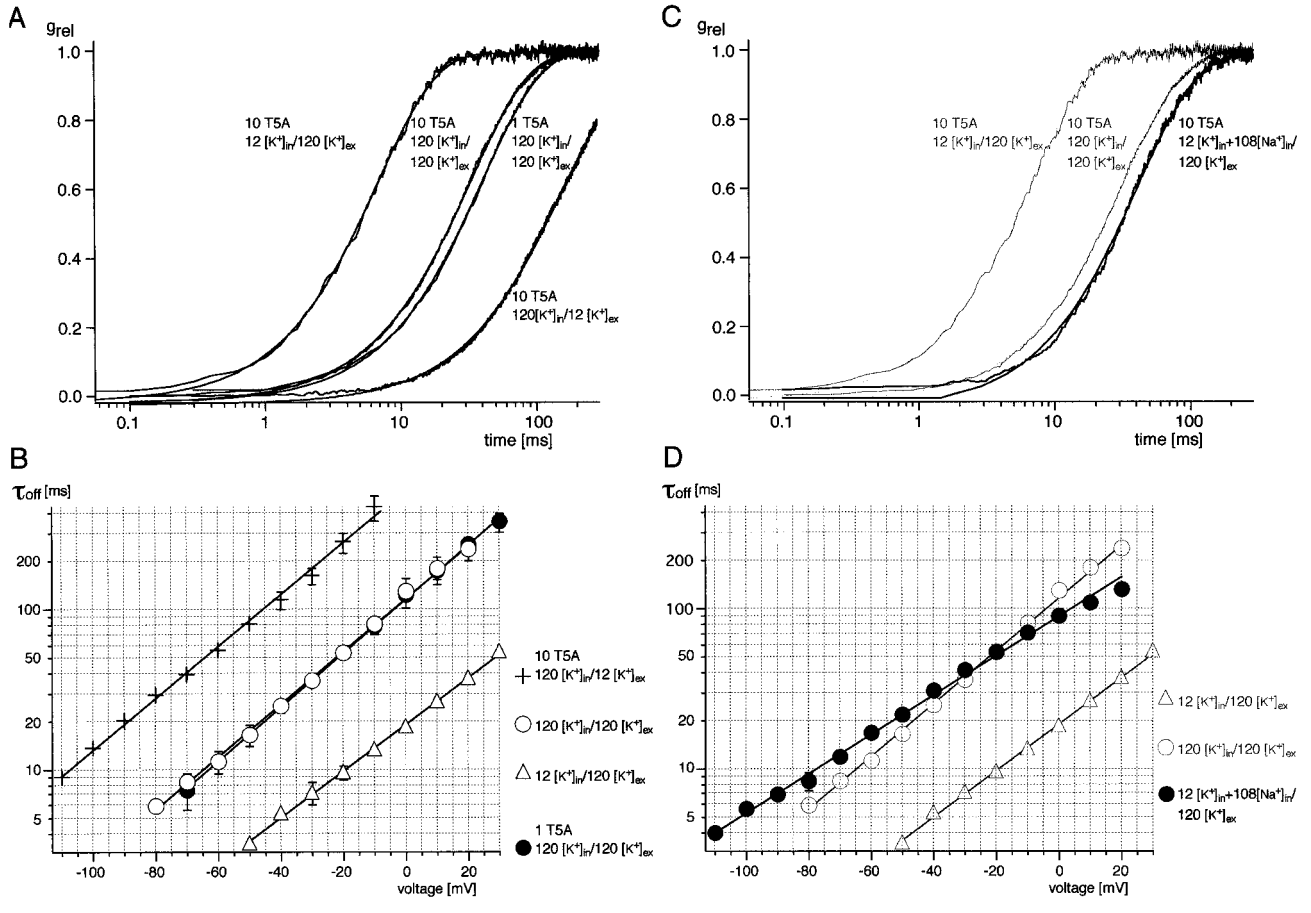

FIGURE 4 Time constants for release of T5A at various potentials and K^+ concentrations. (*A*) Time course of block release determined in an experiment in which membrane potential and intracellular solution were changed simultaneously. Voltage was stepped from 80 mV where channels were blocked completely to −30 mV, concentration of T5A in the intracellular solution (composition as indicated) was changed from 1 or 10 mM to 0 mM. Time course of block-release is plotted on a logarithmic scale together with the result of monoexponential fits; conductance was normalized to the asymptotic value at the end of the wash-out. External (pipette) solution was as indicated. (*B*) Voltage-dependence of the mean of τ_{off} obtained from four experiments as in (*A*) at the conditions indicated. Standard deviations of all means were <10%. Data points were fitted with exponential functions displayed as straight lines on the logarithmic scale. (*C*) Experiment as in (*A*) but with Na^+ substituted for the omitted K^+. (*D*) Voltage-dependence of the mean of τ_{off} obtained for the 12 mM $[K^+]_{in}$ + 108 $[Na^+]_{in}$ condition at 120 mM $[K^+]_{ex}$. Results from (*A*) and (*B*) for the conditions indicated were added in (*C*) and (*D*) for better comparison.

room temperature. It is striking that the sensitivity to changes in $[K^+]_{in}$ is at least as high as for changes in $[K^+]_{ex}$, apparently contrasting the observation that rectification in K_{ir} channels is strictly coupled to E_K only as long as $[K^+]_{ex}$ is changed, but not $[K^+]_{in}$. Coupling of τ_{off} to E_K does, however, not result in an equal shift of the steady-state block (Fig. 2 *C*) because reduction in $[K^+]_{in}$ does also cause an increase in on-rate (Fig. 2 *A*), which compensates for the increase in off-rate.

