Inward Rectification of the IRK1 K^+ Channel Reconstituted in Lipid Bilayers

Andrei Aleksandrov, Bratislav Velimirovic, and David E. Clapham
Department of Pharmacology, Mayo Foundation, Rochester, Minnesota 55905

ABSTRACT Inwardly rectifying potassium (K^+) channels (IRK1) were incorporated into lipid bilayers to test the relative contributions of various mechanisms to inward rectification. IRK1 channels were expressed in *Xenopus laevis* oocytes and oocyte membrane vesicles containing the channels were fused with lipid bilayers. The major properties of the IRK1 channel were similar whether measured in the oocyte membrane or lipid bilayer; the single channel conductance was 21 pS in 140 mM symmetrical [K^+] and varied as a square root of external [K^+]. Importantly, IRK1 channels display voltage-dependent inward rectification in the absence of divalent ions or charged regulators such as spermine, indicating that they possess an intrinsic rectification mechanism. Although rectification was significantly increased by either Mg^{2+} or spermine added to the cytoplasmic face of the channel, their effects could not be explained by simple block of the open pore. The Hille and Schwartz (1978) model, originally proposed to explain inward rectification by singly charged blocking particles, cannot be used to explain rectification by multiply charged blocking particles. As an alternative, we propose that in addition to a slow gating mechanism producing long lasting open and closed states, there is a distinct, intrinsic fast gating process amplified by cytoplasmic Mg^{2+} and/or polyamine binding to the channel.

INTRODUCTION

Inwardly rectifying K^+ channels differ from voltage-gated outward rectifiers in structure and function. The inward rectifier is presumably a tetramer composed of subunits with two transmembrane-spanning domains surrounding a K^+-selective pore (H5). In contrast, voltage-gated K^+ channels are tetramers of six transmembrane-spanning segments in which the pore region lies between the domains S5 and S6 (Kukuljan et al., 1995). Inward rectification in native channels occurs through voltage-dependent block by cytoplasmic Mg^{2+} (Matsuda et al., 1987; Logothetis et al., 1987; Vandenberg, 1987) and voltage-dependent intrinsic gating (Ishihara et al., 1989; Silver and DeCoursey, 1990). The expression-cloning of the IRK1 inward rectifier K^+ channel from a mouse macrophage cell line (Kubo et al., 1993) enabled structure/function studies to be performed. Mutagenesis studies have shown that amino acids in the M2 domain (D172) and C-terminal domain (E224) play an important role in the mechanism of inward rectification. Mutations in these residues dramatically reduce Mg^{2+}- and polyamine-induced rectification, presumably by lowering the affinity of this negatively charged domain for these positively charged open channel blockers (Stanfield et al., 1994; Taglialatela et al., 1994; Wible et al., 1994; Fakler et al., 1994; Yang et al., 1995; Lopatin et al., 1994; Fakler et al., 1995). It has been suggested that open channel block by polyamines accounts for an additional voltage-gating mechanism, complementary to the Mg^{2+} block. Block by both Mg^{2+} and polyamines has been proposed to obviate the need for any other mechanism for inward rectification (Lopatin et al., 1994; Fakler et al., 1995; Yang et al., 1995).

Hille and Schwartz' theory (1978) explained inward rectification on the basis of the block of an invariant, conductive pore without an intrinsic channel gating mechanism. We present evidence that this simple mechanism cannot explain the experimental data without the inclusion of a mechanism for fast intrinsic gating. The fast gating process of IRK1 is enhanced by Mg^{2+} and polyamines, but it is not caused by voltage-dependent block of the open channel. A preliminary report of this work has appeared previously in abstract form (Aleksandrov et al., 1995).

MATERIALS AND METHODS

The cDNA encoding the murine inward rectifying potassium channel, IRK1 (Kubo et al., 1993), was transcribed in vitro (mCAP, Stratagene, La Jolla, CA). *Xenopus* oocytes were surgically removed, defolliculated in 2 mg/ml collagenase and injected with 2 ng/oocyte of IRK1 cRNA. Injected oocytes were screened for inwardly rectifying K^+ current 48–72 h later. Two-electrode voltage clamp recordings were performed with a Turbo TEC 01C (NPI Instruments, Tamm, Germany). The bath solution (K-96) contained in mM: 96 KCl, 2 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 K-HEPES (pH 7.4). Inside out patch clamp recordings were filtered with an 8-pole Bessel filter (cutoff frequency 2 kHz), sampled at 10 kHz through a DigiData 1200 A/D converter (Axon Instruments, Sunnyvale, CA), and analyzed with pCLAMP 6.0 software. Amplitude histograms were fitted by multiple Gaussian functions using Origin software (MicroCal Software, Inc., Sunnyvale, CA).

For lipid bilayers, plasma membranes from oocytes were isolated according to Bretzel et al. (1986), and plasma membranes were detached from the cortical vesicles according to Kinsey et al. (1980). Water-injected oocytes served as controls. The probability of inward rectifier K^+-channel incorporation after fusion was <1% in controls compared to membrane

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The permanent address for Dr. Aleksandrov is the Institute of Cell Biophysics, Russian Academy of Science, Puschino, Moscow Region, 142292, Russia.

Address reprint requests to Dr. David E. Clapham, Department of Pharmacology, Mayo Foundation, Rochester, MN 55905. Tel: 507-284-5881; Fax: 507-284-9111; E-mail: clapham@mayo.edu.

