External Barium Influences the Gating Charge Movement of *Shaker* Potassium Channels

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ABSTRACT External Ba^{2+} speeds the *OFF* gating currents (I_g^{OFF}) of *Shaker* K^+ channels but only upon repolarization from potentials that are expected to open the channel pore. To study this effect we used a nonconducting and noninactivating mutant of the *Shaker* K^+ channel, ShH4-IR (W434F). External Ba^{2+} slightly decreases the quantity of *ON* gating charge (Q_{ON}) upon depolarization to potentials near −30 mV but has little effect on the quantity of charge upon stepping to more hyperpolarized or depolarized potentials. More strikingly, Ba^{2+} significantly increases the decay rate of I_g^{OFF} upon repolarization to −90 mV from potentials positive to ~−55 mV. For Ba^{2+} to have this effect, the depolarizing command must be maintained for a duration that is dependent on the depolarizing potential (>4 ms at −30 mV and >1 ms at 0 mV). The actions of Ba^{2+} on the gating current are dose-dependent ($EC_{50} \approx 0.2$ mM) and are not produced by either Ca^{2+} or Mg^{2+} (2 mM). The results suggest that Ba^{2+} binds to a specific site on the *Shaker* K^+ channel that destabilizes the open conformation and thus facilitates the return of gating charge upon repolarization.

INTRODUCTION

The regulation of ionic conductance by membrane potential was originally interpreted by Hodgkin and Huxley to imply the existence of charged or polar "gating particles" within the membrane electric field (1952). As the membrane potential changes, this charge redistributes across the electric field and generates a transient "gating current" that can be measured in voltage clamp experiments (e.g., Armstrong and Bezanilla, 1973; Schneider and Chandler, 1973; Keynes and Rojas, 1973). It is generally believed that gating currents of voltage-gated ion channels reflect the structural rearrangements that impart the voltage dependence on channel activity (for review see Almers, 1978).

Channel-blocking compounds can alter or interfere with the opening and closing conformational changes of ion channels (e.g., Armstrong, 1971; Århem 1980; Armstrong and Palti, 1991; Cahalan and Almers, 1979). One of the best-described examples is that of internal tetraethylammonium (TEA) and related quaternary ammonium ion block of the squid axon K^+ channel (Armstrong, 1966, 1969, 1971). Internally applied quaternary ammonium ions can only reach the binding site after the channel has opened and can cause a rapid inactivation of the ionic current (Armstrong, 1969). Once at the internal blocking site, quaternary ammonium ions impede the closing of the channel presumably by sterically hindering the closing conformational change (Armstrong, 1971). TEA also slows the return of gating charge to its resting state, suggesting that channel closing is coupled to the return of gating charge (Bezanilla et al., 1991; see also Stühmer et al., 1991). In contrast, internally applied 4-aminopyridine (4-AP), which also blocks the open channel, prevents closed-blocked channels from opening as evidenced by a 10–15% decrease in total charge movement and an increased decay rate of *OFF* gating current (McCormack et al., 1994).

Divalent cations can also modify the gating behavior of ion channels (Clay, 1995; Frankenhaeuser and Hodgkin, 1957; Gilly and Armstrong, 1982a,b; Spires and Begenisich, 1994, 1995; Harrison et al., 1993; Davidson and Kehl, 1995; Talukder and Harrison, 1995). In the case of the squid giant axon, external Zn^{2+} greatly slows the activation of Na^+ (Gilly and Armstrong, 1982a) and K^+ currents (Gilly and Armstrong, 1982b; Spires and Begenisich, 1995) while having much smaller effects on the kinetics of ionic current deactivation. Consistent with this, external Zn^{2+} slows the *ON* gating currents of the squid Na^+ channel while little affecting the *OFF* gating currents (Gilly and Armstrong, 1982a). However, Zn^{2+} causes only a modest change of the K^+ channel gating currents when added to either side of the membrane (Spires and Begenisich, 1995). Based on this observation, Spires and Begenisich (1995) proposed that Zn^{2+} modifies conformational changes of the squid K^+ channel that are only weakly voltage-dependent, most likely occurring in the final opening transition (Conti et al., 1984; Schauf and Bullock, 1979; Zagotta and Aldrich, 1990; Koren et al., 1990).

The present work addresses the influence of external Ba^{2+} on the gating charge movement of *Shaker* K^+ channels. Ba^{2+} blocks many classes of K^+ channels and has been widely used to probe the structure of K^+ selective pores (Armstrong and Taylor; 1980; Armstrong et al., 1982; Eaton and Brodwick, 1980; Miller et al., 1987; Neyton and

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Miller, 1988; Grissmer and Cahalan, 1989; Neyton and Pelleschi, 1991; Taglialatela et al., 1993; Slesinger et al., 1993; Wollmuth, 1994; Lopez et al., 1994). Our previous work suggests that external Ba^{2+} blocks the *Shaker* K^+ channel at two distinct and sequential binding sites as evidenced, in part, by both a fast and a slow component of ionic current block (Hurst et al., 1995). The present work demonstrates that external Ba^{2+} can significantly speed the return of gating charge to its resting state, but only upon repolarization from potentials that are expected to populate the open state or states very near the open state. The results can be accounted for by a simple mechanism whereby Ba^{2+} binding destabilizes the open state by modifying transition rates between states that occur late in the activation pathway.

