

## Interaction of $\text{Ba}^{2+}$ with the Pores of the Cloned Inward Rectifier $\text{K}^+$ Channels Kir2.1 Expressed in *Xenopus* Oocytes

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**ABSTRACT** Interactions of  $\text{Ba}^{2+}$  with  $\text{K}^+$  and molecules contributing to inward rectification were studied in the cloned inward rectifier  $\text{K}^+$  channels, Kir2.1. Extracellular  $\text{Ba}^{2+}$  blocked Kir2.1 channels with first-order kinetics in a  $V_m$ -dependent manner. At  $V_m$  more negative than  $-120$  mV, the  $K_d$ - $V_m$  relationship became less steep and the dissociation rate constants were larger, suggesting  $\text{Ba}^{2+}$  dissociation into the extracellular space. Both depolarization and increasing  $[\text{K}^+]_i$  accelerated the recovery from extracellular  $\text{Ba}^{2+}$  blockade. Intracellular  $\text{K}^+$  appears to relieve  $\text{Ba}^{2+}$  blockade by competitively slowing the  $\text{Ba}^{2+}$  entrance rate, instead of increasing its exit rate by knocking off action. Intracellular spermine ( $100\text{ }\mu\text{M}$ ) reduced, whereas  $1\text{ mM}$   $[\text{Mg}^{2+}]_i$  only slightly reduced, the ability of intracellular  $\text{K}^+$  to repulse  $\text{Ba}^{2+}$  from the channel pore. Intracellular  $\text{Ba}^{2+}$  also blocked outward  $I_{\text{Kir2.1}}$  in a voltage-dependent fashion. At  $V_m \geq +40$  mV, where intrinsic inactivation is prominent, intracellular  $\text{Ba}^{2+}$  accelerated the inactivation rate of the outward  $I_{\text{Kir2.1}}$  in a  $V_m$ -independent manner, suggesting interaction of  $\text{Ba}^{2+}$  with the intrinsic gate of Kir2.1 channels.

### INTRODUCTION

Inward rectifier  $\text{K}^+$  channels conduct inward currents at potentials more negative than the  $\text{K}^+$  reversal potential but permit much smaller currents at potentials positive to the reversal potential (Hille, 1992). Thus inward rectifier  $\text{K}^+$  channels set the resting membrane potential and control the excitability of many cell types. Two mechanisms have been proposed to account for inward rectification of  $\text{K}^+$  channels. The first involves the voltage-dependent blockade of outward  $\text{K}^+$  currents by both intracellular  $\text{Mg}^{2+}$  (Matsuda et al., 1987; Vandenberg, 1987) and polyamines (Fickler et al., 1994; Lopatin et al., 1994). These observations are consistent with the hypothesis that inward rectification is caused by intracellular pore blocking particle(s) (Hille and Schwarz, 1978). The second mechanism is a novel  $\text{pH}_i$ -dependent gating mechanism that is involved in the inactivation of the outward currents and is independent of intracellular  $\text{Mg}^{2+}$  and polyamines (Shieh et al., 1996).

$\text{Ba}^{2+}$  is known to inhibit several types of  $\text{K}^+$  channels expressed in different tissues, including delayed rectifier  $\text{K}^+$  channels of squid giant axon (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982), native inward rectifier  $\text{K}^+$  channels of starfish eggs and frog skeletal muscle (Hagiwara et al., 1978; Standen and Stanfield, 1978), and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (Vergara and Latorre, 1983; Neyton and Miller, 1988a,b). The inhibitory effects of extracellular  $\text{Ba}^{2+}$  have also been demonstrated in different cloned inward rectifier  $\text{K}^+$  channels, including those cloned from mouse macrophage (Kubo et al., 1993),

human and mouse brain (Makhina et al., 1994; Morishige et al., 1994; Perier et al., 1994), rat kidney (Zhou et al., 1996), and human heart (Ashen et al., 1995). Application of extracellular  $\text{Ba}^{2+}$  to either native or cloned inward rectifier  $\text{K}^+$  channels resulted in voltage- and time-dependent inhibitions of the  $\text{K}^+$  currents. In contrast, the application of extracellular  $\text{Ba}^{2+}$  to the delayed rectifier  $\text{K}^+$  channels from squid axon reduced the magnitude of  $\text{K}^+$  current without changing its kinetics, whereas intracellular  $\text{Ba}^{2+}$  induced time-dependent inhibition of the channels (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982). However, detailed characterization of  $\text{Ba}^{2+}$  blockade in the cloned inward rectifier  $\text{K}^+$  channels has not been carried out.

Because  $\text{Ba}^{2+}$  and  $\text{K}^+$  have similar crystal radii, it has been hypothesized that  $\text{Ba}^{2+}$  ions inhibit  $\text{K}^+$  channels by entering the pores when the channels are opened by changes in membrane potential, as evidenced by the findings that extracellular  $\text{K}^+$  could either knock off the  $\text{Ba}^{2+}$  (Eaton and Brodwick, 1980; Armstrong and Taylor, 1980) or compete for the  $\text{Ba}^{2+}$  binding site (Vergara and Latorre, 1983) in  $\text{K}^+$  channels. These previous studies suggest that the voltage-dependent inhibition of  $\text{K}^+$  channels by  $\text{Ba}^{2+}$  is due to the interaction of  $\text{Ba}^{2+}$  ions with the amino acid residues lining the pores of the channels, which are presumably located within the electrical field. Thus  $\text{Ba}^{2+}$  has been used as a probe to learn about the permeability of the  $\text{K}^+$  channels. For example, from the studies of  $\text{K}^+$  and  $\text{Ba}^{2+}$  interaction in the pores of the high-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, it has been shown that each channel has at least three binding sites for the permeant  $\text{K}^+$  ions (Neyton and Miller, 1988b).

In this study we examined the interactions of  $\text{Ba}^{2+}$  with the permeant  $\text{K}^+$  ion in Kir2.1 channels and found that extracellular  $\text{Ba}^{2+}$  blockade was relieved by intracellular  $\text{K}^+$  through competition for the same binding site.  $\text{Ba}^{2+}$

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thus is a useful probe for studying  $K^+$  binding in Kir2.1 channels. In addition, we identified the interactions of  $Ba^{2+}$  with the rectifying factors, including intracellular  $Mg^{2+}$  and spermine and the "intrinsic" gate. The results demonstrate that although both  $Mg^{2+}$  and spermine induce inward rectification by blocking the Kir2.1 channel, their binding sites are not identical.  $Ba^{2+}$  applied intracellularly appears to accelerate the inactivation of outward current by interfering with the "intrinsic" gating mechanism and thus is a potential tool for unraveling the essence of the intrinsic gating property of the Kir2.1 channel. A preliminary report of these findings has been presented to the Biophysical Society (Shieh et al., 1998).

## MATERIALS AND METHODS

### Molecular biology and preparation of *Xenopus* oocytes

Mouse macrophage Kir2.1 DNA (the original clone in pCDNPAI/Amp was generously provided by Dr. Lily Jan, UCSF) subcloned into Bluescript II SK+ was a generous gift from Drs. Scott A. John and James N. Weiss (UCLA). Purified linear Kir2.1 DNA and *in vitro* transcription were carried out as previously described (Shieh et al., 1996).

*Xenopus* oocytes were obtained by partial ovariectomy from frogs fully anesthetized with 0.1% tricaine and then defolliculated using 2% collagenase as previously described (Shieh et al., 1996). Oocytes were pressure injected 24 h after defolliculation with 10–100 pg of Kir2.1 cRNAs for whole-cell recordings and 1–10 ng for giant patch recordings. Oocytes were maintained at 18°C in Barth's solution containing (in mM) 88 NaCl, 1 KCl, 2.4  $NaHCO_3$ , 0.3  $Ca(NO_3)_2$ , 0.41  $CaCl_2$ , 0.82  $MgSO_4$ , 15 HEPES, and 20  $\mu$ g gentamicin/ml at pH 7.6 and were used 1–3 days after RNA injection.