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vesicles from expressing oocytes, which was in a good agreement with the low density of basal inward rectifying K^+ channels in oocyte plasma membranes. Lipid bilayers were prepared from a phospholipid solution in *n*-decane containing a 1:1 mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3 phosphocholine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (15 mg/ml). The 0.2-mm diameter bilayer capacitance was ~100 pF. The lipid bilayer separated 1.0 ml of solution in the teflon cup from 4.0 ml of a solution in an outer glass chamber. Both chambers were magnetically stirred. The teflon cup will be referred to as the *cis* compartment and the glass chamber as the *trans* compartment. Electrical contact with the solutions was provided by Ag/AgCl electrodes through agar bridges filled with 0.5 M KCl. The membrane potential difference was measured as the difference between *trans* and *cis* potentials. The *trans* side was grounded and electrical measurements of the single channel current were performed under voltage clamp using an Axopatch 200A (Axon Instruments) amplifier. The output signal was filtered with an 8-pole low pass Bessel filter with the cutoff frequency set at 200 Hz. Membrane vesicles were added as a concentrated stock solution to the teflon chamber only (*cis* side) for a final protein concentration of 15–30 μg/ml. Spontaneous fusions of the vesicles with the preformed bilayer were recorded as fast transient capacitance currents in the voltage-clamp mode and were followed by the appearance of rectangular current jumps typical for single ionic channels. The best results were achieved in symmetrical salt solutions containing, in mM: 120 KCH_3SO_3, 5 EGTA, 2 $MgCl_2$, 10 HEPES, and 20 KOH. Without free Ca^{2+}, and with low concentrations of Cl^- ions on both sides of the membrane, inwardly rectifying K^+ channels appeared in <10% of the fusions. An unrelated, high-conductance, nonselective, voltage-dependent channel was also observed. Fusions were accomplished by intensive stirring of the *cis* chamber, and further fusion events were prevented by termination of the stirring.

RESULTS

Recording of IRK1 in Xenopus oocyte

In two-electrode voltage clamp recordings from *Xenopus* oocytes expressing IRK1, barium (Ba^{2+}) block of IRK1 was strongly voltage- and time-dependent, with the highest level of block at more negative voltages (Fig. 1 *a*). $BaCl_2$ (0.2 mM) blocked >95% of the current at −140 mV. At negative potentials, Ba^{2+} block was time-dependent (Fig. 1 *a*, *inset*) with time constants in the range of 100 ms. Cs^+ block of the channel was voltage-dependent (90% block by 0.8 mM Cs^+) and appeared instantaneously with hyperpolarization (Fig. 1 *b*). Single channel recordings of IRK1 from inside-out oocyte patches revealed a conductance of 21 pS in equimolar $[K^+]$ (140 mM; Fig. 1 *c*). With 1.8 mM $MgCl_2$ in the bath solution, the channel rectified strongly in the inward direction (Fig. 1, *c* and *d*). The dwell times were fit by a single exponential for mean open times (τ_o = 510 ± 40 ms, n = 4) and a double-exponential for mean closed times (τ_{c1} = 75 ± 15 ms, n = 4 and τ_{c2} = 550 ± 50 ms, n = 4) (Fig. 1 *e*). These data do not differ substantially from the electrophysiological properties of the IRK1 channel reported by Kubo et al. (1993).

Giant patch current recordings were performed on oocytes expressing IRK1 channels (Fig. 2, *a* and *b*). At the beginning of the experiment, pipette and bath solution were identical (140 mM KCl, 2 mM $MgCl_2$; Fig. 2 *a* trace 1). When the bath was perfused with the Mg^{2+}-free solution, an increase in inward current was observed (Fig. 2 *a* trace 2), which increased further after excision (Fig. 2 *a* trace 3). Excision in the Mg^{2+}-free solution also caused a large increase in the outward current, but inward rectification at potentials more positive than +60 mV was still present. After extensive washout of the intracellular side of the patch and removal of the oocyte from the recording bath, spermine added to the bath solution blocked the outward current (Fig. 2 *a*, trace 4 and Fig. 2 *b*, curve 4). The zero current potential was shifted by −32 mV by the addition of 400 mM KCl to the bath solution (Fig. 2 *a*, trace 5 and Fig. 2 *b*, curve 5), corresponding to the predicted shift of E_K. The relative conductance (G/G_{max}) plotted as a function of the membrane potential demonstrated the characteristic feature of inward rectification; the midpoint of the curve shifted with E_K (Fig. 2 *c*).

Recording of IRK1 in lipid bilayer membranes

Membrane vesicles were prepared from IRK1-expressing oocytes and fused with the lipid bilayer. We observed that the cytoplasmic face of IRK1 channels oriented to the *trans* compartment, as determined by the direction of rectification. Functional IRK1 channels did not run down for more than 1 h after fusion into the bilayer. The electrophysiological properties of these channels closely corresponded to the properties of native channels measured in patches. In solutions with equimolar 140 mM $[K^+]$ and 2 mM $[Mg^{2+}]$, small but measurable single channel currents were recorded in the outward directions (Fig. 3, *a* and *b*). The single channel conductance in the inward direction was 21 pS and varied in direct proportion to the square root of *cis* $[K^+]$ (corresponding to $[K^+]_o$) up to 600 mM (Fig. 3 *g*). The slope of the relation between the IRK1 channel reversal potential and *cis* $[K^+]_o$ was 55 mV/decade, consistent with a K^+-selective pore (Fig. 3f).

Although removal of Mg^{2+} from the *trans* (cytoplasmatic) side increased the amplitude of the outward single channel current, it did not completely remove rectification (Fig. 3, *c* and *d*). We did not obtain a linear single channel I-V relation in symmetrical Mg^{2+}-free solution in contrast to published data from inside-out patches (Matsuda et al., 1987; Vandenberg, 1987). The likely explanation for this discrepancy is that a rapid (150 ms) ramp protocol (−100 to 100 mV) was used in the patch clamp experiments whereas a slow (4 s) ramp protocol (−100 to 80 mV) was used for the lipid bilayer. Use of the slow ramp protocol or measurement of steady-state single channel currents, revealed the nonlinearity in the current-voltage relation. The nonlinearity developed as a smooth transition without any apparent changes in single channel kinetics. The same residual rectification was reported for recombinant IRK1 channels in giant patch experiments under steady-state conditions (Fakler et al., 1994). In cardiac inward rectifying potassium channels, three subconductance levels recorded in the outward direction (steady state; low $[Mg^{2+}]_i$; Matsuda, 1988) were used to justify a triple barrel model. For IRK1 reconsti-