METHODS

Molecular biology

The amino terminal deletion *Shaker* H4 (Δ6-46) (Hoshi et al., 1990), also called ShH4-IR (Inactivation Removed), containing the (W434F) mutation (Perozo et al., 1993) was the primary construct used in this study. RNA was transcribed in vitro with T7 RNA polymerase (Pharmacia, Piscataway, NJ) according to published methods (Stefani et al., 1994). RNA (25–50 ng) was injected into collagenase-treated *Xenopus laevis* oocytes (stage V-VI) in 50 nl water. Oocytes were maintained at 18°C in a saline solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Na HEPES at pH 7.6) supplemented with gentamycine (50 μg/ml). Voltage clamp recordings were performed at room temperature 2–5 days following injection.

Solutions

Bath solutions were made by mixing stock isotonic solutions (240 mOsm) of the main cation buffered with 10 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) at pH 7.0; methanesulfonate (MES) was the main anion. The external solution in control experiments using the nonconducting clone was 115 mM sodium methanesulfonate (Na-MES), 2 mM calcium methanesulfonate [Ca-$(MES)_2$], 2 mM potassium methanesulfonate (K-MES), 0.1 mM EGTA, and 10 mM HEPES. The external Ba^{2+}-containing solution was composed of the following: 115 mM Na-MES, 2 mM barium methanesulfonate [Ba-$(MES)_2$], 2 mM K-MES, and 10 mM HEPES. Solutions for Ba^{2+} dose response measurements had a constant divalent concentration of 2 mM with Ba-$(MES)_2$ replacing Ca-$(MES)_2$, e.g., the solution containing 0.1 mM Ba^{2+} was as follows: 0.1 mM Ba-$(MES)_2$, 1.9 mM Ca-$(MES)_2$, 2 mM K-MES, and 115 mM Na-MES. The solution in contact with the intracellular compartment in most experiments was 120 mM potassium glutamate buffered with 10 mM HEPES. To measure gating currents from the conducting clone, the intracellular compartment was perfused with 120 mM *N*-methyl-D-glucamine methanesulfonate (NMG-MES) and 10 mM HEPES through a small glass cannula inserted into the bottom of the oocyte. In those experiments, the external solution was 117 mM NMG-MES and either 2 mM Ca-$(MES)_2$ or 2 mM Ba-$(MES)_2$.

Electrophysiology

Macroscopic ionic and gating currents were recorded using the cut-open oocyte Vaseline gap (COVG) technique (Stefani et al., 1994). Negative subtraction pulses (P/−4) were given from a subtraction holding potential (SHP) of −120 mV or positive subtraction pulses (P/4) were given from a SHP of 20 mV. At potentials positive to 20 mV and negative to −120 mV, the capacity is a linear function of voltage. The subtraction protocol had little effect on the kinetic parameters of the gating currents; however, different protocols were used depending on the batch of oocytes which differentially expressed endogenous currents that can contaminate the subtraction pulses. When possible, i.e., with sufficiently high expression, no subtraction pulses were used. All data were filtered at 1/5 the sampling frequency. Gating charge was quantified and analyzed by integrating the gating current using the Clampfit software (Axon Instruments Inc., Foster City, CA).

Simulation

Gating current traces were generated according to the kinetic scheme shown in Fig. 7 using the SCoP 3.5 simulation package (Simulation Resources, Inc., Berrien Springs, MI) and the DJGPP C/C++ compiler (Delorie Software, Rochester, NH) running on an IBM PC-compatible computer. The generated data were filtered at the same cutoff frequency as the experimental data using a Gaussian filter (Colquhoun and Sigworth, 1983). The voltage-dependent rates are assumed to be of the form $\alpha = \alpha_0 \cdot \exp(z_\alpha eV/kT)$ for the forward rates α and $\beta = \beta_0 \cdot \exp(-z_\beta eV/kT)$ for the backward rates β, where α_0 and β_0 are the rates at 0 mV, z_α and z_β are the equivalent charges moving up to the transition state. The kinetic parameters used in the simulation were obtained from an independent fit of gating current and ionic current traces recorded in absence of Ba^{2+}. They are presented in Table 1.

RESULTS

External Ba^{2+} speeds the return of gating charge

When applied externally to noninactivating *Shaker* Δ6-46 K^+ channels (Hoshi et al., 1990; also called ShH4-IR for inactivation removed), Ba^{2+} does not alter the kinetics of ionic current during a brief depolarization or the gating current evoked at potentials negative to channel opening (Hurst et al., 1995). To determine whether Ba^{2+} influenced gating charge movement of *Shaker* H4-IR at more depolarized potentials, the intracellular compartment of the oocyte was perfused with a K^+-free solution. This allowed measurement of gating current at potentials that are otherwise expected to evoke ionic current. As illustrated in Fig. 1, Ba^{2+} had little if any effect on the gating current evoked by depolarizations to potentials negative to ~−50 mV, i.e., potentials that do not open the channel pore. In contrast, upon repolarization from more positive potentials, Ba^{2+} increased the rate at which the *OFF* gating current decayed. The accelerated charge return can be seen as an increased peak amplitude of I_g^{OFF} and a faster return to baseline upon repolarization to −90 mV (Fig. 1).