### Electrophysiology techniques

Extracellular  $Ba^{2+}$  blockade of whole-cell  $I_{Kir2.1}$  was examined at room temperature (21–24°C) using a two-electrode voltage-clamp amplifier (Ca-1 clamp; Dagan, Minneapolis, MN). Oocytes were bathed in a solution containing (in mM) 98 KCl, 2 KOH, 1.8  $CaCl_2$ , and 5 HEPES at pH 7.4. Both the voltage-sensing and current-injecting electrodes were filled with 3 M KCl (resistance 0.2–1 M $\Omega$ ). Voltage steps were applied from a holding potential of 0 mV to various test voltages ranging from –160 to +40 mV in 10-mV increments. To ensure that  $Ba^{2+}$  blockade reached steady state, the effects of  $[Ba^{2+}] \leq 30 \mu$ M were studied using 10-s depolarizing pulses, whereas those of  $[Ba^{2+}] \geq 100 \mu$ M were examined using 2-s pulses.  $Ba^{2+}$  was added directly to the bath solution until the desired concentration was reached, and the pH was adjusted to 7.4.

To avoid inadequate voltage clamping in whole cells caused by large currents, we used oocytes expressing an absolute value of less than –10  $\mu$ A at –160 mV. The time constants for the blockade ( $\tau_{block}$ ) of the  $I_{Kir2.1}$  recorded at –120 mV by 10  $\mu$ M  $[Ba^{2+}]$  in whole cells ( $123.6 \pm 8.8$  ms,  $n = 13$ ) were not significantly different ( $p = 0.19$ ) from those recorded in cell-attached patches ( $104.3 \pm 10.4$  ms,  $n = 8$ ). Furthermore, the recovery time constants in cell-attached patches ( $\tau_{recov} = 412.3 \pm 33.6$  ms,  $n = 8$ ) were not significantly different ( $p = 0.49$ ) from those obtained in whole cells ( $\tau_{recov} = 385.9 \pm 9.8$  ms,  $n = 7$ ) at  $V_r = +100$  mV. These data suggest that the voltage clamping in whole cells was as adequate as that in patches.

In experiments where intracellular ionic conditions needed to be changed,  $I_{Kir2.1}$  was recorded using the inside-out giant patch-clamp technique (Hilgemann, 1995; Shieh et al., 1996) and an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA). Patch electrode solution contained (in mM) 98 KCl, 2 KOH, 1.8  $CaCl_2$  (or 5  $MgCl_2$ ), and 5 HEPES at pH 7.4.

Bath solution (control intracellular solution) contained (in mM) 82 KCl, 18 KOH, 5 EDTA, 2  $K_2ATP$ , and 5 HEPES at pH 7.2. Concentrations of  $K^+$  from 200 mM down to 20 mM were obtained by changing the concentrations of KCl while the concentrations of HEPES, EDTA, and KOH were kept constant. The rundown of channel activity was avoided by treating the patches with 25  $\mu$ M L- $\alpha$ -phosphatidylinositol-4,5-bisphosphate ( $PIP_2$ ) (Sigma Chemical Co., St. Louis, MO) (Huang et al., 1998) for 20–60 s.  $PIP_2$  stock solution (1 mM) was prepared by dissolving  $PIP_2$  in chloroform. On the day of experiments, 125  $\mu$ l of the stock solution was  $N_2$ -dried and resuspended in 5 ml of control intracellular solution by sonication for 15 s three times, separated by 30-s intervals. Although the amplitude of outward  $I_{Kir2.1}$  was increased by 25  $\mu$ M  $PIP_2$ , the recovery from the extracellular  $Ba^{2+}$  blockade and the intrinsic inactivation of the outward currents were not significantly different ( $p > 0.1$ ) between patches treated with and without  $PIP_2$  (data not shown). To wash out the residual intracellular  $Mg^{2+}$  and polyamines, experiments were carried out after at least 5 min of continuous perfusion of  $Mg^{2+}$ - and polyamine-free solution after patch excision. Free  $[Ba^{2+}]$  and  $[Mg^{2+}]$  in the intracellular solution were calculated with the MaxC program (Chris Patton, Stanford University) and stability constants by Martell and Smith (1974).

The command voltage pulses and data acquisition functions were processed using a Pentium 100 computer, a DigiData board, and pClamp6 software (Axon Instruments, Burlingame, CA). Data were filtered at 1 kHz by a 8-pole low-pass filter (Frequency Devices, Rochester, NY). Frequencies of stimulation and data sampling are described in the figure legends.

### Data analysis

Students' independent or paired *t*-test was used to assess statistical significance. Results are presented as mean  $\pm$  SEM.

## RESULTS

### The blockade of inward $I_{Kir2.1}$ by extracellular $Ba^{2+}$

Fig. 1 *A* shows current traces recorded at –120 mV from a representative oocyte in the absence and in the presence of the indicated  $[Ba^{2+}]_o$ . The addition of  $Ba^{2+}$  to the extracellular side of the membrane induced a time-dependent decrease of the inward currents. The rate of inhibition of  $I_{Kir2.1}$  was accelerated by increasing  $[Ba^{2+}]_o$ , and the currents reached steady state at the end of hyperpolarization.  $I_{Kir2.1}$  at the onset of hyperpolarization was little affected by  $Ba^{2+}$ . For example, 10  $\mu$ M  $[Ba^{2+}]_o$  reduced the instantaneous  $I_{Kir2.1}$  recorded at –120 mV by only  $5 \pm 2\%$  ( $n = 6$ ), despite the fact that at the end of the pulse less than 5% of the current remained. This observation suggests that the Kir2.1 channels are not affected by  $Ba^{2+}$  at 0 mV, and channels must open before  $Ba^{2+}$  inhibition takes place.

Normalized steady state current-voltage (*I*-*V*) relationships in the presence of various  $[Ba^{2+}]_o$  are summarized in Fig. 1 *B*, which shows that the effects of extracellular  $Ba^{2+}$  on  $I_{Kir2.1}$  were voltage dependent. Extracellular  $Ba^{2+}$  preferentially affected Kir2.1 channels at more negative potentials. This voltage-dependent effect of  $[Ba^{2+}]_o$  (1 or 10  $\mu$ M) on Kir2.1 channels resulted in a negative conductance at negative potentials (Fig. 1 *B*), as has previously been observed in Kir2.1 channels (Kubo et al., 1993).

Fig. 1 *C* shows, from left to right, the dose-response curves for the blockade of the steady-state  $I_{Kir2.1}$  by extra-