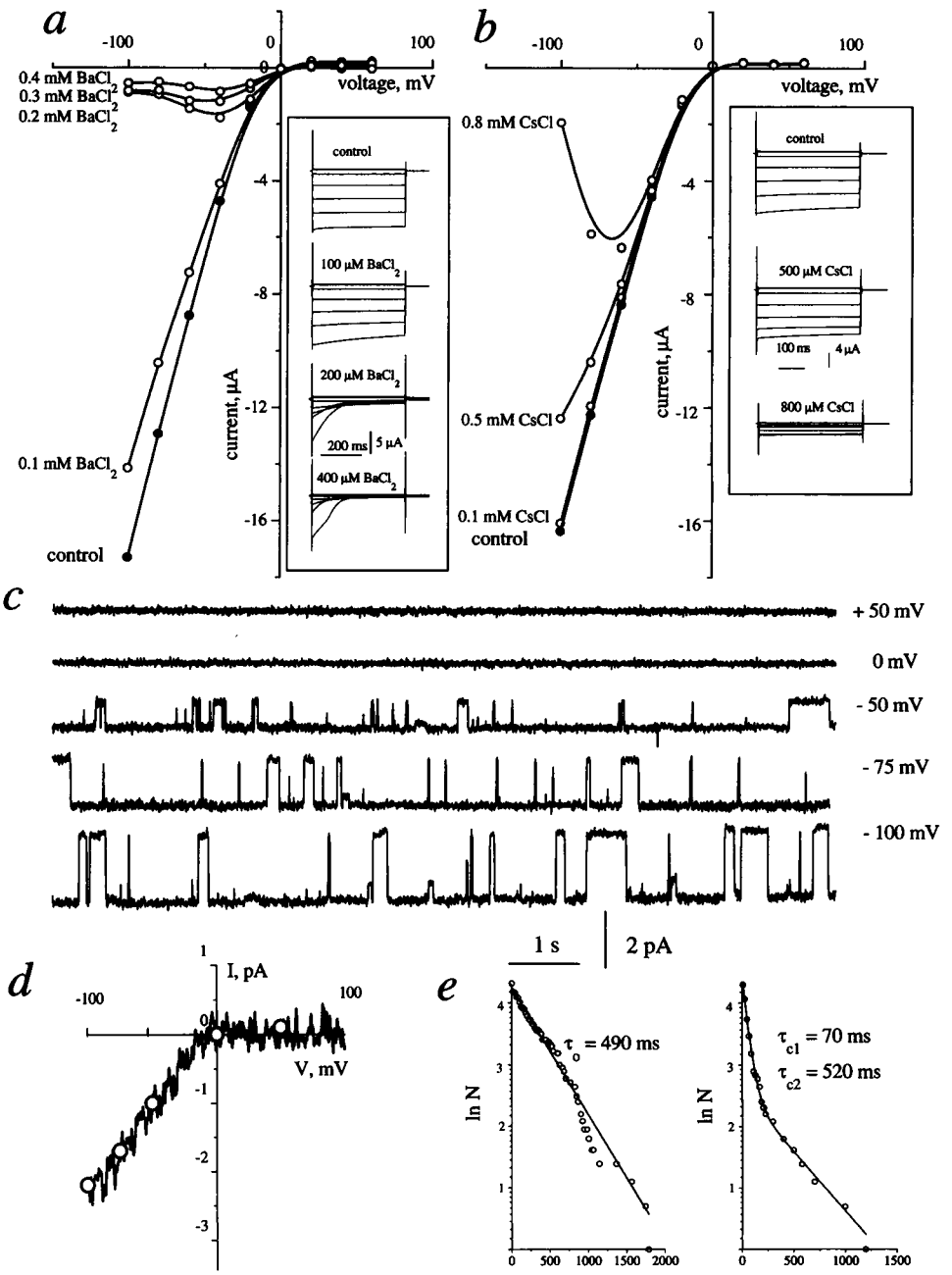

FIGURE 1 IRK1 expressed in *Xenopus* oocytes. Expressed IRK1 current exhibited strong inward rectification under voltage clamp conditions and was blocked by external Ba^{2+} and Cs^+ (*a,b*). Currents were measured at the end of the 400-ms voltage steps (−100 mV to +60 mV in 20-mV steps). Holding potential was −10 mV. Bath solution contained in mM: 96 KCl, 2 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-KOH (pH = 7.4). *a*) Ba^{2+} block of expressed IRK1 current. *b*) Cs^+ block of expressed IRK1 current. *c*) Single IRK1 channel records from excised inside-out oocyte patches. Records were filtered at 500 Hz (low pass Gaussian filter). Pipette and bath solutions contained in mM: 120 KCl, 5 EGTA/10 KOH, 2 $MgCl_2$,10 HEPES-KOH (pH = 7.2). *d*) *i*-V relation for a single IRK1 channel expressed in an oocyte. IRK1 single channel current was recorded during a ramp protocol from −100 mV to +100 mV (200 mV/300 ms). Capacitance and leakage currents were subtracted using blank records. The superimposed open circles indicate the current values calculated from amplitude histograms of single channel records at the appropriate voltages shown in *c*. *e*) Distribution of the dwell-times for the IRK1 single channels shown in *c*. Open times were well-fitted by a single exponential of τ_o = 490 ms, and by a double-exponential for closed time constants τ_{c1} = 70 ms and τ_{c2} = 520 ms.

tuted in the lipid bilayer, outward currents under steady-state conditions (+100 mV; Fig. 3) were threefold smaller than currents recorded at −100 mV. This could be accounted for by channels opening to the first putative subconductance level (Fig. 3, *c* and *d*). But, in contrast to the data from ventricular myocytes, our reconstituted IRK1 did not display a linear I-V relation in the outward direction. For example, the single channel current at +50 mV was only twofold smaller than at −50 mV. Therefore our data do not support the triple barrel model for IRK1.