When experiments as in Fig. 4, *A* and *B* (12 mM $[K^+]_{in}$) were performed under conditions of constant ionic strength, i.e., where the omitted K^+ was replaced by an equal amount of Na^+ (12 mM $[K^+]_{in}$ + 108 mM $[Na^+]_{in}$), τ_{off} shows no more coupling to E_K. In the presence of internal Na^+, τ_{off} at −30 mV was even sightly slower than in 120 mM $[K^+]_{in}$ (Fig. 4 *C*; 41 ± 1 ms; $n = 5$). As shown in Fig. 4 *D*, the voltage-dependence of block release under these conditions is in a similar voltage range as for 120 mM $[K^+]_i$ but it is less steep, resulting in an electrical distance of only 0.71. In addition, similar to what was shown for Rb^+ (Fig. 2 *B*), substitution with internal Na^+ also slowed down the on-rate of T5A block (data not shown). Thus, under conditions of 12 mM $[K^+]_{in}$ + 108 mM $[Na^+]_{in}$, the decrease in on-rate is compensated by a decrease in off-rate resulting in an almost unchanged steady-state block (data not shown). It should be noted, however, that the substitution with 108 mM Na^+ blocks ~80% of the conductance at 60 mV and an interaction of the two blocking ions might be difficult to interpret.

Block of K_{ir}4.1 channels by SPM

Among the strongly rectifying K_{ir} channels cloned so far K_{ir}4.1 exhibits the slowest time constants for onset and release of block by SPM. Therefore, we chose SPM-block in K_{ir}4.1 to verify the results obtained for the "artificial" K_{ir}1.1-T5A system in a "physiological" channel-blocker combination. Fig. 5 characterizes the block of K_{ir}4.1 channels by 3 μM SPM in a way similar to that shown above for K_{ir}1.1 and T5A. The steady-state block fitted with a single Boltzmann function (Fig. 5 *A*) was half maximal at −9.7 ±