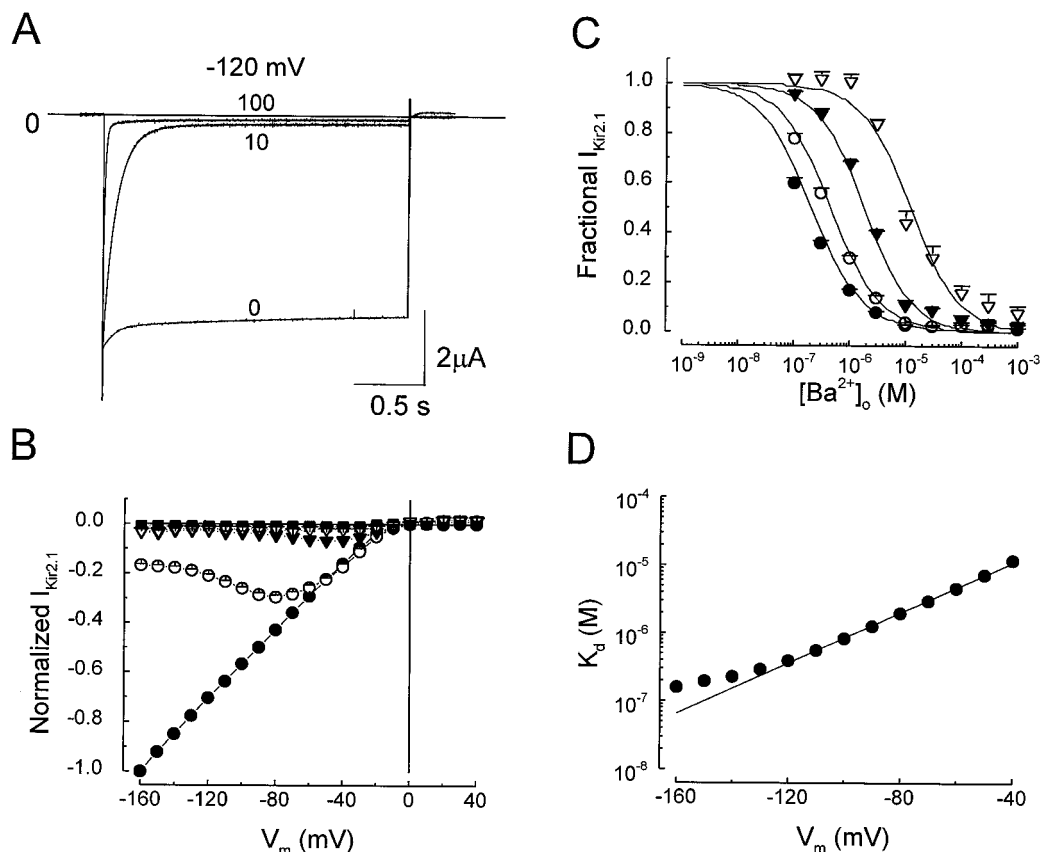


FIGURE 1 Blockade of inward  $I_{Kir2.1}$  by extracellular  $Ba^{2+}$ . (A) Representative currents elicited by voltage steps to  $-120$  mV for 2 s from a holding potential of 0 mV in the presence of the indicated  $[Ba^{2+}]_o$  ( $\mu M$ ). The horizontal line indicates the zero current level. Current traces recorded in the presence of 5 mM  $[Ba^{2+}]_o$  were used to eliminate the linear leak currents. (B) Normalized current-voltage ( $I-V$ ) relationships for the steady-state inward  $I_{Kir2.1}$ . Data are the averages from 4–12 cells in the presence of 0 (●), 1 (○), 10 (▼), 100 (▽), and 1000  $\mu M$   $Ba^{2+}$  (■). Steady-state currents measured at the end of the 2- or 10-s pulses were normalized to that recorded at  $-160$  mV in the absence of  $Ba^{2+}$  in each cell and then averaged. (C) Dose-response curves for extracellular  $Ba^{2+}$  blockade of the steady-state  $I_{Kir2.1}$  at  $-160$  mV (●),  $-120$  mV (○),  $-80$  mV (▼), and  $-40$  mV (▽). Lines are the best fits to the averaged data ( $n = 4-12$  for each point) using the Hill equation (Eq. 1). (D)  $K_d$  values obtained from the concentration-dependent inhibition of the steady-state  $I_{Kir2.1}$  are plotted against membrane potentials. The solid line is the best fit of the data obtained at  $V_m$  ranging from  $-120$  mV to  $-40$  mV, using the Boltzmann equation (Eq. 2). The frequencies of stimulation were 0.05–0.083 Hz, and the sampling rates of the  $I_{Kir2.1}$  were 1.43–1.67 kHz.

cellular  $Ba^{2+}$  at  $V_m = -160$ ,  $-120$ ,  $-80$ , and  $-40$  mV.  $I_{Kir2.1}$  amplitude was normalized to the control amplitude ( $[Ba^{2+}]_o = 0$ ) obtained at the same potential and expressed as fractional  $I_{Kir2.1}$ . The dose-response curves also demonstrate the voltage-dependent inhibition of the inward  $I_{Kir2.1}$  by extracellular  $Ba^{2+}$ . As the membrane potential became more hyperpolarized, the steady-state  $I_{Kir2.1}$  was more sensitive to extracellular  $Ba^{2+}$ , as if the negative electrical field attracted  $Ba^{2+}$  inside the membrane. The dose-response curves at each potential were well described by the Hill equation (Eq. 1):

$$\text{Fractional } I_{Kir2.1} = \frac{1}{1 + \left(\frac{[Ba^{2+}]}{K_d}\right)^n} \quad (1)$$

where  $K_d$  is the apparent dissociation constant and  $n$  is the Hill coefficient. The  $K_d$  values obtained from the fittings were 0.2  $\mu M$ ,  $V_m = -160$  mV; 0.5  $\mu M$ ,  $V_m = -120$  mV; 2.2  $\mu M$ ,  $V_m = -80$  mV; 12.7  $\mu M$ ,  $V_m = -40$  mV. The Hill

coefficients were 0.84,  $V_m = -160$  mV; 0.90,  $V_m = -120$  mV; 1.06,  $V_m = -80$  mV; 0.99,  $V_m = -40$  mV. The corresponding Hill coefficients were close to 1, suggesting one  $Ba^{2+}$  interacted with one channel protein.

To quantitatively assay the voltage dependence of the inhibition of  $I_{Kir2.1}$  by extracellular  $Ba^{2+}$ , the  $K_d$  values obtained from the blockade of the steady-state  $I_{Kir2.1}$  were plotted against the membrane potential (Fig. 1 D). The solid line is the fit to the data points from  $-40$  to  $-120$  mV, using the Boltzmann equation:

$$K_d = K_d(0) \cdot \exp\left[\frac{z\delta F}{RT} (V_m)\right] \quad (2)$$

where  $K_d(0)$  is the dissociation constant at 0 mV,  $z$  is the valence of  $Ba^{2+} = +2$ ,  $\delta$  is the apparent electrical distance between the  $Ba^{2+}$ -channel interacting site and the outer pore of the channel, and  $F$ ,  $R$ ,  $T$ , and  $V_m$  have their usual meanings. The estimated  $K_d(0)$  was 62  $\mu M$ , and  $\delta$  was 0.54 for the blockade of steady-state  $I_{Kir2.1}$ . Note that the  $K_d$ - $V_m$

relationship became less steep at  $V_m$  more negative than  $-120$  mV. This may be due to  $\text{Ba}^{2+}$  dissociation into the intracellular site at very negative  $V_m$ , as previously described in the high-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel (Neyton and Miller, 1988b).

### Kinetics of the extracellular $\text{Ba}^{2+}$ -induced blockade of Kir2.1 channels

To quantitatively examine the kinetics of the blockade of Kir2.1 channels by extracellular  $\text{Ba}^{2+}$ , we obtained the association ( $k_{\text{on}}$ ) and dissociation ( $k_{\text{off}}$ ) rate constants of  $\text{Ba}^{2+}$  interacting with the binding site. Assuming the interaction of  $\text{Ba}^{2+}$  with Kir2.1 channels follows a first-order reaction, as suggested by the dose-response curves (Fig. 1 C), we can then compute  $k_{\text{on}}$  and  $k_{\text{off}}$  using the equations described by Holmgren et al. (1997):

$$k_{\text{on}} = \frac{1-f}{\tau_{\text{block}} \times [\text{Ba}^{2+}]} \quad (3)$$

$$k_{\text{off}} = \frac{f}{\tau_{\text{block}}} \quad (4)$$

where  $f = I_{\text{steady-state}}/I_{\text{ctrl}}$ .

Fig. 2 A summarizes the  $k_{\text{on}}-V_m$  relationship. The solid line is the fit to the data points from  $-40$  to  $-120$  mV, using the Boltzmann equation with a  $k_{\text{on}}$  at  $0$  mV =  $6.8 \times 10^3 \text{ s}^{-1}\text{M}^{-1}$  and a  $\delta = 0.52$ . This value of  $\delta$  was comparable to that (0.54) obtained using stationary analysis of dose-response curves (Fig. 1 C). These results demonstrate that the voltage-dependent interaction of  $\text{Ba}^{2+}$  ions with the binding sites in the channels to induce blockade of Kir2.1 channels is mainly due to the voltage dependence of  $k_{\text{on}}$ . In contrast, the  $k_{\text{off}}-V_m$  relationship (Fig. 2 B) showed little  $V_m$  dependence at  $V_m$  between  $-130$  and  $-50$  mV. However,  $k_{\text{off}}$  increased as  $V_m$  became more negative than  $-130$  mV. These results are indicative of  $\text{Ba}^{2+}$  dissociating into the intracellular space, as previously shown in the high-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (Neyton and Miller, 1988b) and Shaker  $\text{K}^+$  channels (Harris et al., 1998). This is also consistent with the observation that the  $K_d-V_m$  relationship became less steep at  $V_m$  more negative than  $-120$  mV.