The dwell times for both the open and the closed states measured at steady state could be fitted by monoexponential functions with time constants of τ_o = 545 ± 35 ms, n = 6, and τ_c = 3.5 ± 0.7 s, n = 6 (Fig. 3, *e* and *f*), accounting for the observed slow gating process. Neither the mean open times nor mean closed times varied significantly with membrane potential for the slow gating process (Fig. 4 *a*). Mean closed times were slightly longer in the absence of Mg^{2+}, but open times were not significantly different in the presence or absence of Mg^{2+}. Open channel probability (p_o; calculated as $\tau_o/(\tau_o+\tau_c)$) also did not vary as a function of $[K^+]_o$ over the range from 100 to 600 mM $[K^+]_o$(Fig. 4 *b*). In summary, observable slow channel kinetics were independent of both membrane voltage and $[K^+]$. The fast gating process presumed to underlie the rapid decay seen in oocytes at depolarized potentials (Figure 2 *a*, *trace 3*) could not be recorded from the bilayer because of the large bilayer capacitance. The settling time restricted our observations to

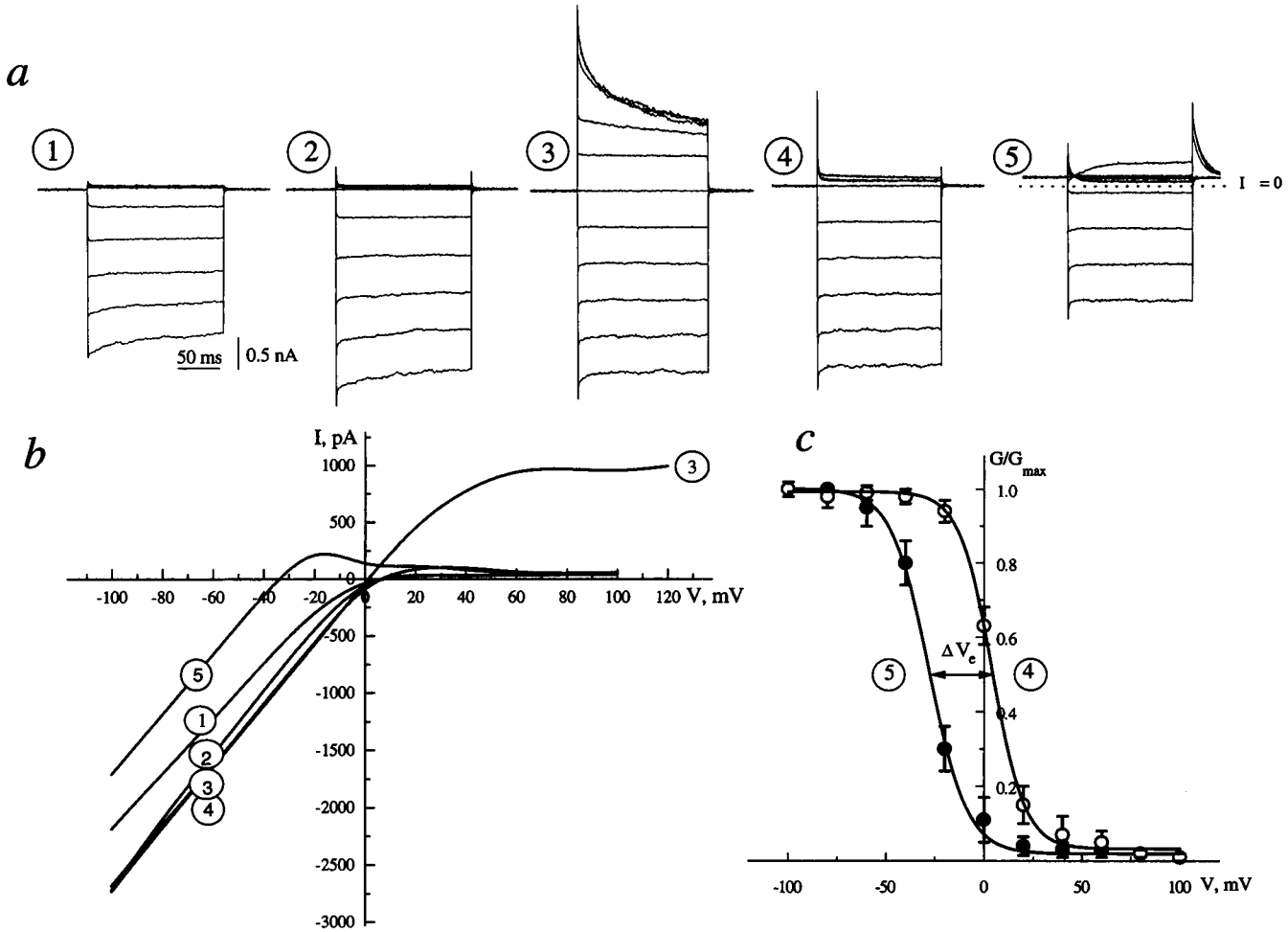

FIGURE 2 IRK1 currents measured from giant patches from *Xenopus* oocytes. The pipette solution in all experiments contained, in mM: 120 KCl, 5 EGTA, 2 $MgCl_2$, 10 HEPES-KOH (pH = 7.2). *a*) Current recordings were elicited by a 400-ms duration rectangular pulse protocol from −100 mV to +100 mV in 20-mV increments. At the beginning of the experiment (1) the pipette and bath solution were identical. In (2) the bath was perfused in the same solution but without Mg^{2+}. Note the small increase in inward current. Outward currents developed after excision of the patch (3). The addition of 25 μM spermine to the bath solution blocked the outward component (4). E_K was shifted to −32 mV by the addition of 400 mM KCl in the bath solution (5). *b*) I-V relations were plotted from the steady-state currents measured at the end of rectangular pulses in *a*. *c*) I-V curves like 4 and 5 were used to plot relative conductance (G/G_{max}) as a function of membrane potential, V_m. Experimental points shown represent mean values ± SEM for three different sets of experiments. The shift of the midpoint of G/G_{max} was accompanied by a shift in the zero current potential, V_r.

the steady state (measured 1 s after the depolarization step) and concomitant bandwidth limitations restricted time resolution to ≥5 ms.