TABLE 1 Voltage-dependent and kinetic parameters used for simulating gating currents

Transition	z_α	z_β	α_0 (ms^{-1})	β_0 (ms^{-1})
C_0–C_1	0.2	0.75	1.16	0.12
C_1–C_2, C_2–C_3, C_3–C_4	0.62	0.64	9.4	1
C_4–C_5	0.7	2	3.2	0.7
C_5–C_6	0.05	0.93	2.6	0.015
C_6–O	0.2	0.38	1.7	0.95

z_α and z_β are the equivalent charge moved in the forward and backward transitions, respectively. α_0 and β_0 are the rates of the forward and backward transitions at 0 mV, respectively.

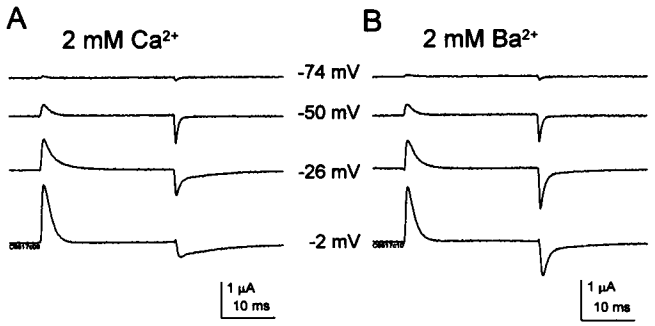

FIGURE 1 External Ba^{2+} speeds gating charge return of ShH4-IR. Gating currents evoked from the same K^+ depleted oocyte expressing *Shaker* H4-IR. Voltage steps were given from a holding potential of −90 mV to −74, −50, −26, and −2 mV in the presence of 2 mM Ca^{2+} (*A*) and 2 mM Ba^{2+} (*B*); the membrane was repolarized to −90 mV. The intracellular compartment of the oocyte was perfused with 120 mM NMG-MES, 10 mM HEPES at 4 μl/h for approximately 30 min. Note that the vertical scale bars are slightly different due to the run-down of gating currents that often occurs in perfused oocytes.

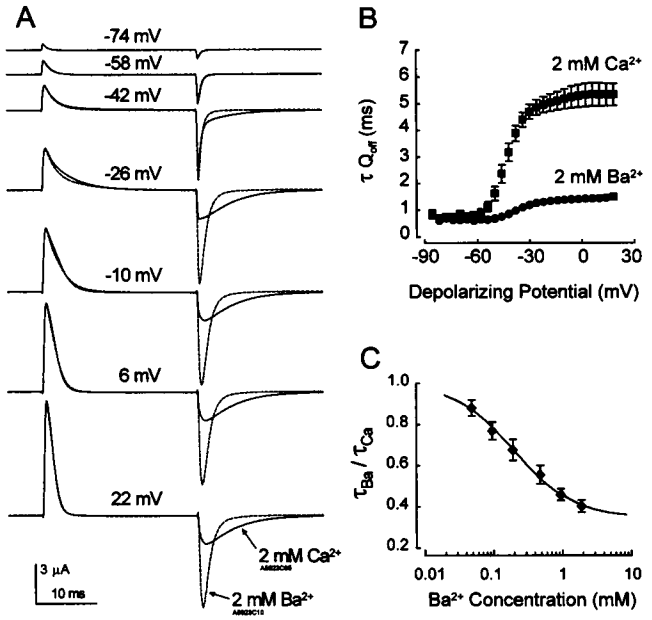

FIGURE 2 The effect of Ba^{2+} on gating currents is voltage- and dose-dependent. (*A*) Superimposed gating currents from the same oocyte with either 2 mM external Ca^{2+} (——) or 2 mM external Ba^{2+} (- - - -). Gating currents were evoked by a step depolarization from −90 mV to the potential indicated above each trace; the membrane was repolarized to −90 mV. (*B*) Plot of the time course of Q_{OFF}, measured at −90 mV, as a function of the preceding depolarizing potential. Gating currents were evoked in either 2 mM Ca^{2+} (■) or 2 mM Ba^{2+} (●). Data points show the time constant (τ) obtained from a single exponential fit of Q_{OFF}, the time integral of I_g^{OFF}. Because Q_{OFF} contains at least two exponential components, these values qualitatively show the conversion of a predominant fast component to a predominant slow component. Date points are the mean ± SEM of 6 oocytes; when not present the error is less than the size of the symbol. (*C*) Ba^{2+} dose response of the gating current. Data points are the ratio of the τ of Q_{OFF} measured at the indicated concentration of Ba^{2+} to the τ of Q_{OFF} in 2 mM Ca^{2+}; τ was obtained from a single exponential fit of Q_{OFF} evoked by a repolarizing to −90 mV from 30 mV. The total divalent cation concentration was maintained constant at 2 mM. Each point shows the average ratio obtained from individual oocytes (± SEM, n = 3). The continuous curve is the best fit to a general logistic function with unit Hill slope.