### Recovery of Kir2.1 channels from extracellular $\text{Ba}^{2+}$ blockade

Fig. 2 B shows that  $k_{\text{off}}$  tends to increase at  $V_m > -50$  mV. Unfortunately, currents recorded at  $V_m > -40$  mV were too small to allow us to estimate  $k_{\text{off}}$  at more positive  $V_m$ . To obtain experimental estimates of  $k_{\text{off}}$  over the positive range of potentials, the rate of recovery from  $10 \mu\text{M}$   $[\text{Ba}^{2+}]_o$  blockade was examined using a two-pulse protocol. The fraction of channels blocked by  $10 \mu\text{M}$   $[\text{Ba}^{2+}]_o$  at  $-120$  mV was tested by the first pulse, whereas the fraction of channels recovered from blockade after a given time interval at various recovery voltages ( $V_r$ ) was recorded by the

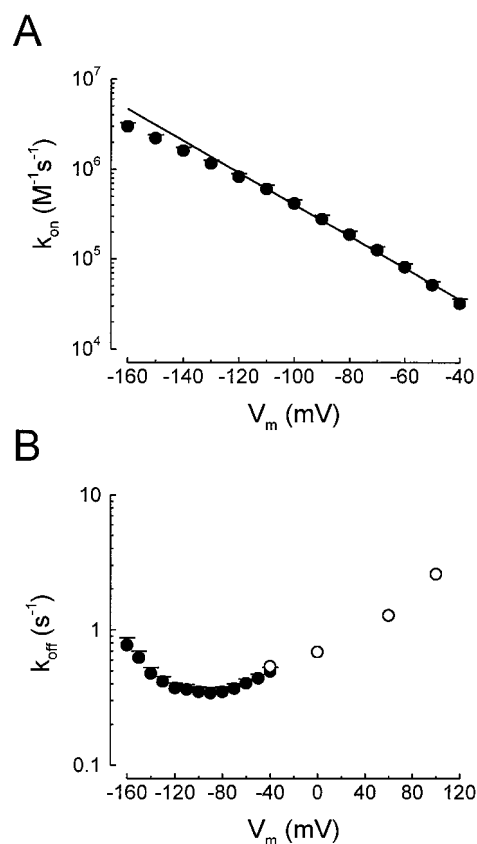
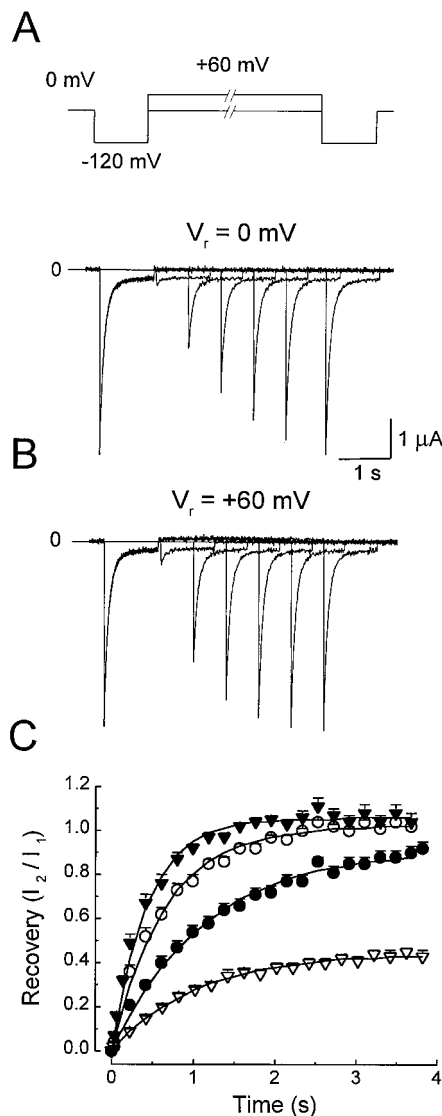


FIGURE 2 Kinetics analysis of inward  $I_{\text{Kir2.1}}$  blockade by extracellular  $\text{Ba}^{2+}$ . (A)  $k_{\text{on}}$  values obtained from Eq. 3 are plotted at the corresponding membrane potentials ( $n = 6$ ). The solid line was the fit of the data using the Boltzmann equation. (B) The voltage dependence of the  $k_{\text{off}}$  values computed from Eq. 4 (●,  $n = 6$ ) and those obtained from recovery from extracellular  $\text{Ba}^{2+}$  blockade experiments (○).  $k_{\text{on}}$  and  $k_{\text{off}}$  values were estimated by using  $f$  and  $\tau_{\text{block}}$  obtained at  $[\text{Ba}^{2+}]_o = 3 \mu\text{M}$ . Similar  $k_{\text{on}}-V_m$  and  $k_{\text{off}}-V_m$  relationships were also observed with  $[\text{Ba}^{2+}]_o = 1, 10$ , and  $30 \mu\text{M}$ .

second pulse. Fig. 3, A and B, shows the voltage protocol (A, top panel) with various recovery time intervals and the corresponding current traces recorded with  $V_r = 0$  (A, lower panel) and  $V_r = +60$  mV (B).  $I_{\text{Kir2.1}}$  activated by the first hyperpolarizing pulse displayed a monoexponential blockade until only 5% of the instantaneous current amplitude remained at the end of the pulse. The instantaneous current recorded with the second hyperpolarizing pulse increased as the interval between the two pulses increased. The recovery from the blockade followed a monoexponential time course and was faster with  $V_r = +60$  mV (time constant = 867 ms) than that with  $V_r = 0$  mV (time constant = 1506 ms). The steady-state  $I_{\text{Kir2.1}}$  at the end of the  $-120$ -mV pulse remained constant, independent of the recovery time interval, the recovery voltage, or the fraction of channels recovered at the beginning of the second hyperpolarizing pulse.

The recovery time courses for the instantaneous current recorded at the second pulse at different recovery voltages are shown in Fig. 3 C. The fractional recovery was calculated as  $I_2/I_1$ , where  $I_2$  was the current recorded 4 ms after





**FIGURE 3** Time course of recovery from extracellular Ba<sup>2+</sup> blockade. (A) Upper panel shows the two-pulse voltage protocol. The time interval between the two identical pulses (each for 1 s) was increased by 0.6 s between sweeps (starting at 0.05 s) to assess recovery from blockade induced by extracellular Ba<sup>2+</sup> (10  $\mu$ M) during the first pulse. The corresponding current traces recorded with  $V_r = 0$  mV are shown in the lower panel. (B) Current traces recorded with  $V_r = +60$  mV, using the same protocol as described in A. Currents recorded in the presence of 5 mM [Ba<sup>2+</sup>]<sub>o</sub> were subtracted from the currents recorded in 10  $\mu$ M [Ba<sup>2+</sup>]<sub>o</sub> to eliminate the linear leak currents. The horizontal lines indicate the zero current level. (C) Time courses for recovery from extracellular Ba<sup>2+</sup> blockade with  $V_r = -40$  ( $\nabla$ ),  $0$  ( $\bullet$ ),  $60$  ( $\circ$ ), and  $100$  ( $\blacktriangledown$ ) mV. The recovery time constants ( $\tau_{\text{recov}}$ ) were  $1160 \pm 53$  ms,  $V_r = -40$  mV ( $n = 5$ );  $1315 \pm 85$  ms,  $V_r = 0$  mV ( $n = 6$ );  $775 \pm 48$  ms,  $V_r = +60$  mV ( $n = 6$ );  $386 \pm 10$  ms,  $V_r = +100$  mV ( $n = 7$ ). Continuous lines are single-exponential function fits to the data. The estimated maximum fractional recoveries obtained from fittings were  $0.43 \pm 0.03$ ,  $V_r = -40$  mV ( $n = 5$ );  $0.94 \pm 0.03$ ,  $V_r = 0$  mV ( $n = 6$ );  $1.01 \pm 0.01$ ,  $V_r = +60$  mV ( $n = 6$ );  $1.06 \pm 0.05$ ,  $V_r = +100$  mV ( $n = 7$ ). The frequency of stimulation was 0.04 Hz, and the data sampling rate was 0.5 kHz.

the onset of the second pulse minus the steady-state current, and  $I_1$  was the current recorded 4 ms after the onset of the first pulse minus the steady-state current. The fraction of

Kir2.1 channels recovered from extracellular Ba<sup>2+</sup> blockade followed an exponential time course, as shown by the fits to monoexponential functions. The  $\tau_{\text{recov}}$  values calculated from the monoexponential fits, the  $k_{\text{on}}$  values described in Fig. 2 A, and the function  $1/\tau_{\text{recov}} = k_{\text{on}} \times [\text{Ba}^{2+}]_o + k_{\text{off}}$  were used to estimate  $k_{\text{off}}$  at positive voltages. The  $k_{\text{on}}$  values at 0, +60, and +100 mV were obtained from the extrapolation of the  $k_{\text{on}}-V_m$  relationship shown in Fig. 2 A. The calculated  $k_{\text{off}}$  values were plotted against  $V_m$  in Fig. 2 B.  $k_{\text{off}}$  at  $-40$  mV is very close to  $k_{\text{off}}$  determined from the kinetic analysis of the extracellular Ba<sup>2+</sup> blockade.  $k_{\text{off}}$  obtained from the recovery for the extracellular Ba<sup>2+</sup> blockade at potentials positive to 0 mV increased significantly ( $p < 0.0005$ ) when compared to that calculated from the kinetic analysis of the extracellular Ba<sup>2+</sup> blockade at  $-40$  mV. The voltage dependence of  $k_{\text{off}}$  at positive voltages determined from recovery of blockade is probably due to the effects of the electrical field on the dissociation of Ba<sup>2+</sup> into the extracellular side.