Comparison of experimental data with theory

Hille and Schwarz (1978) proposed that the K^+-selective inward rectifier channel is a multi-ion single-file pore and inward rectification could be explained by a cytoplasmic charged blocking particle. Three main assumptions of the model were 1) comparable binding site affinities for blocking and permeating ions in the channel; 2) equal charges for blocking particle and permeating ion; and 3) sufficient concentrations of permeating ions to produce multiple occupancy of the pore along with comparable concentrations of the blocking particle and permeating ions.

We converted the distribution coefficient between blocked and unblocked states (Eq. 20 in Hille and Schwarz, 1978) to the relative conductance, G/G_{max}, as a function of membrane potential, V_m (Fig. 5). We used a three-binding-site model as the simplest explanation for the observed sensitivity of G/G_{max} to the reversal potential. By assuming a ratio of blocking particles/permeating ions (polyamines/$[K^+]$ = 0.001) as a typical range under experimental conditions, rectification was observed at membrane potentials more positive than +75 mV (instead of 0 mV, the zero current potential under our experimental conditions; Fig. 5). This deviation from theory was not surprising given the underlying assumption that comparable concentrations did not exist for blocking and permeating ions (Hille and Schwartz, 1978). The experimental data shown in Fig. 5 demonstrate that rectification takes place at potentials near

