Barium Inhibition of the Collapse of the *Shaker* K^+ Conductance in Zero K^+

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ABSTRACT In the absence of K^+ on both sides of the membrane, delivery of standard activating pulses collapses the *Shaker* B K^+ conductance. Prolonged depolarizations restore the ability to conduct K^+. It has been proposed that the collapse of the conductance results from the dwelling of the channels in a stable closed (noninactivated) state (Gómez-Lagunas, 1997, *J. Physiol. (Lond.).* 499:3–15). Here it is shown that 1) Ba^{2+} impedes the collapse of the K^+ conductance, protecting it from both sides of the membrane; 2) external Ba^{2+} protection (K_d = 63 μM at −80 mV) decreases slightly as the holding potential (HP) is made more negative; 3) external Ba^{2+} cannot restore the previously collapsed conductance; on the other hand, 4) internal Ba^{2+} (and K^+) protection markedly decreases with hyperpolarized HPs (−80 to −120 mV), and it is not dependent on the pulse potential (0 to +60 mV). Ba^{2+} is an effective K^+ substitute, inhibiting the passage of the channels into the stable nonconducting (noninactivated) mode of gating.

INTRODUCTION

Permeation and gating were once considered two independent processes. However, recent observations have shown that permeant and/or blocking ions strongly modulate the gating of ion channels. For example, external K^+ modulates the entry into and the recovery from inactivation (e.g., see Demo and Yellen, 1991; Ruppersberg et al., 1991; López-Barneo et al., 1993; Gómez-Lagunas and Armstrong, 1994; Baukrowitz and Yellen, 1995; Levy and Deutsch, 1996) and the rate of deactivation of voltage-dependent K channels (Kv channels) (Swenson and Armstrong, 1981; Matteson and Swenson, 1986; Sala and Matteson, 1991; Demo and Yellen, 1992). Furthermore, removal of the extracellular K^+ renders Kv1.3 and Kv1.4 channels unable to conduct K^+, until the external K^+ is added back (Pardo et al., 1992; Levy and Deutsch, 1996; Jäger et al., 1998). On the other hand, with zero-K^+ solutions on both sides of the membrane, the squid delayed rectifier (DR) K channel undergoes an irreversible run down (Almers and Armstrong, 1980; Khodakha et al., 1997); in contrast, some mammalian DRs remain operational and permit a stable flow of Na^+ through them (Zhu and Ikeda, 1993; Callahan and Korn, 1994; Korn and Ikeda, 1995).

Recently, the behavior of *Shaker* B K channels in zero-K^+ solutions on both sides of the membrane was studied (Gómez-Lagunas, 1997). Briefly it was reported that 1) In 0 K^+ the K^+ conductance collapses with the delivery of activating pulses; the extent of collapse depends on the number, but not on the frequency of the pulses, and it is fully prevented if the channels are kept closed while the membrane is in zero K^+. 2) Depolarized holding potentials (HPs) avoid the drop in conductance. 3) The lost conductance recovers after prolonged depolarizations. 4) This behavior is observed with or without N-type inactivation. These results were interpreted as meaning that the channels normally close with a K^+ ion(s) bound(ed) to a "gating site(s)" located toward the extracellular side of the pore. The bound K^+ ion(s) would serve a "gating function," keeping the channels prone to opening by a brief depolarization, as observed under physiological conditions. Closing without K^+ sinks the channels into a stable closed (noninactivated) conformation that requires prolonged depolarizations to be overcome (Gómez-Lagunas, 1997). The present work extends the study of the nonconducting (noninactivated) state of *Shaker* B (Gómez-Lagunas, 1997), using divalent cations, particularly Ba^{2+}, as a tool to further analyze this state. Ba^{2+} has nearly the same crystal radius as K^+ and blocks the pore of Kv channels (e.g., Armstrong et al., 1982; Vergara and Latorre, 1983; Slesinger et al., 1993; Tagliatela et al., 1993; Lopez et al., 1994; Hurst et al., 1995; Harris et al., 1998). It is shown that Ba^{2+} can replace K^+, impeding the collapse of the K^+ conductance. Ba^{2+} protects from both sides of the membrane, and the characteristics of its protective action are investigated. A preliminary account of this work was reported in abstract form (Gómez-Lagunas, 1998, 1999).

MATERIALS AND METHODS

Cell culture and *Shaker* B channel expression

The insect cell line Sf9, from *Spodoptera frugiperda*, was kept in culture at 27°C in Graces' media (Gibco BRL). The cells were transfected by infection with a recombinant baculovirus, *Autographa californica* nuclear polyhedrosis virus, containing the cDNA of *Shaker* B, and were used for the experiments 2 days later (Klaiber et al., 1990). The recombinant baculovirus was kindly provided by Dr. C. M. Armstrong (University of Pennsylvania, Philadelphia).

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Electrophysiology

Macroscopic currents were recorded under whole-cell patch clamp (Hamill et al., 1981) with an Axopatch-1D (Axon Instruments). The currents were sampled at 100 μs per point and filtered in line at 5 kHz. Except where indicated, the leak conductance was subtracted with a P/-4 protocol. The electrodes were pulled from borosilicate glass (KIMAX 51) to a 1.2–2.0-MΩ resistance; 80% of the series resistance was electronically compensated.

Solutions

The solutions will be named by their main cation and will be represented as external/internal, e.g., K_o/Na_i. The internal (Na_i) solution was composed of (mM) 90 NaF, 30 NaCl, 10 EGTA, 10 HEPES-Na (pH 7.2). In the experiments with intracellular Ba^{2+}, the amount of $BaCl_2$ required to get the desired free [Ba^{2+}] was estimated with the program MaxC (C. Patton, Hopkins Marine Station, Stanford University) and added to the Na_i solution (named Na_i-Ba). MaxC does not take into account the presence of F^- ions in the buffer (needed for stable K^+ currents); therefore the internal [Ba^{2+}] values are approximate and were not used for quantitative assessments. Where indicated, the proteolytic enzyme papain (Boehringer Mannheim GmbH) or trypsin (type XIII; Sigma) was added to the Na_i-Ba solution.

The external control (K_o) solution was composed of (mM) 100 KCl, 15 NaCl, 10 $CaCl_2$, 10 MES-Na (pH 6.4). The external test (Na_o) solution was composed of (mM): 115 NaCl, 10 $CaCl_2$, 10 Mes-Na, pH 6.4; or 115 NaCl, 10 $CaCl_2$, 10 HEPES-Na, pH 7.1. Most experiments were done at pH 6.4 (the phenomenon under study shows no differences in the pH_o range of 6.4–7.1; Gómez-Lagunas, 1997). Where indicated, the chloride salt of Ba, Sr, Mg, Mn, Cd, Co, and Ni and the sulfate salt of Zn were added to the Na_o solution (e.g., Na_o-Ba).