To study the influence of Ba^{2+} on the gating current more carefully we used the W434F nonconducting mutant of ShH4-IR, which produces gating currents similar to the conducting clone but obviates the removal of intracellular K^+ (Perozo et al., 1993). As in the case of ShH4-IR, external Ba^{2+} most notably increased the decay rate of I_g^{OFF}; the extent of this effect was dependent on the potential of the preceding depolarizing command (Fig. 2 *A*). Fig. 2 *A* compares two families of gating currents recorded from the same oocyte with either 2 mM Ca^{2+} (——) or 2 mM Ba^{2+} (- - - -) as the total external divalent component. Depolarizations to potentials less positive than ~−55 mV evoked gating currents that were essentially identical in Ca^{2+} and Ba^{2+}. However, as the depolarization was made more positive, the slowing of I_g^{OFF} that normally occurs (see below) was substantially reduced by Ba^{2+}, and consequently the *OFF* gating currents decayed faster in Ba^{2+} than Ca^{2+}.

The slowing of I_g^{OFF} that is observed upon repolarizing from progressively more depolarized potentials reflects the voltage-dependent population of a state that introduces a rate-limiting transition in the closing reaction (Bezanilla et al., 1991; Stühmer et al., 1991; Perozo et al., 1992,1993; Bezanilla et al., 1994; Zagotta et al., 1994; McCormack, 1994). To address the voltage-dependence of the effect of Ba^{2+} on the return of gating charge, Q_{OFF}, the quantity of *OFF* gating charge moved as a function of time, was fit to a single exponential term. Fig. 2 *B* qualitatively shows that the primary effect of Ba^{2+} was to reduce the slowing of Q_{OFF} that is otherwise observed as the depolarizations become positive to ~−55 mV. It should be noted that the time constants shown are only an approximation of the overall time course. At intermediate potentials, Q_{OFF} contained at least two major components that varied in amplitude with the potential of the depolarizing command. In this potential range, the time constant should not be taken literally and the single exponential fit simply reflects the conversion of a predominant fast component to a predominant slow component. At the two ends of the voltage range, where the time constants approach a limiting value, the time-dependent development of Q_{OFF} was predominated by a component reasonably well described by a single exponential term.

The dose response for the effect of Ba^{2+} on the gating current was assayed by fitting Q_{OFF}, evoked by repolarizing to −90 mV from 30 mV, to a single exponential term (Fig. 2 *C*). The values plotted in Fig. 2 *C* are the ratio of the time constant (τ) of Q_{OFF} at the indicated concentration of Ba^{2+} to τ in 2 mM Ca^{2+}; data points are the mean of ratios obtained from single oocytes. In each case the total divalent cation concentration was maintained constant at 2 mM by replacing Ca^{2+} with Ba^{2+}. The dose response data were fitted to a general logistic equation with unit Hill slope (——), and the derived half-maximal concentration was 0.23 ± 0.1 mM (± SD, n = 3, Fig. 3*C*). Accordingly, 2 mM

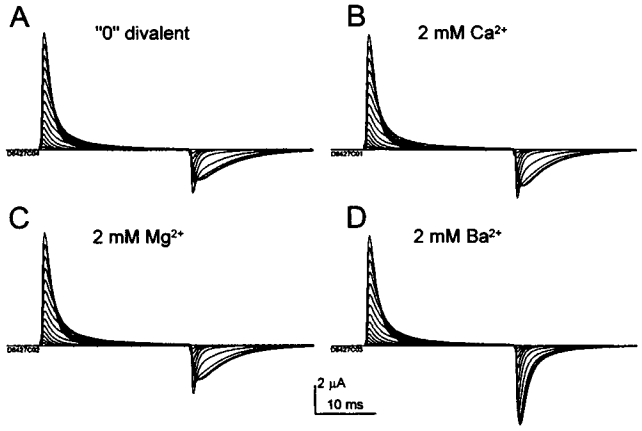

FIGURE 3 The effect of Ba^{2+} on I_g^{OFF} is not produced by Ca^{2+} or Mg^{2+}. Gating currents from the same oocyte expressing ShH4-IR (W434F) with either no added divalent cation (*A*), 2 mM Ca^{2+} (*B*), 2 mM Mg^{2+} (*C*), or 2 mM Ba^{2+} (*D*) in the external solution. Voltage steps were from −90 mV to 22 mV in 8-mV increments; the holding and repolarizing potentials were −90 mV.

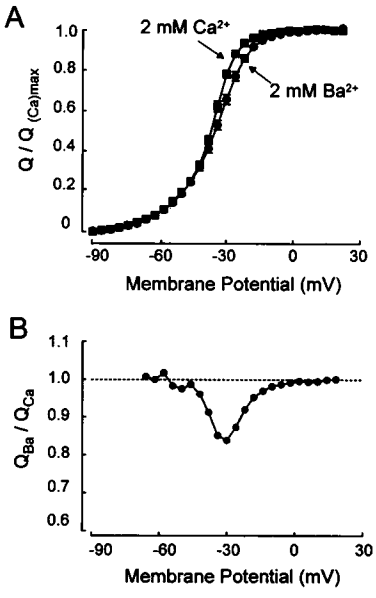

FIGURE 4 Ba^{2+} does not change the maximum amount of charge. (*A*) Q_{ON} as a function of membrane potential for a series voltage steps from a holding potential of −90 mV. Q_{ON} from individual oocytes was measured in 2 mM Ca^{2+} (■) and 2 mM Ba^{2+} (●) and normalized to the maximum charge measured in Ca^{2+}; data points indicate the average of the ratios obtained from individual oocytes (± SEM, n = 5). (*B*) Plot of the ratio of Q_{ON} measured in Ba^{2+} to Q_{ON} obtained in Ca^{2+}. Date points show the mean of 5 oocytes.