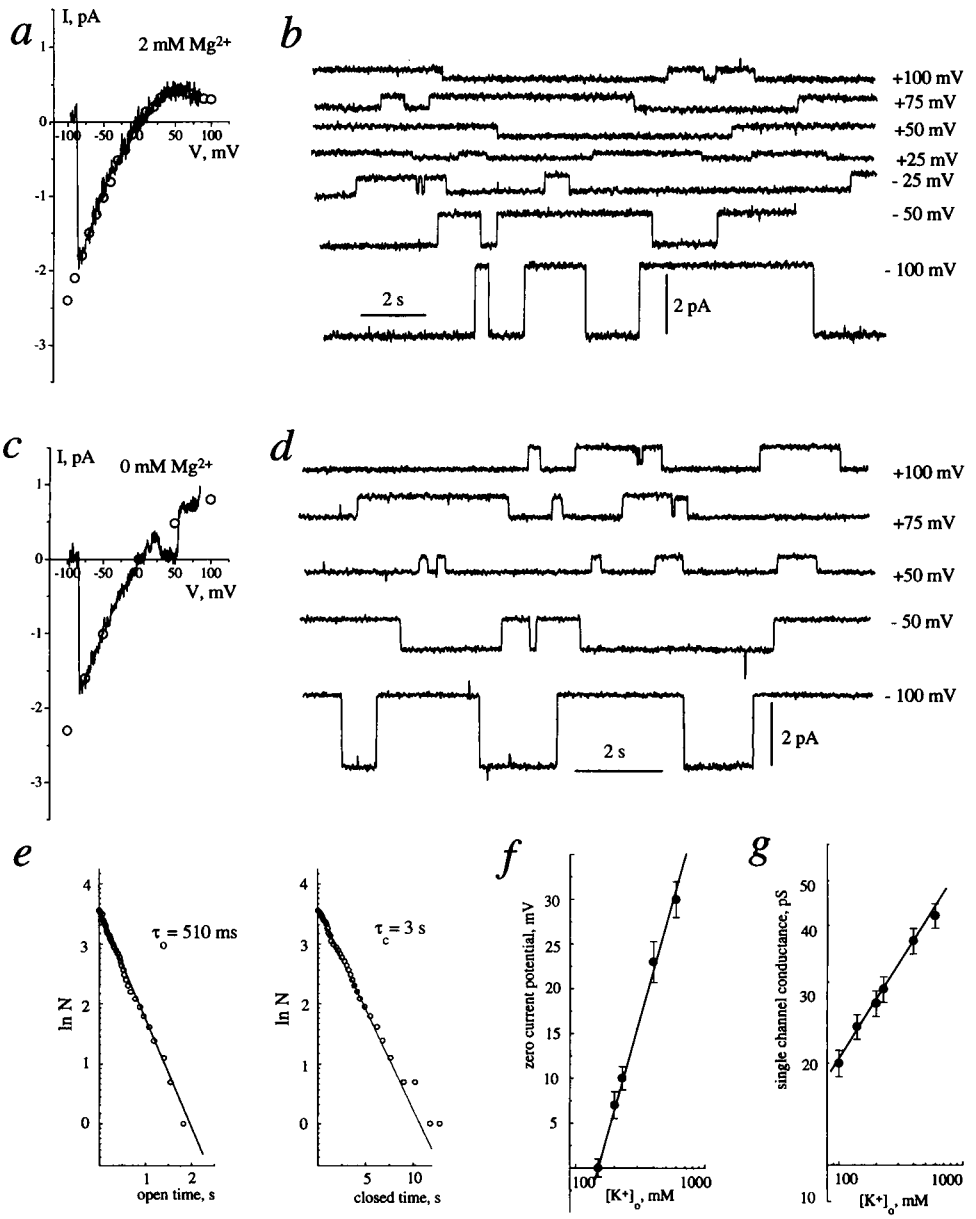

FIGURE 3 Single IRK1 channels incorporated in lipid bilayers. *a*) Ramp protocol (−100 mV to +80 mV at 40 mV/s) reveals strong inward rectification (2 mM $[Mg^{2+}]_i$) of an IRK1 channel in a lipid bilayer. The superimposed open circles indicate the current values calculated from amplitude histograms of single channel currents at the indicated voltages. *b*) IRK1 single channel currents. The capacitance and leakage currents were subtracted using blank records and filtered at 100 Hz. *c, d*) Weak inward rectification of single IRK1 channel incorporated in the bilayer after removing $[Mg^{2+}]_i$ (addition of 20 mM EDTA-KOH to the *trans* chamber). *e*) Distribution of the dwell-times. Experimental data were fit by a single exponential with time constants τ_o = 530 ms for mean open time and τ_c = 3 s for mean closed time. *f*) Reversal potential for single IRK1 channel as a function of $[K^+]_o$ (*cis*); $[K^+]_i$ (*trans*) was kept constant at 140 mM. The best fit is a straight line with a slope of 55 mV/decade. *g*) IRK1 single channel conductance in the inward direction as a function of $[K^+]_o$ (*cis*). The best fit of the slope was 0.42, close to the expected square root dependence of inward rectifier conductances on $[K^+]_o$.

the zero current potential (V_r) and that the shift in V_r (ΔV_r) by increasing $[K^+]_i$ leads to the parallel shift (ΔV_e) of G/G_{max}. The blocking particle model predicts less than half the observed shift of G/G_{max} (ΔV_t; Fig. 5 *a*). The same blocking particle model predicts an equal shift in G/G_{max} if ΔV_r is shifted by an appropriate change in $[K^+]_o$. In other words, for the single charge blocking particle model, the shift of the relative conductance depends on the mechanism by which V_r is shifted—it depends not only on $[K^+]_i/[K^+]_o$, but also on the [blocking particle]/[permeating ion] ratio. The data shown on Fig. 5 *a* cannot be fit by the singly charged blocking particle model, regardless of the number of binding sites.

The midpoint of G/G_{max} can be shifted by increasing the charge of the blocking particles (Fig. 5 *b*), but electrical forces on the blocking particle and permeating ion would no longer match and ΔV_t will always be much less than ΔV_r (Hille and Schwartz, 1978). The position of the midpoint of G/G_{max} on the V_m axis will be governed by V_m to a greater extent than V_r. The blocking particle model for inward rectification assumes equal charges for the blocking particle and permeant ions. But block by Mg^{2+} or any other multiply charged cation would cease at negative potentials for any reasonable ratio of blocking particle/permeating ion concentration and thus cannot explain the rectification observed at potentials approaching E_K under physiological conditions.

If we suppose a higher binding site affinity for the blocking particle, we would expect to detect the block of both outward and inward currents in contrast with experimental results. A multiply charged blocking particle with higher binding site affinity results in pure block rather than inward

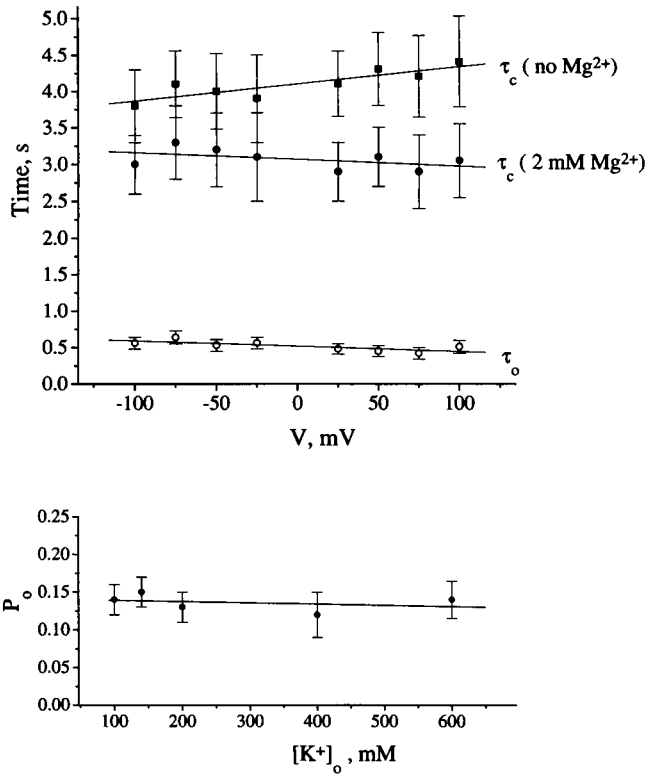

FIGURE 4 Slow (>5 ms) IRK1 channel kinetics are independent of V_m and $[K^+]_o$. *a*) Mean open and closed times of IRK1 channels incorporated into lipid bilayers do not vary significantly over the physiologic voltage range. Single channels were recorded in symmetrical 140 mM $[K^+]$, ±2 mM Mg^{2+}. Closed times averaged slightly longer in the absence of Mg^{2+}. Each point is the mean ± SEM from three to five separate experiments. *b*) Channel open probability (P_o) is independent of $[K^+]_o$. Mean open times averaged 620 ± 90 ms and mean closed times (in 2 mM Mg^{2+}) averaged 3.1 ± 0.1 s at −100 mV.