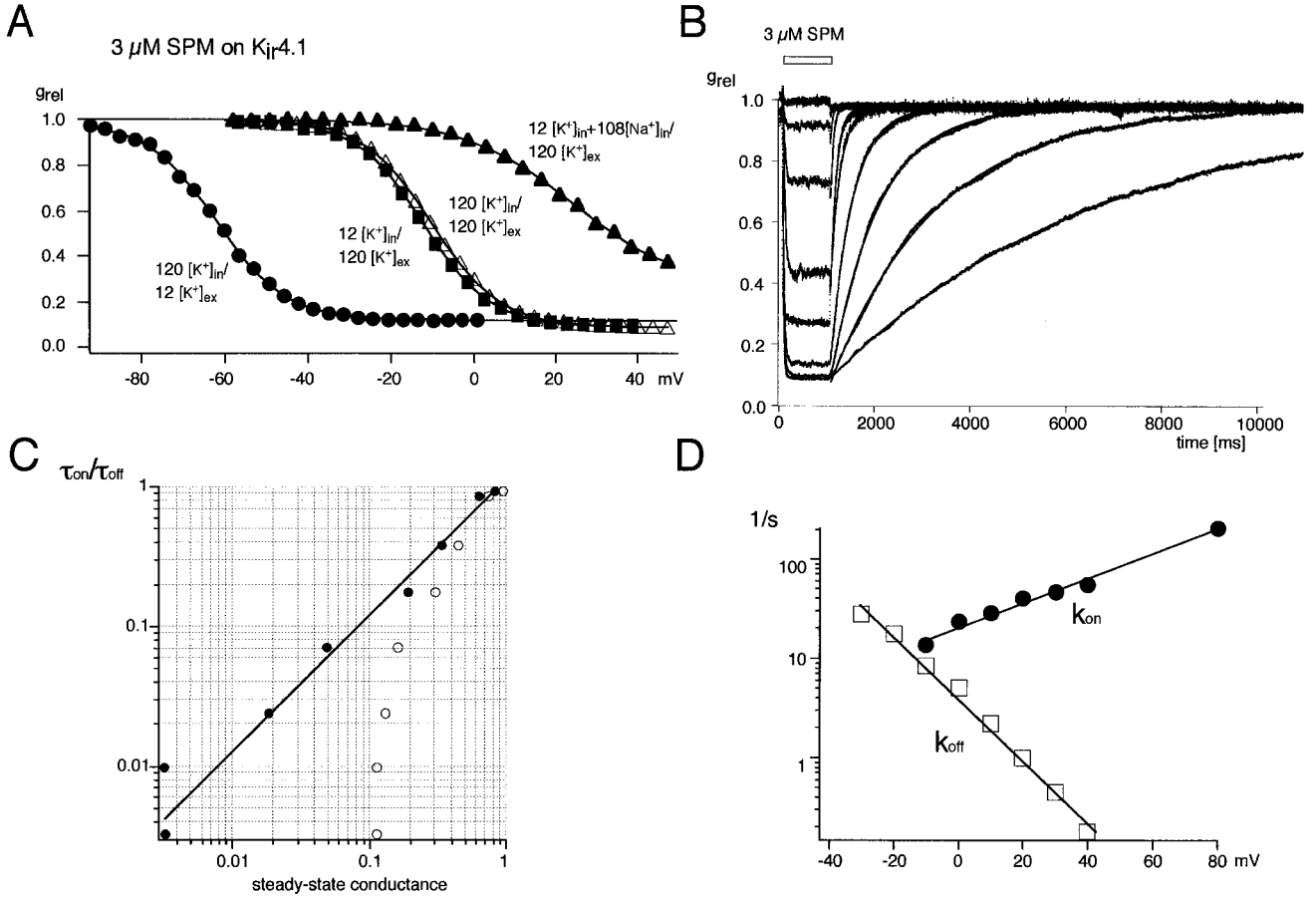

FIGURE 5 Block of K_{ir}4.1 channels by SPM. (*A*) Steady-state block obtained and plotted as in Fig. 1, *A* and *B* measured in the presence of 3 μM SPM under the conditions indicated. (*B*) Fast application of 3 μM SPM in symmetrical 120 mM K^+ at potentials of −30 mV to 40 mV in 10-mV increments plotted and fitted as in Fig. 3 *A*. (*C*) Ratio τ_{on}/τ_{off} obtained from the fits in (*B*) plotted on a logarithmic scale versus steady-state block obtained from the asymptotic value of the exponential function fitted to the time course of block (*open circles*). Filled circles represent correction of steady-state block for the residual conductance calculated by subtracting a constant conductance as described in the text. Data points were fitted by a straight line with logarithmic weight. (*D*) Voltage dependence of k_{on} and k_{off} obtained as in Fig. 3 *D*.

1.3 mV ($n = 4$) in symmetrical 120 mM K^+ and showed a voltage-dependence of 8.27 ± 0.28 mV. Assuming a valence for SPM of 4 this resulted in a δ of 0.76 ± 0.02, which is about half the electrical distance obtained for T5A in $K_{ir}1.1$.

In all experiments performed with $K_{ir}4.1$ and SPM there was a residual conductance of ~10% in the presence of SPM even at highly positive voltages (Fig. 5 *A*), an observation which was never made in experiments with SPM in $K_{ir}2.1$ channels (Fakler et al., 1995).