When the concentration of the test cation was above 1 mM the [NaCl] was adjusted to keep the osmolarity constant.

Data analysis

The dose-response curve in Fig. 3 was fitted with Sigmaplot 5 (Jandel Scientific). Student's *t*-test was used to evaluate statistical significance.

RESULTS

To study the behavior of *Shaker* B in zero K^+ solutions, the activity of the channels was recorded, under whole-cell patch clamp, with a Na^+-containing, zero-K^+, internal solution (Na_i), and the channels were alternately activated in both a control (100 mM K^+) external solution (K_o) and a test Na^+-containing (zero-K^+) external solution (Na_o) (see Materials and Methods), as illustrated below.

Fig. 1 introduces the basic features of the collapse of the conductance, produced by gating the channels in 0 K^+. Fig. 1 *A* shows two control K^+ currents, recorded with a 2-min difference in K_o/Na_i. The currents were elicited by 30-ms pulses to +20 mV from the HP of −80 mV (henceforth the +20 mV/30 ms pulses will be referred to as activating pulses).

After the control was recorded, the cell was bathed in 0 K^+ solutions on both sides of the membrane, by perfusing the Na_o solution (Na_o/Na_i), and 15 activating pulses were delivered from −80 mV (without P/-4 subtraction); this is shown in Fig. 1 *B*. Only the leak current is seen (see Discussion).

Afterward, the cell was brought back to the control K_o solution, and the state of the channels was tested with the delivery of five activating pulses at a rate of 0.02 Hz (in K_o/Na_i). The traces in Fig. 1 *C* show that the ability of the channels to conduct K^+ was completely abolished. The reluctance of the channels to conduct is overcome by prolonged depolarizations, as illustrated below.

After the traces in Fig. 1 *C* were recorded, the HP was changed to 0 mV for 2 min, then it was brought back to −80 mV, and, 1 min later, the state of the channels was tested with the delivery of activating pulses. The traces in Fig. 1 *D* show that the ability of the channels to conduct K^+ was restored. In Fig. 1 *E*, the control currents in Fig. 1 *A* and those recorded after the depolarization to 0 mV in Fig. 1 *D* are superimposed; there was a complete recovery.

The currents in Fig. 1, *A–D*, were recorded with 10 mM Ca^{2+} in the external solution. In the range of 5–40 mM, external Ca^{2+} has no effect on either the collapse or the recovery of the K^+ conductance. Fig. 1 *F* presents the extent of recovery as a function of the time spent at 0 mV in K_o/Na_i (as in Fig. 1 *D*), in a cell where the conductance had previously been turned off, with either 5 or 40 mM Ca^{2+} in the test Na_o solution. There is no difference in the time course of recovery.

The extent of collapse of the conductance depends on the number of pulses delivered in 0 K^+. Fifteen pulses produce a 100% collapse (Gómez-Lagunas, 1997), as illustrated in Fig. 1. Therefore, throughout this work, the role of Ba^{2+} (and of the other divalent cations tested) was studied with the delivery of 15 activating pulses in 0 K^+ (this procedure will be referred to as pulsing).

Among divalent cations, external Ba^{2+} specifically inhibits the collapse of K^+ conductance

Ba^{2+} added to the external Na_o solution (Na_o-Ba) effectively replaces K^+, impeding the drop of the conductance (Fig. 2). Fig. 2 *A* shows five control (I_0) inward K^+ currents in K_o/Na_i. Once the stability of the currents was checked, the cell was superfused with the Na_o solution containing 50 μM Ba^{2+} (Na_o-Ba), and 15 activating pulses were delivered, from −80 mV, in Na_o-Ba/Na_i (Fig. 2 *B*).

Afterward, the cell was extensively superfused for 2 min with the K_o solution, and then the state of the channels was tested with the delivery of activating pulses, in K_o/Na_i. Fig. 2 *C* shows that 1) a significant fraction of the channels were still able to conduct K^+ (compare with the effect of pulsing without Ba^{2+} in Fig. 1); 2) the current elicited by the first pulse (labeled I_1) is notably smaller (including the tail) than that elicited by the following pulses, which then have a constant amplitude (collectively labeled I_2), and, in addition, the time to peak of I_1 is slightly lengthened compared to that of I_2 ($\Delta t_{peak} = 0.8 \pm 0.02$ ms, $n = 22$). In Fig. 2 *D* the control currents in *A* and those after pulsing in Na_o-Ba/Na_i in *C* are superimposed. With 50 μM Ba^{2+} only ~50% of the channels became resistant to conduction of K^+.