### Effects of intracellular K<sup>+</sup> on the recovery from extracellular Ba<sup>2+</sup> blockade

To further investigate whether extracellular Ba<sup>2+</sup> enters the pore of Kir2.1 channels to induce blockade, we examined the effects of intracellular K<sup>+</sup> concentration ([K<sup>+</sup>]<sub>i</sub>) on the recovery from extracellular Ba<sup>2+</sup> blockade, as previously described for delayed rectifier K<sup>+</sup> channels of squid giant axon (Armstrong and Taylor, 1980). To control the intracellular ionic compositions,  $I_{\text{Kir2.1}}$  was recorded using a giant patch technique and a voltage protocol similar to that described in Fig. 3 A. The hyperpolarizing pulses were set at  $V_m = -120$  mV,  $V_r$  was set at +100 mV, and the holding potential was set at  $V_m = E_K$  throughout this series of experiments. Fig. 4 A shows a set of  $I_{\text{Kir2.1}}$  currents recorded from a cell-attached patch, with a pipette solution containing 10  $\mu$ M [Ba<sup>2+</sup>]<sub>o</sub> used to induce blockade. The overall characteristics ( $\tau_{\text{block}}$  and  $\tau_{\text{recov}}$ ) of  $I_{\text{Kir2.1}}$  recorded in cell-attached patches were similar to those recorded in whole cells (compare to Fig. 3). When the same patch was excised (inside out) into a Mg<sup>2+</sup>-free and polyamine-free control intracellular solution, both the rate of the blockade for the inward  $I_{\text{Kir2.1}}$  and the rate of the recovery from extracellular Ba<sup>2+</sup> blockade were increased (Fig. 4 B), and outward currents were observed during the recovery interval.  $\tau_{\text{block}}$  decreased from 106 ms to 47 ms, and  $\tau_{\text{recov}}$  decreased from 452 ms to 42 ms. When the same patch was perfused with an intracellular solution containing only 20 mM [K<sup>+</sup>], the outward current decreased at  $V_r = +100$  mV,  $\tau_{\text{recov}}$  increased from 42 to 62 ms, and  $\tau_{\text{block}}$  decreased from 47 to 22 ms (Fig. 4 C).

It was previously considered that there are two possible mechanisms for relief of the channel blocker by intracellular K<sup>+</sup> (Yellen, 1984). One is that K<sup>+</sup> relieves the block by competing with the blocker for the same binding site in the channel. The other is that K<sup>+</sup> accelerates the exit of the

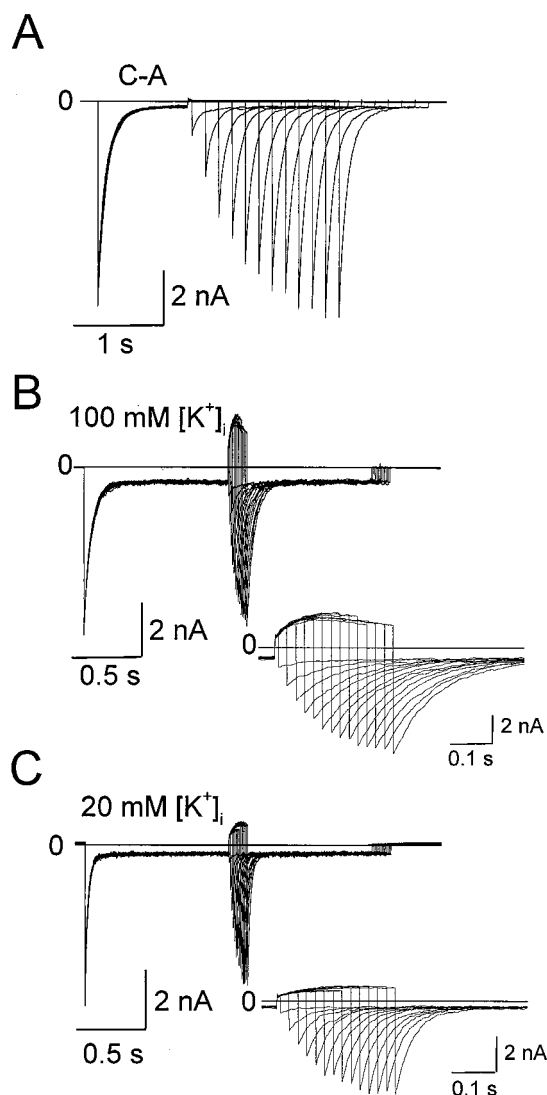


FIGURE 4 Effects of  $[K^+]_i$  on the time course of recovery from extracellular  $Ba^{2+}$  blockade of the inward  $I_{Kir2.1}$ . (A) Current traces were recorded at  $V_m = -120$  mV from a holding potential of 0 mV and  $V_r = +100$  mV in a cell-attached patch. (B and C) Currents were recorded from the same patch shown in A after excising into the  $Mg^{2+}$ - and polyamine-free solution containing 100 mM  $[K^+]_i$  and 20 mM  $[K^+]_i$ , respectively. The blockade of  $I_{Kir2.1}$  was induced by the presence of  $10 \mu M [Ba^{2+}]_o$  in the pipette solution. The insets in B and C display currents recorded during the second pulse in amplified time scales. The horizontal lines indicate the zero current level. The frequencies of stimulation were 0.067 Hz. The sampling rates were 1 kHz (A) and 2.5 kHz (B and C).

blocker. We can distinguish between these two possibilities by examining the effects of  $[K^+]_i$  on the kinetics of extracellular  $Ba^{2+}$  blockade. If  $K^+$  competes with  $Ba^{2+}$  for a binding site, then  $k_{on}$  should be affected but  $k_{off}$  should not be affected by  $[K^+]_i$ . On the other hand, if  $K^+$  knocks off  $Ba^{2+}$ ,  $k_{off}$  should be affected.

Table 1 summarizes the effects of various  $[K^+]_i$  on the extracellular  $Ba^{2+}$  blockade of the Kir2.1 channels with  $V_r = +100$  mV.  $\tau_{recov}$  was about one order of magnitude faster in the inside-out excised patches perfused with the control intracellular solution compared to cell-attached

patches. Furthermore,  $\tau_{block}$  was smaller in the inside-out patches than in the cell-attached patches. When  $[K^+]_i$  was increased from 20 to 200 mM,  $\tau_{recov}$  decreased, whereas  $\tau_{block}$  increased. In addition, the fractions of nonblocked channels at the end of the  $-120$ -mV pulse increased as intracellular  $[K]$  increased. These results indicate that  $K^+$  ions interfere with extracellular  $Ba^{2+}$  blockade of the Kir2.1 channels at  $V_m = -120$  mV. To examine how  $[K^+]_i$  affects the kinetic parameters for the extracellular  $Ba^{2+}$  blockade of  $I_{Kir2.1}$  recorded at  $-120$  mV, we estimated  $k_{on}$  and  $k_{off}$  using Eqs. 3 and 4, respectively. Note that here  $f = I_{ss,Ba}/I_{inst,Ba}$ ;  $I_{ss,Ba}$  and  $I_{inst,Ba}$  are the steady-state and instantaneous currents recorded at  $-120$  mV in the presence of  $10 \mu M [Ba^{2+}]_o$ . The accurate expression of  $f$  should be  $I_{ss,Ba}/I_{ss,ctrl}$ , where  $I_{ss,ctrl}$  is the steady-state current recorded at  $-120$  mV in the absence of extracellular  $Ba^{2+}$ . Because currents were recorded in inside-out patches, we were not able to obtain both  $I_{ss,Ba}$  and  $I_{ss,ctrl}$  in the same patch. However, we consistently observed that the inward currents recorded in inside-out patches perfused with  $Mg^{2+}$ -free and polyamine-free solution did not decrease over time when there was no  $Ba^{2+}$  in the pipette (Fig. 6, A and B), and that  $10 \mu M [Ba^{2+}]_o$  only inhibited  $5 \pm 2\%$  of the instantaneous inward  $I_{Kir2.1}$  in whole-cell experiments. These observations support our assumption that  $I_{inst,Ba} \approx I_{ss,ctrl}$ . The averaged  $k_{on}$  and  $k_{off}$  values at different  $[K]_i$  are summarized in Table 1. The results show that increasing  $[K^+]_i$  decreased  $k_{on}$  without significantly affecting  $k_{off}$  for the binding of  $Ba^{2+}$  to the channels at  $-120$  mV. Thus intracellular  $K^+$  appears to relieve  $Ba^{2+}$  blockade by competing for the  $Ba^{2+}$  binding site in the channel.