Ba^{2+}, the concentration used in the majority of the experiments of this study, was sufficient to produce >90% of the predicted maximal effect.

Unlike Ba^{2+}, external Ca^{2+} and Mg^{2+} had little influence on the movement of gating charge of ShH4-IR (W434F) when compared to gating currents recorded in the absence of any divalent cation ($n \geq 4$) (Fig. 3). This suggests that the actions of Ba^{2+} on the gating current result from a relatively specific binding site. Consistent with this idea, a single point mutation in the putative pore-forming region of this channel, D447N, dramatically reduces the sensitivity of the gating currents of Ba^{2+} (Hurst et al., 1996).

Ba^{2+} does not change the maximum amount of charge available to move

Fig. 4 *A* shows the relationship between *ON* gating charge and membrane potential with either 2 mM Ca^{2+} or 2 mM Ba^{2+} in the external solution. The charge-voltage ($Q_{(V)}$) relationships were obtained in both conditions from individual oocytes and were normalized to the maximal charge measured in Ca^{2+}. Thus, Ba^{2+} did not change the amount of charge available to move (upper limit) and produced only a slight shift in the midpoint potential of the $Q_{(V)}$ relationship. The main difference was that the $Q_{(V)}$ relationship was consistently shallower near the upper inflection when Ba^{2+} replaced Ca^{2+} (n = 5). For example, a depolarization to −30 mV in the presence of Ba^{2+} moved 16% ± 4% (±SD) less charge than the same depolarization in Ca^{2+}. The difference in charge moved was maximal at −30 mV and diminished as the membrane potentials approached the hyperpolarized and depolarized limits of the experiment (Fig. 4 *B*). This suggests that Ba^{2+} modifies conformational changes that are most likely to occur with depolarizations near −30 mV. In other words, with less positive depolarizations (negative to −55 mV) channels fail to reach the Ba^{2+}-modified transition, and therefore the same charge moves in the presence of Ca^{2+} and Ba^{2+}. Moderate depolarizations (~−30 mV) heavily populate a state that, when Ba^{2+}-bound, has a decreased likelihood of proceeding past that state and therefore move less charge. Sufficiently strong depolarizations (positive to ~0 mV) appear to "push" the channel through the Ba^{2+}-modified transition and consequently move the same total charge in the absence and presence of Ba^{2+}.

Ba^{2+} does not influence I_g^{OFF} following a very brief depolarization

In the absence of Ba^{2+}, the decay rate of I_g^{OFF} is not only dependent on the potential of the preceding depolarizing command but also on the duration that the membrane remains depolarized (Bezanilla et al., 1991, 1994; Perozo et al., 1992, 1993; Zagotta et al., 1994a; McCormack, 1994). This can be seen in Fig. 5 *A* of gating currents evoked by depolarizing to 0 mV for various durations and repolarizing to −90 mV in the absence of Ba^{2+}. The dependence on the depolarizing duration reflects the kinetics of all the conformational changes that lead to the state associated with the rate-limiting transition in the closing reaction (Bezanilla et al., 1991, 1994; Perozo et al., 1992, 1993; Zagotta et al., 1994a; McCormack, 1994). Ba^{2+} was only able to influence I_g^{OFF} if the preceding depolarization was long enough to induce a slow return of gating charge regardless of the

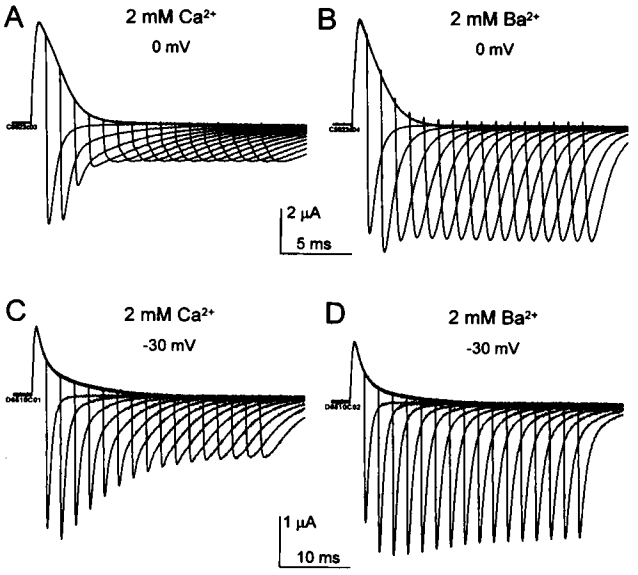

FIGURE 5 Influence of Ba^{2+} on Q_{OFF} is time-dependent. Gating currents evoked with either 2 mM Ca^{2+} (*A* and *C*) or 2 mM Ba^{2+} (*B* and *D*) in the external solution. The membrane was stepped from −90 mV to 0 mV (*A* and *B*) or −30 mV (*C* and *D*) and then repolarized to −90 mV; depolarizing durations were in increments of 1 and 2 ms, respectively. (*A*) and (*B*) were from the same oocyte and (*C*) and (*D*) were from the same oocyte.