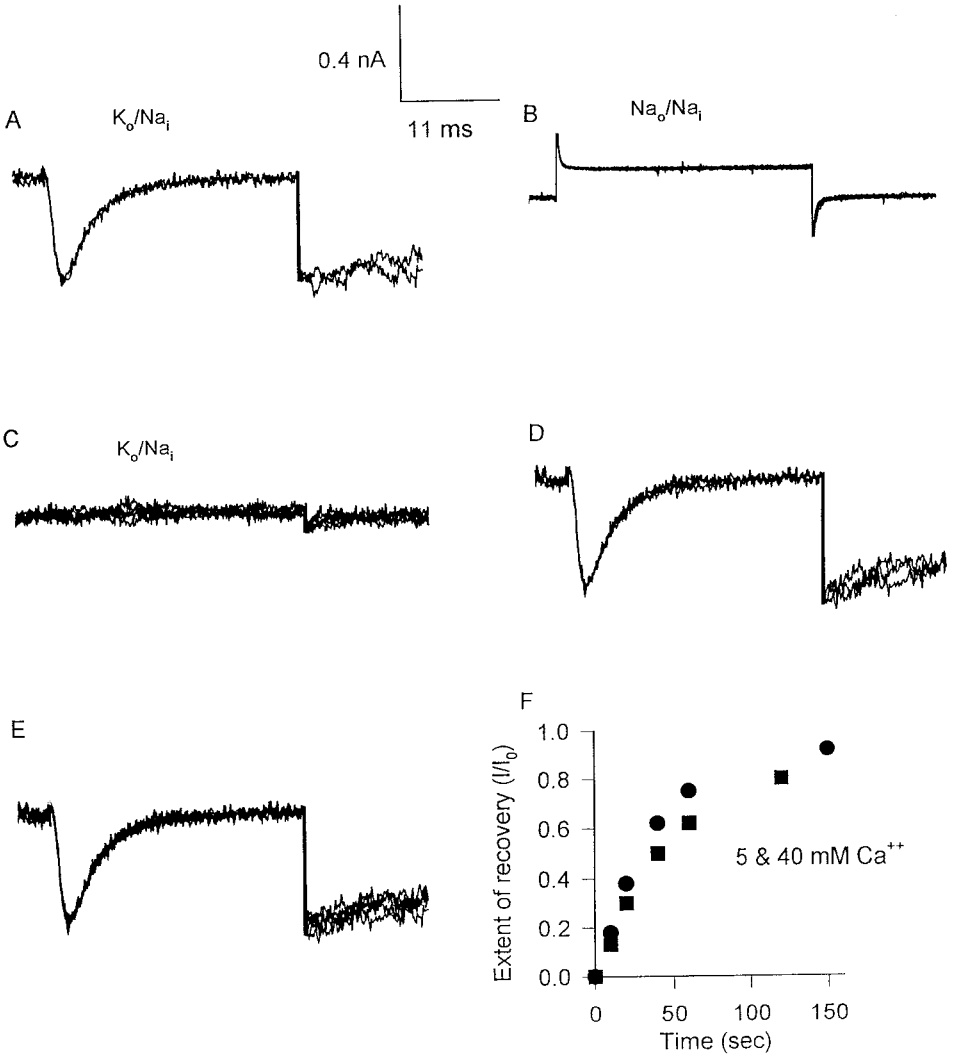

FIGURE 1 Collapse-recovery cycle of the K^+ conductance in 0 K^+ solutions. (*A*) K^+ currents, elicited by two +20 mV/30 ms pulses delivered, with a 2-min difference, in K_o/Na_i (see Materials and Methods). HP = −80 mV. (*B*) Currents evoked by 15 activating pulses, delivered at 1 Hz from −80 mV, in Na_o/Na_i (without P/-4 subtraction). (*C*) Currents evoked by five activating pulses delivered every minute, with the cell back in K_o/Na_i (after the pulses in *B*). (*D*) Current recovery by a depolarization to 0 mV: after the traces in *C* were recorded, the HP was changed to 0 mV for 2 min; then it was brought back to −80 mV, and, 1 min later, four +20 mV/30 ms pulses were delivered to test the state of the channels. (*E*) The currents in *A* and those in *D* are superimposed. (*F*) Current recovery as a function of the time spent at the HP of 0 mV, in K_o/Na_i, as in *D*. The conductance was first abolished by pulsing in Na_o/Na_i with either 5 mM ● or 40 mM Ca^{2+} ■ in the Na_o solution (not shown).

The missing conductance in Fig. 2 *C* was recovered by a 2-min depolarization to 0 mV (as in Fig. 1); this is shown in Fig. 2 *E*, which presents two currents recorded after the depolarization. In Fig. 2 *F*, the currents in Fig. 2 *E* are shown superimposed on those in the control in Fig. 2 *A*. There was a complete recovery; i.e., the lacking conductance in Fig. 2 *C* corresponds to the fraction of channels that were not protected by Ba^{2+} and therefore became nonconducting.

$I_1 \neq I_2$ in all of the cells that were treated with Ba^{2+} (see Discussion).

In summary, a comparison of Figs. 1 and 2 shows that external Ba^{2+} (Ba_o^{2+}) is able to replace K^+, impeding the collapse of the conductance. It is important to point out the following: 1) Ba_o^{2+} protects at micromolar concentrations in a background of 10 mM Ca^{2+}. 2) At the HP of −80 mV, Ba^{2+} protects with the same potency if the pulses start 1 or 6 min after the cell is placed in Na_o-Ba, for pulses delivered at a rate of 0.03–1 Hz (not shown). This indicates that Ba^{2+} equilibrates fast in the site where it protects. 3) Among divalent cations the capacity of external Ba^{2+} to protect the conductance seems to be unique: Ca^{2+} has no effect in the range of 5–40 mM (Fig. 1 *F*). Similarly, Zn^{2+} (200 μM) and Sr^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} (up to a concentration of 5 mM) added to the external Na_o solution do not protect the K^+ conductance (not shown).

Concentration and voltage dependence of external Ba^{2+} protection

The concentration dependence of Ba^{2+} protection was assayed as in Fig. 2, by pulsing in Na_o solutions containing several levels of [Ba^{2+}] (in Na_o-Ba/Na_i). Fig. 3 *A* shows that as the [Ba_o^{2+}] increases, the ratio of the stable K^+ current left after pulsing in 0 K^+ (I_2) to that in the control (I_0) increases, following a saturation curve with a Hill coefficient of 1.4 and a K_d of 63 μM at −80 mV. The inset shows the linear double-reciprocal plot of the data.

Ba^{2+} protects with an affinity higher than that of K^+ and the other monovalent cations previously tested in the external solution: Rb^+, NH_4^+, Cs^+, and TEA^+, all of which protect with millimolar affinity (Gómez-Lagunas, 1997).