#### Spermine contributes to the slow recovery from extracellular $Ba^{2+}$ blockade in cell-attached patches

Fig. 4 demonstrates that the rates of recovery from  $Ba^{2+}$ -induced blockade were faster in inside-out patches exposed to  $Mg^{2+}$ -free and polyamine-free control solution containing 100 mM  $[K]_i$  than those observed in cell-attached patches. To examine whether intracellular  $Mg^{2+}$  and spermine were responsible for the difference between the recovery rates obtained in the whole cells (or cell-attached patches) and the inside-out patches, we examined the effects of perfusing patches with the control intracellular solution containing  $Mg^{2+}$  or spermine on the recovery from the extracellular  $Ba^{2+}$  blockade. Fig. 5 A shows a set of current traces recorded from an inside-out patch exposed to the control intracellular solution, using the two-pulse voltage protocol. Perfusing this patch with the control intracellular solution containing 1 mM free  $[Mg^{2+}]$  induced a slight increase in the recovery time constant from the control value of 45 ms (Fig. 5 A) to 99 ms (Fig. 5 B). Note that outward  $I_{Kir2.1}$  at  $V_r = +100$  mV was blocked. However, when the same patch was exposed to the control solution containing 100  $\mu M$  spermine, the block time constant was

**TABLE 1** Effects of various intracellular cations on the kinetics of the extracellular Ba<sup>2+</sup> blockade of K<sub>ir2.1</sub> channels

	C-A (n = 8)	100 mM [K <sup>+</sup> ] <sub>i</sub> (n = 14)	200 mM [K <sup>+</sup> ] <sub>i</sub> (n = 4)	20 mM [K <sup>+</sup> ] <sub>i</sub> (n = 3)	+1 mM [Mg <sup>2+</sup> ] <sub>i</sub> (n = 4)	+100 μM [spm] <sub>i</sub> (n = 3)
τ <sub>block</sub> (ms)	104.3 ± 10.4 <sup>§</sup>	53.3 ± 2.3	65.0 ± 7.7*	24.0 ± 1.5 <sup>#</sup>	70.8 ± 0.8 <sup>#</sup>	122.7 ± 5.9 <sup>#</sup>
τ <sub>recov</sub> (ms)	412.3 ± 33.6 <sup>§</sup>	38.1 ± 1.9	32.3 ± 3.4*	57.1 ± 6.0*	82.8 ± 5.9 <sup>#</sup>	2300 ± 503 <sup>§</sup>
f	0.05 ± 0.00 <sup>§</sup>	0.12 ± 0.01	0.20 ± 0.03*	0.08 ± 0.01*	0.07 ± 0.01 <sup>#</sup>	0.05 ± 0.03 <sup>#</sup>
k <sub>on</sub> (10 <sup>6</sup> × s <sup>-1</sup> M <sup>-1</sup> )	1.00 ± 0.15 <sup>§</sup>	1.71 ± 0.12	1.31 ± 0.23*	3.85 ± 0.25*	ND	ND
k <sub>off</sub> (s <sup>-1</sup> )	0.54 ± 0.11 <sup>§</sup>	2.31 ± 0.11	3.17 ± 0.47	3.49 ± 0.65	ND	ND
			(p = 0.24)	(p = 0.17)		

Paired *t*-tests were carried out by comparing data in various groups to the control 100 mM K<sub>i</sub> solution group.  
<sup>\*,#</sup>§The groups were statistically different at *p* < 0.05, *p* < 0.005, and *p* < 0.0005, respectively, unless otherwise indicated.  
+1 mM [Mg<sup>2+</sup>]<sub>i</sub> = control 100 mM [K<sup>+</sup>]<sub>i</sub> solution containing 1 mM free [Mg<sup>2+</sup>]; +100 μM [spm]<sub>i</sub> = control solution containing 100 μM spermine.  
τ<sub>recov</sub> was obtained from I<sub>Kir2.1</sub> recorded with V<sub>r</sub> = +100 mV at V<sub>m</sub> = -120 mV; τ<sub>block</sub>, f, k<sub>on</sub>, and k<sub>off</sub> were obtained at V<sub>m</sub> = -120 mV.  
ND, not determined.

increased, the recovery time course was dramatically retarded (Fig. 5 C, τ<sub>recov</sub> = 3071 ms), and the outward I<sub>Kir2.1</sub> was completely blocked. The effects of 1 mM free [Mg<sup>2+</sup>]<sub>i</sub> and 100 μM spermine on the extracellular Ba<sup>2+</sup> blockade of the Kir2.1 channels are also summarized in Table 1. These results suggest that spermine, but not Mg<sup>2+</sup>, is one of the major factors that contribute to the slow recovery from extracellular Ba<sup>2+</sup> blockade observed in whole cells and cell-attached patches, possibly by prohibiting the interaction

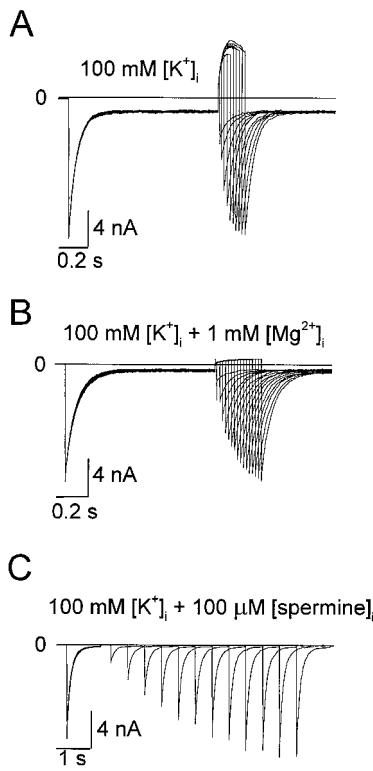
between the bound Ba<sup>2+</sup> and K<sup>+</sup>. Because intracellular Mg<sup>2+</sup> and spermine inactivated the inward I<sub>Kir2.1</sub> recorded in the absence of extracellular Ba<sup>2+</sup> (data not shown), k<sub>on</sub> and k<sub>off</sub> were not determined in patches treated with Mg<sup>2+</sup> or spermine.

### Blockade of Kir2.1 channels by intracellular Ba<sup>2+</sup>

To examine whether Kir2.1 channels are inhibited by intracellular Ba<sup>2+</sup>, we recorded I<sub>Kir2.1</sub> from excised inside-out giant patches. The I<sub>Kir2.1</sub> at positive and negative voltages were recorded in the absence of intracellular Mg<sup>2+</sup> and polyamines. Fig. 6 shows the effects of 0, 0.1, and 3 μM [Ba<sup>2+</sup>]<sub>i</sub> on the I<sub>Kir2.1</sub> recorded at +50 and -50 mV (Fig. 6 A) and at +30 and -30 mV (Fig. 6 B) from a holding potential of 0 mV and a prepulse voltage = -40 mV. As previously described (Shieh et al., 1996), control I<sub>Kir2.1</sub> ([Ba<sup>2+</sup>]<sub>i</sub> = 0) at +50 mV showed inactivation. In contrast, the current recorded at -50 mV was nearly constant throughout the 1.2-s period. The perfusion of the intracellular side of the membrane with 0.1 and 3 μM [Ba<sup>2+</sup>]<sub>i</sub> resulted in reductions of the currents at the end of depolarization to +50 or +30 mV and acceleration of the inactivation of the outward I<sub>Kir2.1</sub>. In contrast to the effects on outward I<sub>Kir2.1</sub>, 0.1 and 3 μM [Ba<sup>2+</sup>]<sub>i</sub> caused no change in the inward I<sub>Kir2.1</sub> recorded at -50 or -30 mV. All of these effects were reversible upon Ba<sup>2+</sup> removal.

The normalized current-voltage relationships in the presence of 0, 0.1, and 3 μM [Ba<sup>2+</sup>]<sub>i</sub> are shown in Fig. 6 C. The normalized I<sub>Kir2.1</sub> under control condition showed inward rectification at positive potentials, reflecting the presence of intrinsic inactivation. Intracellular Ba<sup>2+</sup> induced a dose-dependent and V<sub>m</sub>-dependent inhibition of the outward currents.