As shown in Fig. 5 *B* rapid application experiments revealed a time course for onset and release of block that were again nicely fitted with single exponentials. On- and off-rates calculated from the fitted time constants were in good agreement with the steady-state block (*filled circles* in Fig. 5 *C*; fitline with a slope of 1.15 and an offset of 0.0005) once the residual (unblocked) conductance determined as $g_{rel} - \tau_{on}/\tau_{off}$ for the data point at 40 mV was subtracted. On- and off-rates were about twice as voltage-dependent as the rates determined for T5A and showed electrical distances of 0.18 and 0.49, respectively. Similar to what was observed for T5A the sum of electrical distances of on- and off-rates was slightly less than the distance determined from the steady-state block. The time constant of block measured in response to a voltage jump to 80 mV (4.9 ± 0.2 ms; $n = 4$) was accelerated by a factor of 7.8 when the SPM concentration was increased 10-fold (0.63 ± 0.08 ms; $n = 5$). This corresponds nicely to the shift in $V_{1/2}$ by −17.9 mV (to −27.6 ± 0.6 mV; $n = 5$; data not shown) for an electrical distance of 0.76 assuming first-order kinetics [exp(17.9 mV * $\partial zF/RT$) = 8.6]. Taken together, these results strongly suggest that, unlike in $K_{ir}2.1$, SPM-block of $K_{ir}4.1$ channels exhibits simple first-order kinetics, which makes it suitable for a more detailed analysis as carried out above for block of $K_{ir}1.1$ channels by T5A.

Dependence of SPM block on ion-ion interaction

To test whether SPM-block of $K_{ir}4.1$ resembles block of $K_{ir}1.1$ channels by T5A with respect to ion-ion interactions, we first examined the steady-state block mediated by 3 μM SPM under various conditions of intra and extracellular monovalent cations. When $[K^+]_{ex}$ was reduced from 120 to 12 mM, the steady-state block was shifted to more negative potentials by 55 mV, a value exactly corresponding to the calculated change in E_K ($V_{1/2}$: −64.8 ± 2.6 mV, $n = 3$; Fig. 5 *A*). Reduction of $[K^+]_{in}$ by the same amount resulted in a steady-state block that strongly depended on the total concentration of monovalents in the cytoplasmic solution. Steady-state block observed in 12 mM $[K^+]_{in}$ + 108 mM $[Na^+]_{in}$ was shifted to more positive potentials by 30.6 mV relative to the symmetrical K^+ condition ($V_{1/2}$: 20.9 ± 4.2 mV, $n = 3$; Fig. 5 *A*). This suggests coupling of SPM-block to E_K even for changes in $[K^+]_{in}$. However, SPM-block determined in 12 mM $[K^+]_{in}$ without correction for ionic strength even showed a moderate shift to the left ($V_{1/2}$: −16.3 ± 3.8 mV, $n = 6$; Fig. 5 *A*), suggesting that the increase in $[Na^+]_{in}$ rather than the decrease in $[K^+]_{in}$ is responsible for the right-shift in steady-state block.

To further investigate this differential shift in steady-state block, time constants for onset and release of block were measured under various conditions. Similar to block of $K_{ir}1.1$ by T5A, the 12 mM $[K^+]_{in}$ + 108 mM $[Na^+]_{in}$ condition significantly slowed τ_{on} to a value of 49.0 ± 5.1 ms ($n = 3$) at a membrane potential of 80 mV. In contrast, a fivefold decrease in τ_{on} at 80 mV (τ_{on}: 1.1 ± 0.3 ms, $n = 3$) was found when $[K^+]_{in}$ was reduced without correction for ionic strength, while changes in $[K^+]_{ex}$ did not affect τ_{on} (data not shown). As shown in Fig. 6 *A*, τ_{off} displays a similar dependence on intra and extracellular ion concentrations as found for the T5A-block of $K_{ir}1.1$ channels. Decrease in $[K^+]_{in}$ to 12 mM without replacement by Na^+ decreases τ_{off} ~6-fold (from 24.1 ± 2.6 ms, $n = 4$, to 4.0 ± 1.7 ms, $n = 5$, at −30 mV), while the same decrease in