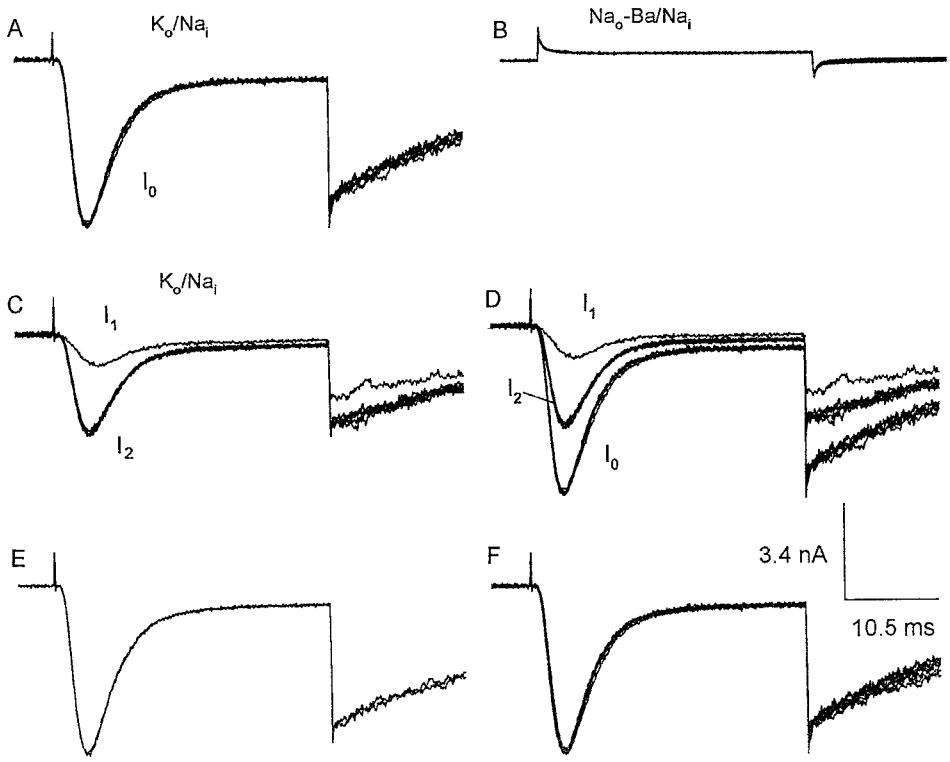

FIGURE 2 External Ba^{2+} inhibition of the collapse of the K^+ conductance. (*A*) Control K^+ currents in K_o/Na_i (I_0). The channels were activated by five +20 mV/30 ms pulses, delivered at 0.02 Hz. HP = −80 mV. (*B*) Currents evoked by 15 +20 mV/30 ms pulses, delivered at 0.5 Hz from the HP of −80 mV (without P/-4 subtraction) in Na_o-Ba/Na_i, [Ba^{2+}] = 50 μM. Pulsing started 2 min after the perfusion of the Na_o-Ba solution. (*C*) K^+ currents recorded back in K_o/Na_i. The channels were activated every 30 s. The current evoked by the first pulse (I_1) is smaller than those evoked by the following pulses (I_2), HP = −80 mV. (*D*) The K^+ currents in *A* (I_0) and after pulsing in *C* (I_1, I_2) are superimposed. (*E*) Current recovery by a 2-min depolarization to 0 mV. (*F*) The control currents in *A* and after the depolarization to 0 mV in *E* are superimposed.

Both Ba^{2+} block of *Shaker* and the collapse of the K^+ conductance are voltage dependent (Hurst et al., 1995; Harris et al., 1998; Gómez-Lagunas, 1997; Melishchuk et al., 1998); therefore it was of interest to look at the voltage dependence of external Ba^{2+} protection. This was done by pulsing from different holding potentials. Fig. 3 *B* illustrates the extent of protection (I_2/I_0) exerted by 100 μM Ba^{2+} as a function of the HP during pulsing in Na_o-Ba/Na_i. For a reference, the figure includes the intrinsic voltage dependence of the conductance drop (*curve labeled* Na^+; see Introduction) (Gómez-Lagunas, 1997; see also Melishchuk et al., 1998); in the absence of K^+ (and Ba^{2+}), in Na_o/Na_i, pulsing from the HP of −80 mV or hyperpolarized potentials completely turns off the K^+ conductance. On the other hand, depolarized HPs avoid the drop in conductance.

When the same measurements are done in the presence of 100 μM Ba^{2+} (Na_o-Ba/Na_i), the following is observed (*curve labeled* 100 μM Ba^{2+}): 1) Ba_o^{2+} protects in the whole range of voltages that were tested (−60 to −140 mV). 2) Ba^{2+} protection itself is voltage dependent. This is seen in the range of −80 to −140 mV, where in the absence of Ba^{2+} there is a 100% drop in conductance, whereas with Ba^{2+} the ratio of the current left after pulsing (I_2) to that in the control (I_0) still depends on the membrane potential. 3) Ba^{2+} protects less effectively as the HP becomes more negative.

The apparent K_d of Ba^{2+} protection was determined in the range of −80 to −140 mV, from experiments like those in Fig. 3 *A*. The results in Fig. 3 *C* show that the apparent K_d increases exponentially, although slightly, as the HP is made more negative (with an *e*-fold change every 66 mV). Thus, whereas external Ba^{2+} block of *Shaker* K^+ currents becomes stronger with hyperpolarized HPs (Hurst et al., 1995), Ba^{2+} protection in zero K^+ becomes weaker.

Ba^{2+} protection, on the other hand, is not dependent on the pulse potential (V_p), within a moderate range of voltages that fully activate the channels (0 to +60 mV) (e.g., the extent of protection exerted by 50 μM Ba^{2+} with V_p = 0 mV (0.29 ± 0.03, n = 6), is not significantly different ($p <$ 0.01) from that obtained with V_p = +60 mV (0.30 ± 0.05, n = 7) (not shown).

The stable nonconducting state may involve the occlusion of the extracellular side of the pore

External Ba^{2+} blocks closed (Armstrong et al., 1982; Hurst et al., 1995; Harris et al., 1998), as well as C-type inactivated K channels (Basso et al., 1998). Therefore, it was of interest to determine if Ba_o^{2+} could restore the K^+ conductance previously collapsed by pulsing in zero K^+.

Fig. 4 *A* shows superimposed control K^+ currents recorded in K_o/Na_i. After the stability of the current was checked, the cell was superfused with the Na_o solution, and the conductance was collapsed by pulsing in Na_o/Na_i (Fig. 4 *B*). After that, the cell was immediately immersed for 3 min in a Na_o solution containing an excess of Ba^{2+} (10 mM), this time without pulsing, with the membrane potential constant at −80 mV (not shown). Subsequently, the cell was extensively washed with the K_o solution, and the state of the channels was tested by the delivery of 10 activating pulses in K_o/Na_i. The lack of K^+ current in Fig. 4 *C*