Fig. 7 A displays from right to left the dose-response curves for the I<sub>Kir2.1</sub> blockade by intracellular Ba<sup>2+</sup> at +10, +20, +30, +40, +60, and +80 mV. The currents recorded at the end of the 1.2-s pulses in the presence of intracellular Ba<sup>2+</sup> were normalized to the control at each voltage and expressed as fractional I<sub>Kir2.1</sub>. Relations of the fractional I<sub>Kir2.1</sub> versus [Ba<sup>2+</sup>]<sub>i</sub> were fitted by the Hill equation (Eq. 1). Fig. 7 B shows that K<sub>d</sub> obtained from fitting in Fig. 7 A



**FIGURE 5** Effects of intracellular Mg<sup>2+</sup> and spermine on the recovery of the inward I<sub>Kir2.1</sub> from blockade by extracellular Ba<sup>2+</sup>. A, B, and C are the current traces recorded in an inside-out patch perfused with the control solution, the control solution containing 1 mM free [Mg<sup>2+</sup>]<sub>i</sub>, and the control solution containing 100 μM spermine, respectively. The same voltage protocol as described in Fig. 4 was applied. The frequencies of stimulation were 0.067 Hz in A and B and 0.04 Hz in C. Sampling rates were 2.5 kHz (A and B) and 0.5 kHz (C).

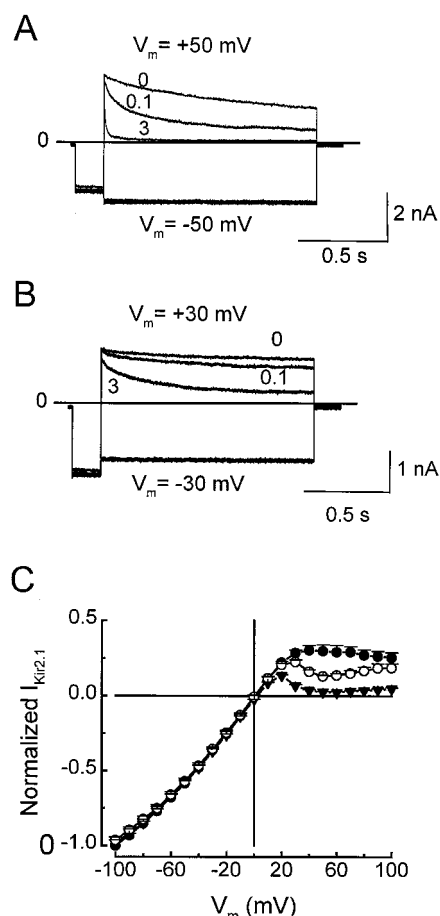


FIGURE 6 Blockade of Kir2.1 channels by intracellular  $Ba^{2+}$ .  $I_{Kir2.1}$  was recorded at +50 and -50 mV (A) and at +30 and -30 mV (B) in the presence of 0, 0.1, and 3  $\mu$ M  $[Ba^{2+}]_i$ . The residual current obtained in the presence of 30 mM intracellular TEA was used to subtract leak and capacitive components. The horizontal lines indicate the zero current level. (C) Normalized  $I$ - $V$  relationships in the presence of 0 (control,  $\bullet$ ,  $n = 5$ ), 0.1 ( $\circ$ ,  $n = 5$ ), and 3  $\mu$ M  $[Ba^{2+}]_i$  ( $\blacktriangledown$ ,  $n = 5$ ).  $I_{Kir2.1}$  measured at the end of the pulse was normalized to the values obtained at -100 mV in the absence of  $Ba^{2+}$ . The frequency of stimulation was 0.15 Hz, and the sampling rate was 5 kHz.

was steeply dependent on  $V_m$  between +10 and +40 mV, and the  $K_d$ - $V_m$  relationship fit well with the Boltzmann equation (Eq. 2). The  $K_d(0)$  was 91  $\mu$ M, and the  $\delta$  value was 1.79, indicative of the multiionic pore feature of the Kir2.1 channel (Armstrong et al., 1982; Cecchi et al., 1987). In contrast,  $K_d$  showed a slight tendency to increase at  $V_m \geq +40$  mV.

It was previously shown that the inactivation of outward Kir2.1 currents recorded at  $V_m \geq +40$  mV followed a double-exponential time course and that this inactivation may be due to a pH<sub>i</sub>-sensitive intrinsic gating movement (Shieh et al., 1996). To analyze the effects of intracellular  $Ba^{2+}$  on the two time constants that describe the inactivation of the outward  $I_{Kir2.1}$ , currents recorded at  $V_m \geq +40$  mV were fitted with a double-exponential function, and the time constants were extracted at each potential and  $[Ba^{2+}]_i$ . The time constants  $\tau_1$  (fast, Fig. 8 A) and  $\tau_2$  (slow, Fig. 8 B)

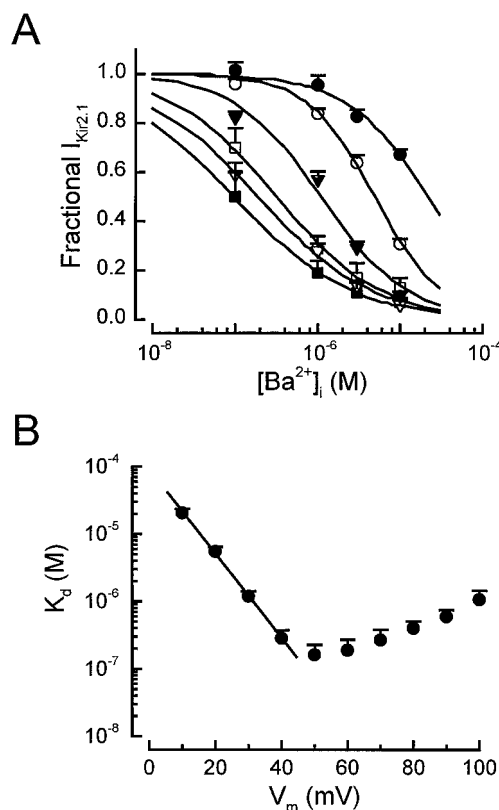


FIGURE 7 Dose-response curves and voltage dependence of Kir2.1 channel blockade by intracellular  $Ba^{2+}$ . (A) Dose-response curves at +10 ( $\bullet$ ), +20 ( $\circ$ ), +30 ( $\blacktriangledown$ ), +40 ( $\nabla$ ), +60 ( $\blacksquare$ ), and +80 ( $\square$ ) mV were constructed for intracellular  $Ba^{2+}$  blockade of steady state  $I_{Kir2.1}$ . Data were fitted with the Hill equation (Eq. 1, continuous lines). The fit  $K_d$  and  $n$  values were 0.32  $\mu$ M, 0.69,  $V_m = +80$  mV; 0.10  $\mu$ M, 0.61,  $V_m = +60$  mV; 0.19  $\mu$ M, 0.63,  $V_m = +40$  mV; 1.09  $\mu$ M, 0.83,  $V_m = +30$  mV; 4.91  $\mu$ M, 1.07,  $V_m = +20$  mV; 22.00  $\mu$ M, 0.87,  $V_m = +10$  mV. (B) Voltage-dependence of  $K_d$ .  $K_d$  at  $V_m$  between +10 and +40 mV were fitted (continuous line) with the Boltzmann equation (Eq. 2) with  $K_d(0) = 91$   $\mu$ M and  $z\delta = 3.57$ .

calculated from currents recorded in various  $Ba^{2+}$  were normalized to the time constants calculated in the absence of  $Ba^{2+}$  and are shown as a function of membrane potential. Both  $\tau_1$  and  $\tau_2$  were decreased by intracellular  $Ba^{2+}$  in a dose-dependent but voltage-independent manner. These results suggest that once intracellular  $Ba^{2+}$  was bound to the channel, it interfered with the intrinsic gating process of the channel in a manner that accelerated the inactivation process.