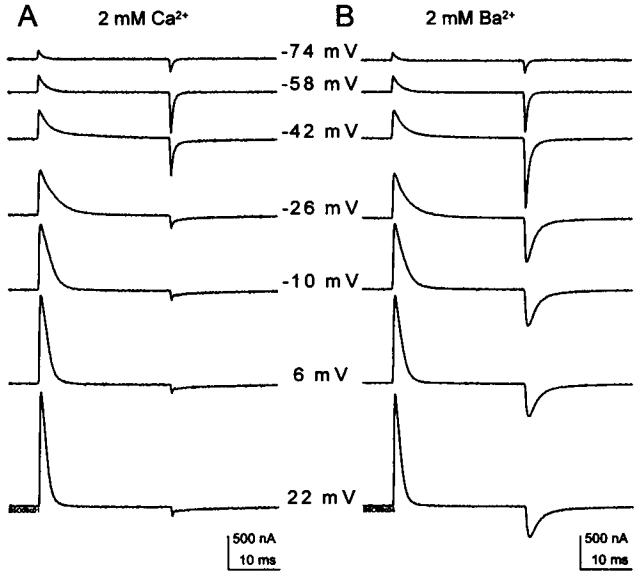

FIGURE 6 External Ba^{2+} inhibits "charge immobilization" produced by N-type inactivation. Gating currents measured from the same oocyte expressing ShH4 (W434F). Currents were measured in the presence of 2 mM Ca^{2+} (*A*) and 2 mM Ba^{2+} (*B*). Note the extremely slow return of gating charge produced by the inactivation domain in the absence of Ba^{2+}. Traces shown are the average of three sweeps.

potential of the depolarizing command (compare Figs. 5 *A* with *B*, and *C* with *D*). For example, a depolarization of 2 ms to 0 mV was sufficient for I_g^{OFF}, evoked upon repolarization, to begin to differ in Ca^{2+} and Ba^{2+}, while the membrane had to be depolarized for ~6 ms to −30 mV for this same effect (Fig. 5, $n > 4$). The lack of effect of Ba^{2+} following shorter depolarizations supports the idea that Ba^{2+} primarily modifies transitions that occur late in the activation pathway, near the open state of the channel.

External Ba^{2+} competes with inactivation-induced "charge immobilization"

It has previously been shown that fast inactivation of ionic current is associated with a dramatic slowing of I_g^{OFF} and gives rise to an apparent "charge immobilization" (Armstrong and Bezanilla, 1977; Bezanilla et al., 1991). Charge immobilization results from the open channel block by the inactivation domain, which hinders channel closing and, consequently, the return of gating charge (Armstrong and Bezanilla, 1977; Bezanilla et al., 1991; Demo and Yellen, 1991). Because Ba^{2+} speeds the return of gating charge, and presumably channel closing, we tested whether extracellular Ba^{2+} could reduce the degree of charge immobilization produced by the inactivation domain. Fig. 6 shows the gating currents of the inactivating but nonconducting mutant ShH4 (W434F) in the absence and presence of external Ba^{2+}. As was shown by Bezanilla and co-workers (1991), I_g^{OFF} in Ca^{2+} becomes extremely slow or "immobilized" for the inactivating clone upon repolarization from potentials sufficient to open the channel pore due to the open channel block by the inactivation domain (Fig. 6 *A*). As illustrated in Fig. 6 *B*, extracellular Ba^{2+} partially prevented the "charge immobilization" produced by the inactivation domain as evidenced by the faster decay of I_g^{OFF} (i.e., larger peak amplitude and faster decay) in Ba^{2+} upon repolarization from potentials positive to −55 mV. Note that I_g^{OFF} still became slower in the presence of Ba^{2+} as the depolarizations were made positive to −42 mV. This suggests Ba^{2+}-occupied ShH4 (W434F) channels are able to reach the inactivated state, and consequently the open state, which results in a slow down I_g^{OFF}. Consistent with this idea, I_g^{OFF} in Ba^{2+} became much slower for ShH4 (W434F) than its noninactivating counterpart, ShH4-IR (W434F), upon repolarization from the more positive potentials [compare Fig. 2 *A* (*dashed trace*) with Fig. 6 *B*].

DISCUSSION

It is not uncommon for channel blocking compounds (e.g., Armstrong, 1971; Århem, 1980; Armstrong and Palti, 1991; Cahalan and Almers, 1979) and conducting ions (e.g., Swenson and Armstrong, 1981; Matteson and Swenson, 1986; Demo and Yellen, 1992) to influence the gating conformational changes of voltage-dependent ion channels. McCormack and co-workers (1994) previously demonstrated that 4-AP alters the gating currents of *Shaker* K^+ channels in ways similar to what we show here for Ba^{2+}, and they proposed that 4-AP bound channels were not able to obtain the open conformation. However, there are some distinct differences between the actions of 4-AP and Ba^{2+}.

First, 4-AP reduces the total gating charge available to move by about 10–15%, suggesting that 4-AP bound channels do not reach the final open state (McCormack et al., 1994). In contrast, Ba^{2+} did not change the amount of gating charge available to move, although it did slightly alter the voltage dependence (Fig. 4 *A*). Second, unlike the present experiments, 4-AP acts from the cytoplasmic side of the membrane and can only reach its blocking site after the channel has opened. This feature contrasts the effects of different intracellular open channel blockers. Both internal TEA (Bezanilla et al., 1991; see also Stühmer et al., 1991) and the endogenous inactivation domain of Na^+ (Armstrong and Bezanilla, 1977) and K^+ (Bezanilla et al., 1991) channels greatly slow the return of gating charge, presumably by sterically hindering the closing of the channel pore. In the latter cases, the slowing of charge return takes place without significant change in the quantity of charge moved.