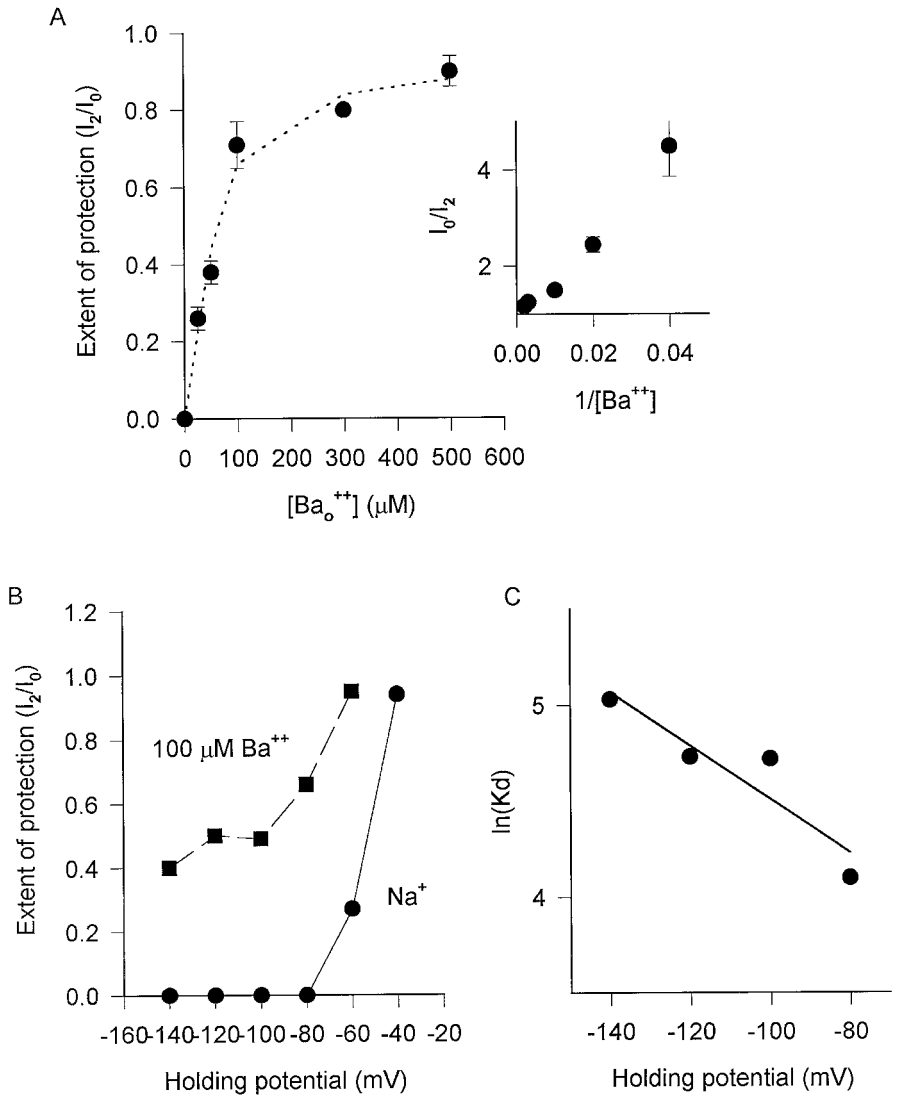

FIGURE 3 Concentration and voltage dependence of external Ba^{2+} protection. (*A*) Extent of Ba_o^{2+} protection, defined as the ratio I_2/I_0 of the peak K^+ currents (in K_o/Na_i) recorded before (I_0) and after (I_2) pulsing from −80 mV at the rate of 0.5 Hz, in Na_o-Ba/Na_i, with the indicated [Ba^{2+}]. The points are the mean ± SEM of at least three experiments. The line through the points is the fit of a Hill equation with $n = 1.4$ and a K_d ($K^{1/1.4}$) of 63 μM (see Materials and Methods). The inset shows the linear double-reciprocal plot of the data ($r = 0.99$). (*B*) Ratio of the K^+ currents (in K_o/Na_i), before (I_0) and after pulsing from the indicated HPs, in Na_o/Na_i (*curve labeled* Na^+) or in Na_o-Ba/Na_i ([Ba^{2+}] = 100 μM) (I_2). The HP was changed from −80 mV to the indicated value 1 min before pulsing. (*C*) Apparent K_d of Ba_o^{2+} protection as a function of the HP during pulsing, determined from complete curves like that in *A*.

demonstrates that Ba^{2+} (10 mM) was unable to restore the conductance.

The absence of K^+ current in Fig. 4 *C* was due to the inability of Ba^{2+} to restore the previously collapsed conductance, and not to an irreversible rundown of the channels. This is demonstrated in Fig. 4 *D*, which shows recovery of the K^+ current brought about by a 1-min change of the HP to 0 mV.

It seems that during the stable nonconducting state there is a high energy barrier toward the external side of the pore, maybe given by a collapse of the extracellular side of the pore, that forbids the entry of either Ba_o^{2+} or K_o^+.

Internal Ba^{2+} inhibits the collapse of the K^+ conductance

Internal Ba^{2+} blocks Kv channels once they open (e.g., see Armstrong et al., 1982). Therefore, the effect of internal Ba^{2+} (Ba_i^{2+}) on the establishment of the nonconducting conformation in zero K^+ was studied. To do this, the currents were recorded with the Na_i internal solution containing the indicated [Ba^{2+}] (Na_i-Ba; see Materials and Methods), and the channels were alternately activated in both the control K_o and in the test Na_o solutions.

Fig. 5 *A* demonstrates that internal Ba^{2+} effectively protects against the development of the nonconducting conformation and that the extent of protection depends markedly on the holding potential during pulsing in zero K^+. The figure presents three panels of currents recorded sequentially in the same cell. Each panel shows two sets of superimposed K^+ currents (in K_o/Na_i-Ba with [Ba^{2+}] ≈ 70 μM; see Materials and Methods), recorded before (I_0) and after (I_1, I_2) pulsing in zero K^+ (in Na_o/Na_i-Ba; not shown) from the indicated HPs. Notice that 1) the currents have a constant amplitude and kinetics (e.g., see I_0) (this indicates that with 100 mM K_o^+, Ba_i^{2+} (≈ 70 μM) does not produce a use dependent block of the inward K^+ current) and that 2) the current evoked by the first pulse applied back in the K_o solution (I_1) after pulsing is again (see Fig. 2) smaller than the currents evoked by the next pulses, that then have