## DISCUSSION

### Blockade of Kir2.1 channels by extracellular $Ba^{2+}$

The blockade of inward rectifier  $K^+$  channels by extracellular  $Ba^{2+}$  has been extensively studied in starfish eggs (Hagiwara et al., 1978) and in frog skeletal muscle fibers (Standen and Stanfield, 1978). These studies showed that extracellular  $Ba^{2+}$  blocked the inward currents in a  $V_m$ - and time-dependent manner. The  $K_d(0)$  values for  $Ba^{2+}$  blockade of the inward rectifier  $K^+$  channels in starfish egg and



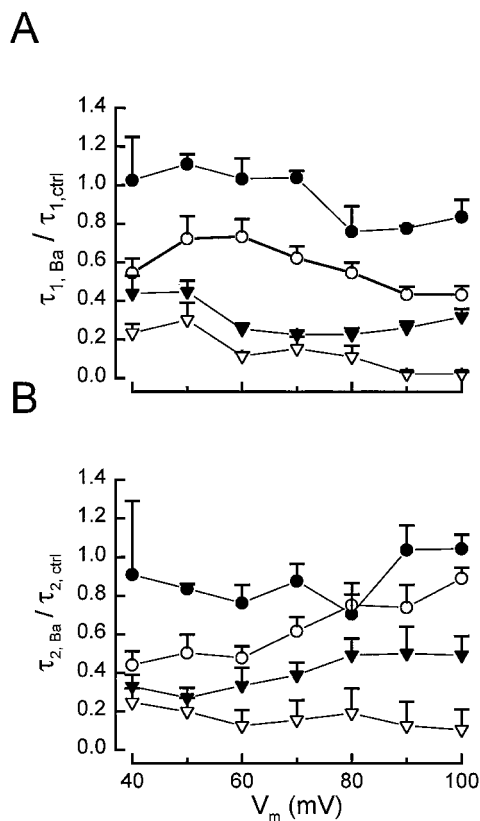


FIGURE 8 Acceleration of outward  $I_{Kir2.1}$  inactivation by intracellular  $Ba^{2+}$ . The  $\tau_{1,Ba}/\tau_{1,ctrl}$ - $V_m$  relationship (A) and  $\tau_{2,Ba}/\tau_{2,ctrl}$ - $V_m$  relationship (B) for 0.03 (●,  $n = 3$ ), 0.1 (○,  $n = 11$ ), 1.0 (▼,  $n = 11$ ), and 10 (▽,  $n = 8$ )  $\mu M$   $[Ba^{2+}]_i$ .

frog skeletal muscle were 560 and 500  $\mu M$ , respectively (Hagiwara et al., 1978; Standen and Stanfield, 1978). The effects of extracellular  $Ba^{2+}$  on these tissues were interpreted as the binding of  $Ba^{2+}$  to sites located within the pores of the channels where they sensed 64–70% of the electrical field (Hagiwara et al., 1978; Standen and Stanfield, 1978). The  $K_d(0)$  value obtained for the extracellular  $Ba^{2+}$  blockage of the Kir2.1 channels was 62  $\mu M$ , suggesting that cloned Kir2.1 channels are about one order of magnitude more sensitive to extracellular  $Ba^{2+}$  than the native inward rectifier  $K^+$  channels in starfish egg or in frog skeletal muscle. Furthermore, the apparent electrical distance ( $\delta = 0.54$ ) in Kir2.1 channels was smaller in the oocyte expressed channels than in the native channels.

Our results demonstrated that extracellular  $Ba^{2+}$  entered the pores to induce blockage of Kir2.1 channels as previously described in cloned and native inward rectifier  $K^+$  channels. In addition, we showed that the  $K_d$ - $V_m$  relationship became less steep as  $V_m < -120$  mV, and  $k_{off}$  values increased when  $V_m < -130$  mV. These observations are similar to those reported in the high-conductance  $Ca^{2+}$ -activated  $K^+$  channels (Neyton and Miller, 1988b) and Skaker  $K^+$  channels (Harris et al., 1998) and suggest that extracellularly applied  $Ba^{2+}$  can dissociate into the intracellular space at very hyperpolarizing potentials. On the

other hand,  $k_{off}$  estimated from recovery of  $Ba^{2+}$  blockade increased as  $V_m$  became more positive. This suggests that the majority of  $Ba^{2+}$  dissociated into the extracellular space in a  $V_m$ -dependent manner.

### Interactions of intracellular cations with $Ba^{2+}$ applied extracellularly

Consistent with the previous finding that permeant  $K^+$  can compete with  $Ba^{2+}$  for binding within the pores of the  $Ca^{2+}$ -activated  $K^+$  channels (Vergara and Latorre, 1983), we demonstrated that in Kir2.1 channels, increasing  $[K^+]_i$  from 20 to 200 mM increased the rate of recovery from extracellular  $Ba^{2+}$  blockade by decreasing the  $Ba^{2+}$  entrance rate (Fig. 3 C and Table 1). We also found that when an inside-out patch was perfused with  $Mg^{2+}$ -free and polyamine-free control solution, the recovery from extracellular  $Ba^{2+}$  (10  $\mu M$ ) blockade was much faster than in whole cells or cell-attached patches. One of the intracellular factors that contributed to the slow recovery from extracellular  $Ba^{2+}$  blockade in cell-attached patches was identified as spermine. We found that although spermine carries a +4 charge, it was not as efficient as intracellular  $K^+$  or  $Mg^{2+}$  in facilitating the dissociation of the bound  $Ba^{2+}$  from the pore of a Kir2.1 channel at  $V_r = +100$  mV. Note that in the presence of extracellular  $Ba^{2+}$ , spermine (100  $\mu M$ ) was still able to completely block the outward  $I_{Kir2.1}$  at  $V_r = +100$  mV, which indicates that spermine bound to its binding site(s) in the pore of the channel. These results provide evidence that a spermine binding site(s) is distinct from that for  $K^+$  or  $Mg^{2+}$ .

In summary, the interactions of extracellular applied  $Ba^{2+}$  with other cations in the pore of the Kir2.1 channel are very complicated. So far we have identified a common binding site for  $K^+$  and  $Ba^{2+}$  and a distinct site for spermine in the Kir2.1 channel. Further investigation is required to determine whether Kir2.1 channels have  $Ba^{2+}$  lock-in and enhancement sites lined up with the  $Ba^{2+}$  binding site similar to those described in the high-conductance  $Ca^{2+}$ -activated  $K^+$  channels (Neyton and Miller, 1988b).

### Blockade of Kir2.1 channels by intracellular $Ba^{2+}$

Intracellular  $Ba^{2+}$  at submicromolar concentrations blocked and accelerated the rate of inactivation of the outward  $I_{Kir2.1}$ .  $[Ba^{2+}]_i$  higher than 10  $\mu M$  resulted in complete inhibition of outward currents with very little blockade of the inward currents activated by hyperpolarization. All of these effects were observed in the absence of intracellular  $Mg^{2+}$  and polyamines. Fig. 7 B shows that  $K_d$  values for the inhibition of the outward currents decreased sharply as the depolarization increased until a minimum value of  $0.16 \pm 0.07$   $\mu M$  was reached at +50 mV. As depolarization became more positive,  $K_d$  for intracellular  $Ba^{2+}$  blockade of the outward  $I_{Kir2.1}$  increased. This may be due to the dissociation of  $Ba^{2+}$  into the extracellular space facilitated by

depolarization, as previously shown for  $\text{Na}^+$  permeating through the  $\text{K}^+$  channels of the squid giant axon (French and Wells, 1977). Alternatively, this may be a result of the limitation of  $\text{Ba}^{2+}$  access to its binding site when the intrinsic inactivation gate closed the channel. Our experimental data also demonstrate that the exposure of the intracellular side of the channels to  $\text{Ba}^{2+}$  results in an acceleration of the inactivation at positive voltages. It is tempting to propose that  $\text{Ba}^{2+}$  may directly interact with the intrinsic gating mechanism responsible for the inactivation of Kir2.1 channels at positive voltages. The possibility of  $\text{Ba}^{2+}$  interaction with the intrinsic gate renders it a useful tool for probing the movement of the intrinsic gate of the Kir2.1 channels.

In conclusion, in this study we report the voltage-dependent blocking effects of both intracellular and extracellular  $\text{Ba}^{2+}$  on the cloned inward rectifier  $\text{K}^+$  channel Kir2.1.  $\text{Ba}^{2+}$  applied extracellularly can enter the pore of a Kir2.1 channel and dissociate into the intracellular space at very negative membrane potentials. Our study of the effects of intracellular cations on the recovery from  $\text{Ba}^{2+}$  blockade suggest that various cations entering the pore of a Kir2.1 channel may occupy different binding sites. Whereas  $\text{Mg}^{2+}$  produced a degree of efficiency similar to that of  $\text{K}^+$  in relieving  $\text{Ba}^{2+}$  blockade, spermine bound in the channel was much less effective in enhancing the dissociation of  $\text{Ba}^{2+}$  from the pore. We also showed that intracellular  $\text{Ba}^{2+}$  closely interacts with the "intrinsic" gate and may be applied to reveal the characteristics of this "intrinsic" gating mechanism in Kir2.1 channels.

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