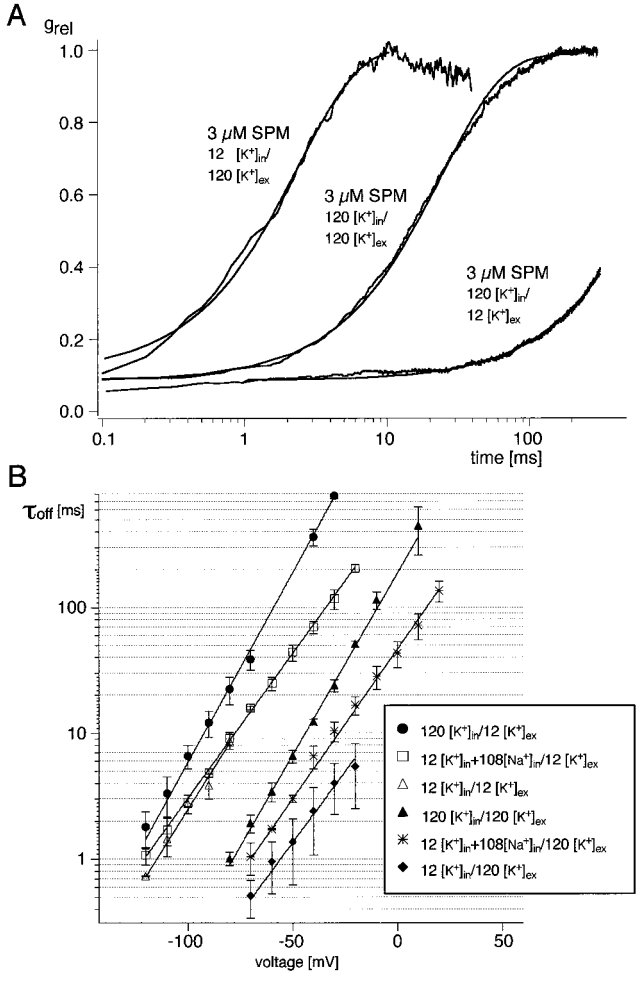

FIGURE 6 Time constants of SPM-release at various potentials and K^+ concentrations. (*A*) Time course of block-release determined in experiments as in Fig. 4 *A* but for 3 μM SPM, voltage step was from 80 mV to −30 mV, $[K^+]_{in}$ and $[K^+]_{ex}$ as indicated. (*B*) Voltage-dependence of the mean of τ_{off} determined from three to five experiments as in (*A*) for all voltages and ion conditions as indicated. Standard deviations indicated by error bars.

$[K^+]_{ex}$ slowed down τ_{off} by as much as a factor of 30 (770 ± 31 ms, $n = 2$, at −30 mV). Analogously with T5A, a 10-fold increase in SPM concentration did not significantly change τ_{off} (20.6 ± 6.0 ms, $n = 8$; at −30 mV in symmetrical 120 mM K^+). Fig. 6 *B* summarizes the values for τ_{off} obtained with 3 μM SPM under various conditions for $[K^+]_{in}$ and $[K^+]_{ex}$ over a wide voltage range. Although the results resemble the data obtained for T5A in $K_{ir}1.1$, it is obvious that the dependence of τ_{off} on $[K^+]_{ex}$ is stronger than that on $[K^+]_{in}$. It is further evident from Fig. 6 *B* that the slope of voltage-dependence of τ_{off} is less steep in the presence of Na^+, resulting in smaller values for the electrical distance. Electrical distances of off-rates were generally slightly more than 50% of the steady-state values, however (not shown).

The off-rates as shown in Fig. 6 *B* together with the on-rates deliver a straightforward explanation for the differential shift in steady-state block with respect to symmetrical K^+ observed for 12 mM $[K^+]_{in}$ with and without correction for ionic strength (Fig. 5 *A*). The only moderate shift in 12 mM $[K^+]_{in}$ results from an equivalent increase of both on- and off-rates, while the large shift in the 12 mM $[K^+]_{in}$ + 108 mM $[Na^+]_{in}$ condition is due to a 10-fold decrease in on-rate in combination with an only moderate increase in off-rate (~2-fold).