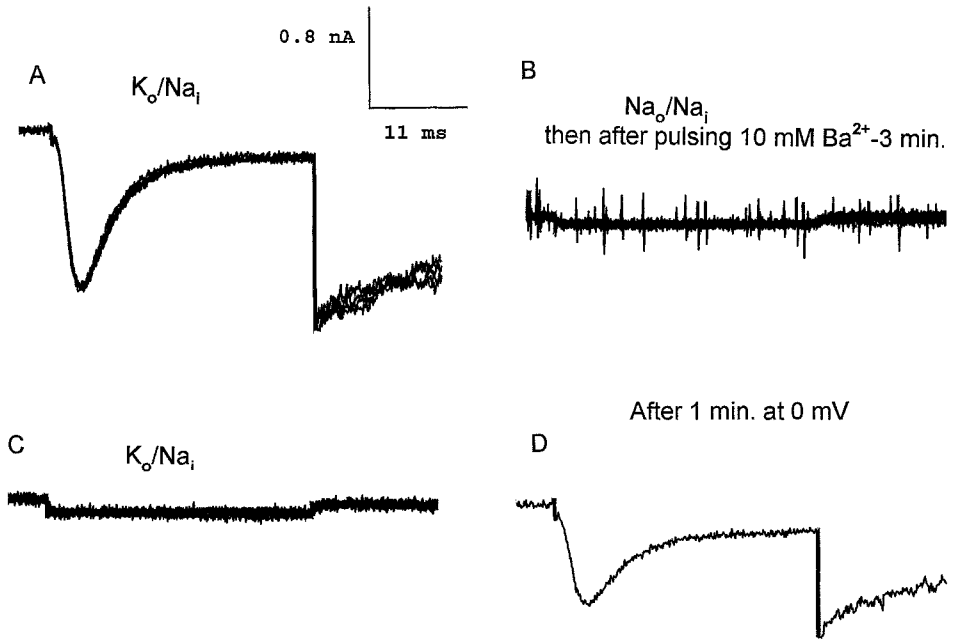

FIGURE 4 The stable nonconducting state might involve a collapse of the extracellular side of the pore. (*A*) Control K^+ currents evoked by +20 mV/30 ms pulses in K_o/Na_i. HP = −80 mV. (*B*) Currents evoked by 15 activating pulses from −80 mV, applied at a rate of 1 Hz, in Na_o/Na_i. The spikes in the traces are due to the flow of the Na_o solution. After pulsing, the cell was immersed for 3 min in a Na_o solution containing 10 mM Ba^{2+} without pulsing (not shown). (*C*) Currents evoked by 10 +20 mV/30 ms pulses delivered at a rate of 0.03 Hz with the cell back in K_o/Na_i. (*D*) Current recovery after a 1-min depolarization to 0 mV.

a constant amplitude (collectively labeled as I_2) (see Discussion).

The traces in the upper panel of Fig. 5 *A* show that Ba_i^{2+} completely blocks the collapse of the K^+ conductance ($I_2 = I_0$) when the pulses in Na_o/Na_i-Ba are delivered from the HP of −80 mV. Ba^{2+} protection, however, is markedly reduced ($I_2 < I_0$) as the HP during pulsing is made more negative; this is shown in the middle and lower panels of Fig. 5 *A*, which present the currents before and after pulsing from −100 and −120 mV, respectively. This behavior is best seen in Fig. 5 *B*, where the extent of protection at three [Ba^{2+}] is plotted against the HP during pulsing.

Fig. 5 *C* shows that, in contrast to its clear variation with the HP during pulsing, Ba_i^{2+} protection is not dependent on the pulse potential, within a moderate range of pulses that fully activate the channels (0 to +60 mV) (e.g., the extent of protection with $V_p = 0$ mV (0.57 ± 0.03, $n = 6$) was not significantly different ($p < 0.01$) from that obtained with +60-mV pulses (0.56 ± 0.07, $n = 3$)). In Fig. 5 *D* it is qualitatively shown that protection tends to saturate as [Ba_i^{2+}] increases (see Materials and Methods).

Is the voltage dependence of Ba_i^{2+} protection a peculiar characteristic of the interaction of this ion with the channels in zero K^+, or is it shared by other internally protective ions, like K^+? Fig. 6 *A* shows that the extent of internal K^+ (5 mM) protection at the HP of −80 mV during pulsing in Na_o (0.75 ± 0.03, $n = 6$) is significantly bigger ($p < 0.01$) than that obtained at the HP of −120 mV (0.48 ± 0.06, $n = 6$). Similarly, Fig. 6 *B* shows that, like Ba^{2+} action, K_i^+ protection does not depend on the amplitude of the pulses delivered in Na_o (0 to +60 mV). In summary, K_i^+ protection has the same qualitative voltage dependence of Ba_i^{2+} protection. This suggests that the two ions protect through the same basic mechanism.

Internal Ba^{2+} protection after removal of the N-type inactivation

Negative HPs speed recovery from N-type inactivation (e.g., see Ruppersberg et al., 1991; Demo and Yellen, 1992; Gómez-Lagunas and Armstrong, 1994); therefore the decrease in Ba_i^{2+} potency as the HP is hyperpolarized (Fig. 5) could indicate that Ba_i^{2+} (and K_i^+) action somehow requires a fast inactivation ball bound to its receptor. To explore this point, the fast inactivation was abolished by adding the proteolytic enzymes papain or trypsin (0.1 mg/ml) to the Na_i-Ba solution, as reported (Gómez-Lagunas and Armstrong, 1995), and the ability of Ba^{2+} to protect the conductance was tested, as described below.

After papain removal of the N-type inactivation, pulsing in 0 K^+ reversibly collapses the conductance (see figure 10 of Gómez-Lagunas, 1997). Fig. 7 *A* presents superimposed K^+ currents in the absence of N-type inactivation, recorded before (I_0) and after (I_1, I_2) pulsing in 0 K^+, with ~23 μM Ba^{2+} in the internal solution (in K_0/Na_i-Ba, with papain at 0.1 mg/ml). Note that 1) as in the WT channel, Ba_i^{2+} prevents the drop of the conductance; 2) the current evoked by the first pulse delivered back in K_o (I_1), after pulsing, has an apparent slower activation than those elicited by the next pulses (I_2), which are faster, and then reaches a slightly bigger amplitude at the end of the pulse (also see figure 1 of Harris et al., 1998; see Discussion). These observations are best seen in Fig. 7 *B*, which presents a plot of the size of the current at the end of each pulse in Fig. 7 *A*, before (*left column*) and after (*right column*) pulsing. Notice that ~48% of the channels remain responsive after pulsing. Ba^{2+} protection in the absence of fast inactivation was verified in three other cells treated with papain and in two cells treated with trypsin (not shown).