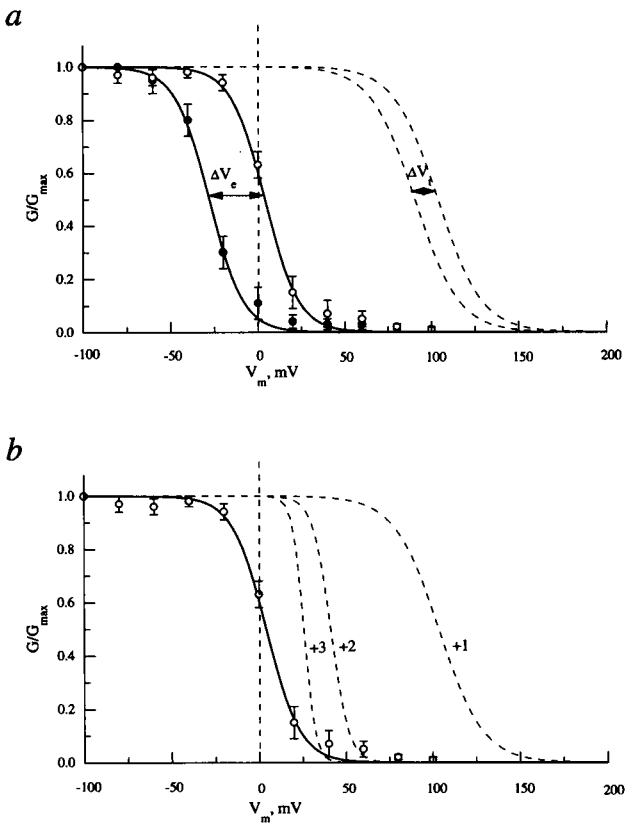

FIGURE 5 Failure of the blocking particle model (for a K^+ channel with three binding-sites) to fit the experimental data. The fraction of unblocked channels, G/G_{max}, was solved using Eq. 20 from Hille and Schwarz (1978). In the Hille and Schwartz nomenclature, R_b is the fraction of blocked channels. We solved for $1 - R_b$, the fraction of unblocked channels, to obtain G/G_{max} as follows:

$$[G/G_{max}]^{-1} = 1 + q_i/s_i \{ 1 + \exp(V_m) * q_i/s_o + \exp(V_m - E_K) + [\exp(V_m) * q_i/s_o + \exp(V_m - E_K)]^2 \}$$

where G/G_{max} = ratio of conductance to the maximum conductance at −100 mV, q_i, s_i, s_o are dimensionless reduced activities (as defined in Hille and Schwartz, 1978) of the internal blocking particles $[K^+]_i$ and $[K^+]_o$, respectively. V_m and E_K are membrane potential and K^+ equilibrium potential, respectively. *a*) Comparison between experimental data (*solid lines*) from Fig. 2 *c* and theory (*dashed lines*) predicted by the blocking particle model with a singly charged blocking particle. Right dashed curve is G/G_{max} calculated for symmetrical conditions $s_i/s_o = 1$, $E_K = 0$, and $q_i/s_i = q_i/s_o = 0.001$ assuming [blocking particle] = 100 μM. The theoretical shift in relative conductance ($\Delta V_r = -12$ mV) is associated with the shift of zero current potential $\Delta V_r = -32$ mV by increasing $[K^+]_i$: $s_i/s_o = 3.9$, $q_i/s_i = 0.00025$, $q_i/s_o = 0.001$, $E_K = -32$ mV or −1.28 kT/e. Experimental data are the same as in Fig. 2 *c*. Experimental data were fitted by a Boltzmann function (*solid line*; $z = 2.6$). The shift between experimental curves at $G/G_{max} = 0.5$ is $\Delta V_e = \Delta V_r = -32$ mV or −1.28 kT/e. *b*) G/G_{max} plotted for multiply (n) charged blocking particles (*dashed line*; e.g., Mg^{2+} and/or polyamines). Experimental data from *a*) fitted by a Boltzmann function ($z = 2.6$), are shown for comparison.

rectification. Under symmetrical conditions most often used in inside-out patches, simple block of the open channel could be mistaken for rectification. Shifting the membrane potential away from zero is necessary to distinguish between open channel block and inward rectification. We conclude that a model of inward rectification based on the blocking particle mechanism with multiple charges and/or high binding site affinity cannot explain our experimental results.

DISCUSSION

Inward rectification in native channels involves both voltage-dependent block of the open channel by cytoplasmic Mg^{2+} and voltage-dependent intrinsic gating (Matsuda et al., 1987; Vandenberg, 1987; Ishihara et al., 1989; Silver and DeCoursey, 1990). Block of the open channel by internal polyamines was proposed as the mechanism of intrinsic gating (Lopatin et al., 1994; Fakler et al., 1995). In this paper we have shown in lipid bilayers, where polyamines are presumably absent, that IRK1 channels still possess intrinsic, voltage-dependent inward rectification.

Point mutations in a region of the second putative transmembrane-spanning domain in IRK1 (amino acid #172) and ROMK1 alter the degree of rectification. For IRK1 (D172N), the instantaneous and steady-state I-V relation

became linear when internal Mg^{2+} was removed (Wible et al., 1994). Inverse point mutations in ROMK1 at the same position (N172D) led to stronger rectification (Lu and MacKinnon, 1994). In both cases, the point mutations did not change single channel conductance or selectivity, implying that the D to N mutation lowered the affinity of the channel for Mg^{2+} and polyamines, but maintained other properties of the open channel as a water-filled pore (Lopatin et al., 1994; Ficker et al., 1994; Yang et al., 1995). This suggests that the M2 domain is not a part of the pore and therefore intrinsic rectification is not a property of the open pore structure. An additional point mutation (E224G) in the carboxyl terminal attenuated the potency of Mg^{2+} and polyamine block, and could not be explained by 1–1 binding (Yang et al., 1995). Yang et al. suggested that part of the hydrophilic C-terminal domain as well as the hydrophobic M2 domain contributed to the channel pore in IRK1. These data suggest a mechanism other than simple open channel block for inward rectification. We hypothesize that inward rectification is an intrinsic property of channel structure and may be modulated by positively charged species such as Mg^{2+} or polyamines.