There are several possible mechanisms that can account for the observed voltage- and time-dependence for the effects of Ba^{2+} on the movement of gating charge. One is that the binding reaction possesses an intrinsic voltage dependence and is enhanced at depolarized potentials. However, this is unlikely because the polarity is opposite to that expected for an external cation. A second possibility is that Ba^{2+} can only bind the open channel and therefore only acts at depolarized potentials. This also seems unlikely because previous studies have shown that Ba^{2+} block of ionic current can occur from the closed state of this channel; in fact, blockade becomes stronger as the membrane is hyperpolarized (Hurst et al., 1995). Therefore, the most likely mechanism by which Ba^{2+} may speed the return of gating charge is that Ba^{2+} occupancy of its binding site destabilizes the open state as was originally proposed by Armstrong and Taylor (1980). Consider that Ba^{2+}, more so than speeding I_g^{OFF}, appears to inhibit the normal slowing of I_g^{OFF} that occurs upon repolarization from moderate to strong depolarizing potentials. This can be seen by plotting the time constant of Q_{OFF} as a function of the depolarizing command potential in Ca^{2+} and Ba^{2+} (Fig. 2 *B*). Following relatively weak depolarizations, I_g^{OFF} decayed with approximately the same time course in Ca^{2+} and Ba^{2+}. However, as the depolarizations were made more positive, the decay of I_g^{OFF} became considerably slower in Ca^{2+}, whereas the slowing is much less pronounced in Ba^{2+}. This suggests that Ba^{2+} hinders the channel from reaching the state associated with the rate-limiting transition in the closing reaction and/or speeds the backward rate of this transition. In either case, the net effect would be that Ba^{2+} occupancy destabilizes any state, including the open state, that follows the Ba^{2+}-modified transition upon a depolarizing voltage step. Consistent with this idea, Fig. 6 shows that Ba^{2+} decreases the stability of the open-blocked state produced by the amino terminal inactivation domain as evidenced by the partial relief of "charge immobilization" when Ba^{2+} replaced Ca^{2+} (Armstrong and Bezanilla, 1977; Bezanilla et al., 1991; Demo and Yellen, 1991).

It has recently been proposed that the nonconducting W434F mutation does not conduct because it is in a permanent state of slow or C-type inactivation (Yan et al., 1996). This raises the possibility that the actions of Ba^{2+} on the gating current of ShH4-IR (W434F) may be related to slow inactivation. We feel that this is not the case because Ba^{2+} had similar effects on the gating current of the conducting clone, ShH4-IR (Fig. 1), and there is no reason to believe that a significant fraction of these channels should become inactivated during the brief depolarizations used in the present experiments.

Model for the effects of Ba^{2+} on *Shaker* gating currents

Several models have been proposed for the voltage-dependent activation of *Shaker* K^+ channels that can account for the major features of the ionic and/or gating currents (e.g., Zagotta and Aldrich. 1990; Koren et al., 1990; Schoppa et al., 1992; Bezanilla et al., 1994; Zagotta et al., 1994b; McCormack et al., 1994). To simulate the actions of external Ba^{2+} we chose to use the linear and sequential model proposed by Bezanilla and co-workers (1994). In this model, shown by the lower sequence in Fig. 7 *A*, the transition rates between each state have varying degrees of voltage dependence (Table 1). Based on the observation that Ba^{2+} blocks ShH4-IR at hyperpolarized and depolarized potentials, we introduced an equivalent Ba^{2+}-bound state for each state in the activation sequence. The only difference between the bound (upper) and unbound (lower) series of states is the transition rates between C_5 and C_6; the forward rate α in the Ba^{2+}-bound series is divided by the factor σ_2 and the backward rate β is multiplied by σ, where σ_2 is 2 and σ is 3. Therefore, Ba^{2+}-bound channels are less likely at a given potential to exist in states to the right $C_{5(Ba)}$ than unbound channels. The rates of Ba^{2+} binding and unbinding between C_6 and $C_{6(Ba)}$ and between O and $O_{(Ba)}$ are also changed by these factors to conserve microscopic reversibility (Colquhoun and Hawkes, 1983; Läuger, 1983; Steinberg, 1987a,b; Song and Magleby, 1994). This implies that both the C_6 and O states have a lower affinity for Ba^{2+} ions than the other conformations of the channel.