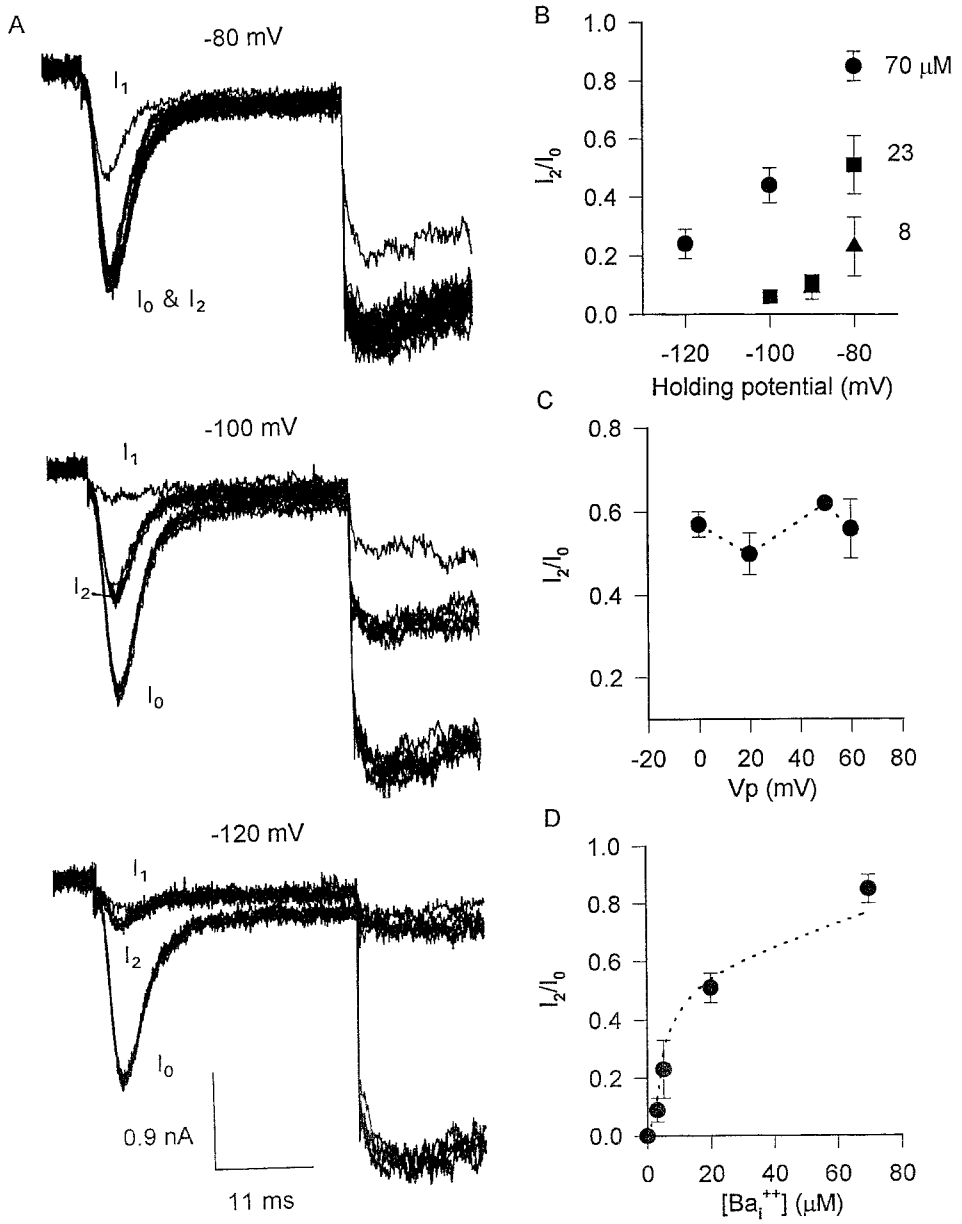

FIGURE 5 Internal Ba^{2+} inhibition of the collapse of the K^+ conductance. (*A*) Superimposed K^+ currents recorded sequentially in the same cell, in K_o/Na_i-Ba with $[Ba^{2+}] \approx 70$ μM (see Materials and Methods). The channels were activated by +20 mV/30 ms pulses delivered at the rate of 0.03 Hz from −80 mV, before (I_0) and after (I_1, I_2) pulsing in Na_o/Na_i-Ba (not shown) from the HP of −80 (*top*), −100 (*middle*), or −120 mV (*bottom*), as indicated. (*B*) Ba_i^{2+} protection (I_2/I_0) as a function of the HP during pulsing, at the indicated $[Ba^{2+}]$. The HP was switched from −80 mV to the indicated values ~15 s before pulsing. (*C*) Ba_i^{2+} protection as a function of the amplitude of the pulses applied in 0 K^+ ($[Ba^{2+}] \approx 23$ μM), HP = −80 mV. (*D*) Extent of protection as a function of $[Ba_i^{2+}]$, HP = −80 mV. The curve through the points has no physical meaning. The points in *B* and *D* are the mean ± SEM of at least four experiments. Rate of pulsing in 0 K^+ = 0.5 Hz.

Therefore, even when negative HPs decrease internal Ba^{2+} protection, Ba^{2+} does not require the interaction of the fast inactivation gate with the channels to be able to protect.

DISCUSSION

With 0 K^+ solutions on both sides of the membrane, the delivery of standard activating pulses collapses the *Shaker* conductance. Prolonged depolarizations are needed to overcome this state. These observations were interpreted as meaning that the channels normally close with K^+ ion(s) bound in a site(s) located toward the extracellular side of the pore, keeping the channels ready to conduct in response to a standard depolarization (Gómez-Lagunas, 1997). Here it has been shown that, among divalent cations, Ba^{2+} specifically replaces K^+, from both sides of the membrane, inhibiting the development of the nonconducting (noninactivated) conformation.

External Ba^{2+} protected at micromolar concentrations in the presence of 10 mM Ca^{2+}, and Ca^{2+} itself was ineffective; this indicates that the site were protection occurs selects Ba^{2+} over Ca^{2+}. The other divalent cations that were ineffective might also have been unable to bind at the site where protection occurs.

Besides Ba^{2+}, external monovalent cations that either permeate or block also protect (Gómez-Lagunas, 1997). Thus the simplest hypothesis is that Ba_o^{2+} protects by binding to an externally located site in the pore of the channels. This hypothesis is strengthened by the lack of effect of Zn^{2+}, which, although it modifies the activation gating, neither permeates nor blocks the pore of the channels (Gilly and Armstrong, 1982; Spires and Begenisich, 1994).