DISCUSSION

Analysis of intracellular block of K_{ir} channels with a rapid application system has not been carried out before. This method allowed reinvestigation of some aspects of the mechanism underlying inward-rectification of these ion channels. Previous studies using voltage-jump protocols could not determine on- and off-rates at identical potentials (Lopatin et al., 1995; Lopatin and Nichols, 1996). Nevertheless, these studies have reached detailed information about possible mechanisms of SPM-block in $K_{ir}2.1$ and 2.3 channels which show rapid and complex block kinetics not investigated here. Since the technique used here is limited to the analysis of processes with time constants slower than the solution exchange at giant inside-out patches (~2 ms) it was not possible to measure on- and off-rates for every blocker at every channel subtype. We therefore restricted this study to the slowest and apparently most simple system of inwardly rectifying channels and blockers that allows rather simple kinetic considerations in order to address very basic questions about interaction of blocking and permeant ions.

The major finding of this study is that voltage-dependent block of K_{ir} channels is greatly affected by concentration and species of monovalent ions on both sides of the membrane. The off-rate of block is accelerated by external and decelerated by internal permeant K^+ ions, which is displayed as a symmetrical shift in voltage dependence of this rate constant to the negative and positive voltage-axis, respectively (see Fig. 4 *B*). The known phenomenon that inward-rectification is controlled by $[K^+]_{ex}$ but not by $[K^+]_{in}$ is explained by two findings adding to each other in their effect on the steady-state block. First, the external side of K_{ir} channels exhibits a high selectivity for K^+ ions with respect to induction of changes in τ_{off}, while on the internal side similar effects on the off-rate were observed for the permeant K^+ and impermeant ions such as Rb^+ and Na^+. Second, internal rather than external ions cause a deceleration of on-rates. As a consequence, a reduction in $[K^+]_{in}$ without correction for ionic strength will increase both on- and off-rates and thus result in only moderate shifts of the steady-state block. A decrease in $[K^+]_{in}$ with replacement of the omitted K^+ ions, however, does not change the off-rate, but it decelerates the on-rate and thus results in a right-shift of the steady-state block. As seen for the SPM-block of $K_{ir}4.1$ channels in 12 mM $[K^+]_{in}$ + 108 mM $[K^+]_{ex}$ (Fig. 5 *A*) this shift can be rather strong. What on a first view might appear as E_K-coupling of the steady-state block is actually the result of an increase in $[Na^+]_{in}$ rather than to a decrease of $[K^+]_{in}$. The missing internal ion selectivity together with the effects on the on-rate produce an asymmetrical response of the steady-state block when E_K is changed by changing composition of internal or external bulk ions.

The kinetic analysis showed first-order kinetics for the blocking reaction of both types of blocker and channel indicating a single blocking molecule per channel. This is further supported by the finding that off-rates were independent of the blocker concentration under all conditions tested. An increase in blocker concentration should increase the average number of blocking ions per channel in any multi-ion block model, which in turn would be expected to affect the off-rate and its dependence on the concentration of bulk ions. Electrical distances greater than unity are not necessarily indicative of the presence of more than one blocking ion within the channel pore at the same time. Large electrical distances can result from any other moveable charge within the channel that interacts with the blocking ion. This charge may be part of the channel protein, may be a second blocking ion, or simply be formed by bulk ions that coexist in the blocked channel together with the blocking ion. Based on the higher concentration of bulk ions the latter possibility seems far more likely than the co-presence of two blocking molecules. Interactions of bulk ions with the blocking ion have, therefore, been discussed to explain the large electrical distances (Ruppersberg et al., 1994). Such interactions are different from competition of bulk ions and blocking ion for a common binding site, since competition would not increase the effective charge moved in the blocking reaction. The physical nature of these interactions may be electrostatics such as changes in the negative surface potential or repulsive short-range ion-ion interactions. One striking argument pointing toward short-range ion-ion interactions is the surprising correlation between volume of the blocker and electrical distance observed for the ammonium derivatives. Such an observation was recently considered in a model for voltage-dependent pore-block (Ruppersberg et al., 1994) in which the energy transmitted between ions of different species by short-range interactions is pos-

itively correlated with the volume of the ion absorbing the energy. Since it seems likely that block by ammonium derivatives is caused by single ions receiving additional energy from other charges within the transmembrane electric field rather than by multi-ion block, this idea may apply to T5A-block. Accordingly, a larger blocking molecule receives more energy from interaction with bulk ions than a smaller one.

Taken together, the steady-state *I-V* of K_{ir} channels is controlled by voltage-dependent on- and off-rates of the block by intracellular cations. The strong dependence of these rate constants on internal and external bulk ions is probably mediated by ion-ion-interactions and explains the degree of coupling of inward rectification to E_K.

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