In contrast to the results of Wible et al. (1994), but in agreement with Fakler et al. (1994), the IRK1 single channel *i*-V measured from the lipid bilayer under steady-state conditions (0 $[Mg^{2+}]_i$) was not linear. A gradual reduction in the open-state conductance was demonstrated by Coronado and Miller (1979) for the Cs^+ block of the sarcoplasmic reticulum K^+ channel. They proposed that internally applied Cs^+ ions move part of the way across the open pore to form the blocking complex and produce extremely brief interruptions of the ion current that can only be detected as an apparent reduction in the level of the open channel current. For IRK1, we have observed a gradual reduction in the open channel conductance for a single reconstituted channel in symmetrical solutions containing no cations other than K^+. To explain this phenomenon in the simplest way, we propose an intrinsic fast gating mechanism with an open/closed ratio dependent on membrane potential, $[K^+]_i$ and $[K^+]_o$. The apparent open channel conductance will correspond to a time-averaged value determined by the rapid open-closed flickering rate. According to our experimental data, neither of the rate constants of the slow gating process are voltage- or K^+-dependent. Thus, there are at least two different gating mechanisms, a slow one that is voltage- and K^+-independent, and the other a fast one that is voltage- and K^+-sensitive.

The simplest kinetic model for two independent gating mechanism has four states: (O) fast gate open, slow gate open; (C) fast gate closed, slow gate open; (I_o) fast gate open, slow gate closed; (I_c) fast gate closed, slow gate closed. Using the kinetic scheme of Fig. 6 we can solve the coupled-state equations to obtain G/G_{max}. Taking into account the fact that the slow equilibrium constant, K_s, is voltage- and $[K^+]$-independent, the equations reduce to $G/G_{max} = 1/(1+K_f)$. The experimental data for single channel and macroscopic current shown in Fig. 6 *b* illustrate

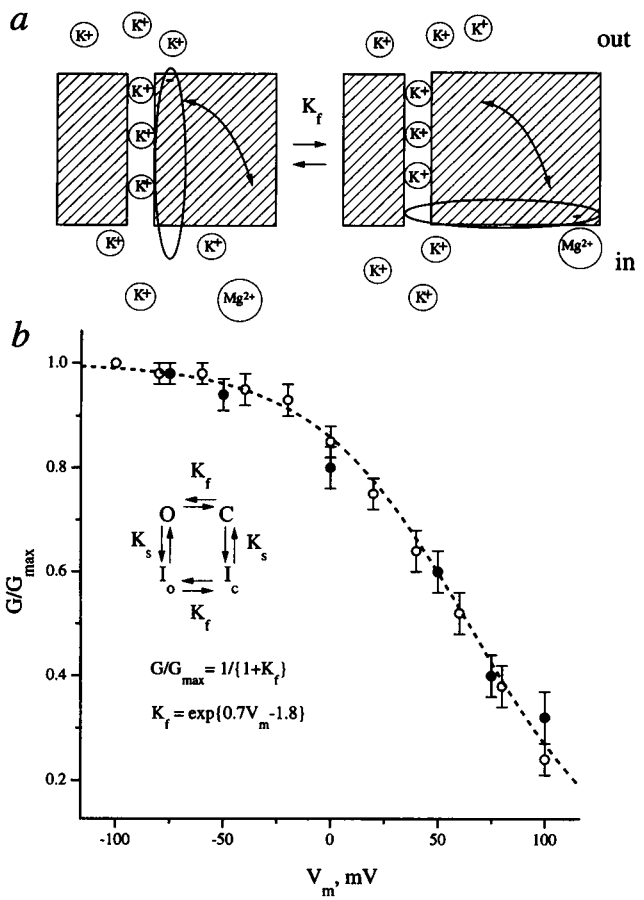

FIGURE 6 Kinetic model of the IRK1 channel. *a*) The fast gate is represented as a hinged oval that is negatively charged at one end. In the open state, the negatively charged end is exposed to the extracellular solution and provides a binding site for K_o^+. In the closed state, the negatively charged domain faces the intracellular solution and is the binding site for K^+_i and polycations like Mg^{2+} and polyamines. Mg^{2+} and polyamines can bind to the fast gate and prolong the closed state. This is the simplest mechanism that demonstrates fast gating, but it is not meant to imply structure. *b*) G/G_{max} was plotted based on measured macroscopic (n = 3) Fig. 2 *b* trace 3 (*open circles*) and single channel (n = 5, bilayer) currents (Fig. 3 *c* (*closed circles*); ±SD). The mean values were fit by a Boltzmann function assuming the kinetic scheme shown in the inset. The fast gating equilibrium constant, K_f, was derived from the best fit. *Inset*: The fast and slow gating mechanisms operate independently. We assume the same equilibrium constant (K_f) for fast (O)-(C) and (I_o)-(I_c) transitions. The equilibrium constant for the slow gating mechanism (K_s), is independent of voltage and thus does not appear in the final equation for G/G_{max}.

good agreement with the proposed kinetic model. The fast gating mechanism does not turn on immediately at potentials more positive than the zero current potential. The speed of the transition between different modes of operation depends on the concentration of free Mg^{2+} and/or polyamines at the cytoplasmic face, becoming faster in solutions with increasing concentrations. The voltage dependence of Mg^{2+} and the polyamine modulation of the fast gating mechanism must be explained by a mechanism other than open channel block. The simplest model assumes voltage-dependent formation of binding site(s) for Mg^{2+} and/or polyamines at the

cytoplasmic face of the channel, correlated with briefly closed or open states. The binding of Mg^{2+} or polyamines to these binding sites changes the rate constants of the fast gating mechanism. Thus, the probability of Mg^{2+} or polyamine binding will depend on the mode of operation of the fast gating mechanism, which is too fast to measure at our recording bandwidth. According to our analysis, rectification is basically an intrinsic property of the channel structure and is enhanced by intracellular Mg^{2+} and/or polyamines binding temporarily to sites on the cytoplasmic face of the channel. These features are consistent with the electrochemical regulation of the gating mechanism as proposed by Ciani et al. (1978) and modified in the K^+-activated K^+-channel model of Pennefather et al. (1992).

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