According to the model proposed by Bezanilla and coworkers (1994), the slowing of Q_{OFF}, seen in the absence of Ba^{2+}, arises from the rate-limiting transition from C_6 to C_5 (Table 1). This still holds for the set of parameters used in this simulation, but unlike the model of Bezanilla and coworkers (1994), the C_5 to C_6 transition is not the most voltage-dependent. Because Ba^{2+} increases the backward rate β from C_6 and C_5 while decreasing the forward rate α, the model in Fig. 7 *A* predicts that I_g^{OFF} is faster for Ba^{2+}-bound channels but only following depolarizations sufficient to populate $C_{6(Ba)}$ (i.e., potentials positive to ~ -55 mV; Fig. 7 *B*). This model can also account for the observation that Ba^{2+} does not change the maximal amount of charge available to move because the modified transition

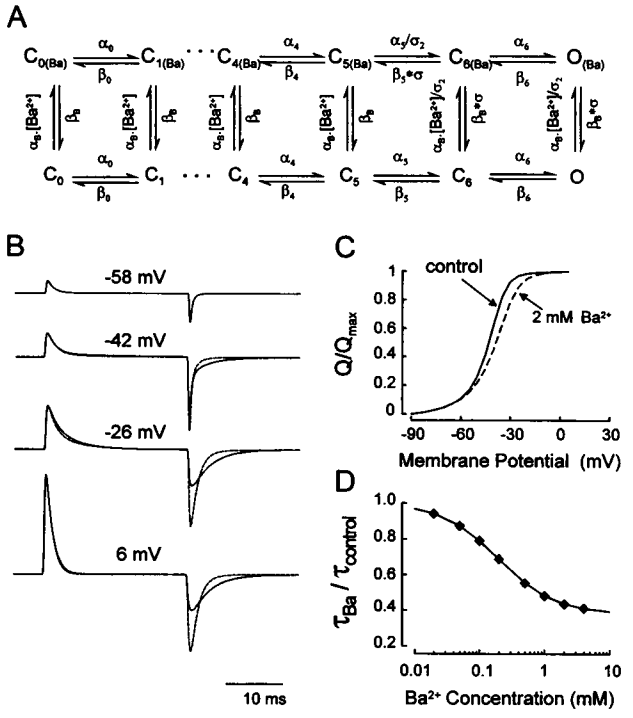

FIGURE 7 Model of the action of Ba^{2+} on the gating current. (*A*) Kinetic scheme for the actions of Ba^{2+} on ShH4-IR (W434F) gating currents. Details of the simulation are described in Methods. Briefly, the horizontal arrows show voltage dependent transitions between states in the activation pathway; vertical arrows are Ba^{2+} binding and unbinding transitions. The only difference in the activation pathway (i.e., horizontal sequences) between the bound (upper) and unbound (lower) series is between $C_5 \rightleftarrows C_6$ and $C_{5(Ba)} \rightleftarrows C_{6(Ba)}$; in the latter, the forward rate α is divided by the factor σ_2 and the backward rate β is multiplied by σ, where σ_2 is 2 and σ is 3. The binding and unbinding rates between C_6 and $C_{6(Ba)}$ and between O and $O_{(Ba)}$ were also modified by σ and $\sigma 2$ to conserve microscopic reversibility. The Ba^{2+} binding parameters were taken from measurements of the slow component of ionic current block (Hurst et al., 1995). However, the unbinding rate was divided by 4 to account for the difference in apparent K_D between the ionic current block and the *OFF* gate acceleration. At 0 mV, the rates of binding (α_B) and unbinding (β_B) were 2.4×10^{-6} (ms^{-1}) and 3.0×10^{-6} (ms^{-1}), respectively, and $z\delta$ was -0.25. (*B*) Simulated gating currents in 2 mM Ca^{2+} (——) and 2 mM Ba^{2+} (- - - -). The holding and repolarizing potentials are -90 mV; the depolarizing potential is indicated above each trace. Compare these simulations with the experimental data shown in Fig. 2 *A*. (*C*) Charge-voltage relation ship predicted by the model in the absence (——) and presence (- - - -) of 2 mM Ba^{2+}. (*D*) Ba^{2+} dose-dependence of the kinetic scheme shown in (*A*). Simulated data points were calculated as in Fig. 2 *C*; the continuous line is the best fit to a logistic function with unit Hill slope (EC_{50} = 0.19 mM).

rates are voltage dependent. Therefore, the effect of Ba^{2+} can become overcome at sufficiently positive potentials (Fig. 7 *C*). Similar modifications of either the pervious (C_4 to C_5) or next (C_6 to O) transition failed to adequately reproduce these features of the Ba^{2+} modified gating current (data not shown).

The σ and σ_2 parameters were adjusted using gating current kinetics for only two concentrations of Ba^{2+}, 0 and 2 mM, as shown in Fig. 7 *B*. As a test of the validity of these values, the dose-dependence of the model (Fig. 7 *D*) was evaluated in the same manner as the experimental data shown in Fig. 2 *C*. The simulated dose response compares well with the experimental results in terms of both the EC_{50} (data, EC_{50} = 0.23 mM ± 0.1 mM, n = 3; model, EC_{50} = 0.19 mM; Fig. 7 *D*) and slope (each set of data were fit to a logistic function with unit Hill slope).

While the model described above predicts the general features of the actions of Ba^{2+} on the on the gating current, the important feature in this context is that extracellular Ba^{2+} destabilizes the open state, consistent with the findings of Armstrong and Taylor (1980). In fact, by invoking a similar mechanism, any model of channel activation that includes a rate-limiting closing transition near the open state should also be able to predict the effects of Ba^{2+}. In summary, we feel that the data presented here provide strong evidence that 1) Ba^{2+} binding decreases the stability of the open state, and 2) Ba^{2+} primarily influences transitions that occur late in the activation pathway, near the open state.

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