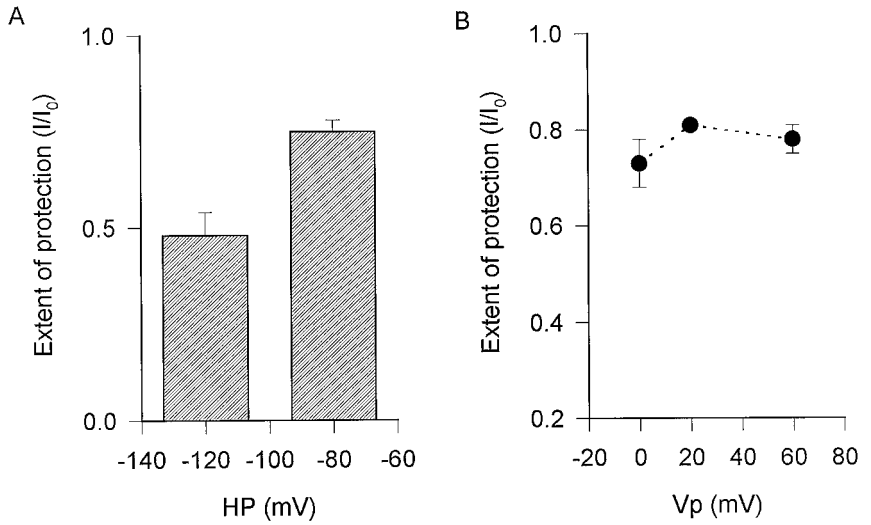

FIGURE 6 Voltage dependence of internal K^+ protection. (*A*) Extent of K_i^+ (5 mM) protection as a function of the HP during pulsing (V_p = +20 mV). (*B*) Extent of protection as a function of the amplitude of the pulses applied in Na_o (HP = −80 mV). Protection with V_p = 0 mV (0.73 ± 0.05, n = 6) was not significantly different ($p <$ 0.01) from that with V_p = +60 mV (0.78 ± 0.03, n = 6). Rate of pulsing in Na_o = 0.5 Hz.

Ba^{2+} stabilizes the closed conformation of Kv channels (Armstrong et al., 1982) and inhibits the slowing of the gating charge return that occurs as the preceding depolarization is made more positive (Hurst et al., 1997). The latter has been interpreted as a Ba^{2+}-induced reduction of the probability of the channels to dwell in states occurring late in the activation pathway (Hurst et al., 1997).

Therefore, one possibility is that Ba^{2+} protection could be related to its reduction of the probability of the final states of the activation pathway. This interpretation is qualitatively consistent with recent observations by Armstrong and co-workers, who have shown that the collapse of the conductance is more likely to occur from intermediate closed states in the activation pathway (Melishchuk et al., 1998).

Whatever the case, it is clear that Ba^{2+} exerts a restriction to the conformational changes leading to the collapse of the K^+ conductance, and that not all of the manipulations that stabilize the closed conformation of the channels impede the collapse of the conductance, as indicated by the lack of effect of Ca^{2+}.

The voltage dependence of external Ba^{2+} protection

The slight reduction of Ba_o^{2+} protection at hyperpolarized HPs is not a characteristic shared by all of the protecting ions (e.g., external K^+ protection is not dependent on the HP (−80 versus −120 mV) or on the V_p during pulsing (0 to +60 mV) (not shown).

The above observation suggests that the voltage dependence of Ba^{2+} protection is not likely to arise from the intrinsic voltage dependence of the conductance collapse. Instead, it could be that protection decreases as the HP becomes more negative because of a Ba^{2+} partition between an external binding site, where Ba^{2+} protects, and an internal site, where Ba^{2+} could also bind but without protecting

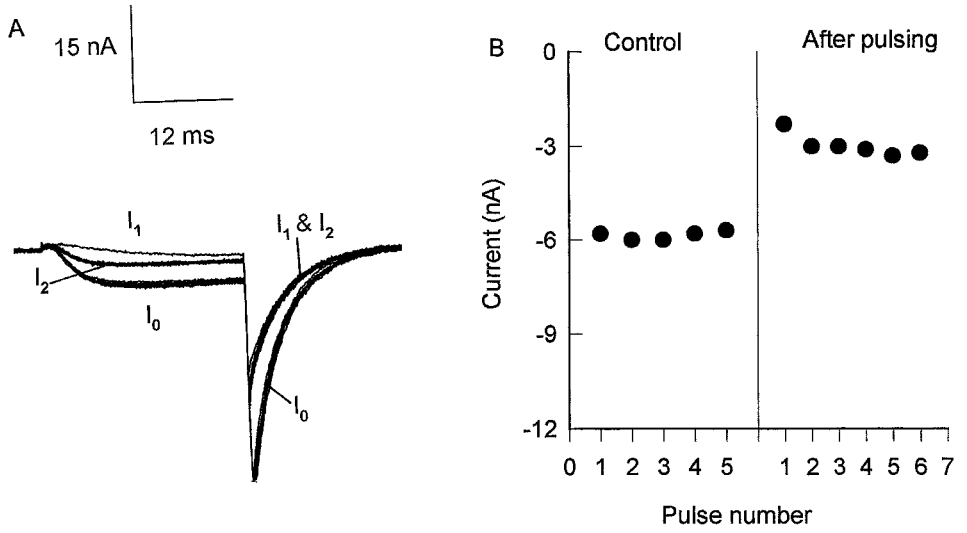

FIGURE 7 Internal Ba^{2+} protection after removal of the N-type inactivation. (*A*) Superimposed K^+ currents recorded after papain removal of the N-inactivation. The channels were activated every 10 s by 0 mV/25 ms pulses in K_o/Na_i-Ba, with $[Ba^{2+}] \approx$ 23 μM and papain at 0.1 mg/ml in the internal solution, before (I_0) and after (I_1, I_2) the delivery of 15 0 mV/25 ms pulses at the rate of 0.5 Hz, from the HP of −80 mV in Na_o/Na_i-Ba (not shown). (*B*) currents measured at the end of each pulse of the traces in *A*.

(or not so well) the K^+ conductance, and/or because an increased Ba^{2+} flow through and exit from some of the channels as the HP is made more negative, the Ba^{2+}-depleted channels then would collapse. The latter possibility is supported by recent observations that show that, depending on the voltage and the $[K^+]$ across the membrane, Ba^{2+} may be able to permeate through K channels (Neyton and Miller, 1988a,b; Harris et al., 1998). It remains to determine the relative weights of these two nonexcluding possibilities.

Finally, the lack of a significant effect of positive pulse potentials, which would have been expected to favor Ba^{2+} exit toward the external solution, suggests that Ba^{2+} dissociation may be slow enough, even at the more positive potential tested (+60 mV), to have a significant effect on protection.

Ba^{2+} access to C-type inactivated and to nonconducting (noninactivated) channels

The inability of Ba_o^{2+} (and K_o^+) to restore the previously collapsed conductance (Fig. 5) suggests that, during the nonconducting state, there is a high energy barrier preventing the access of Ba^{2+} (and K^+) to the pore, maybe caused by a collapse of the extracellular side of the pore.

Interestingly, it has been reported that Ba_o^{2+} is able to block C-type inactivated channels (Basso et al., 1998). This indicates that either the extent of collapse of the pore (magnitude of the energy barrier), occurring during the drop of the K^+ conductance in zero K^+, is bigger than that likely occurring during C-type inactivation (e.g., Liu et al., 1996; Kiss and Korn, 1998), or the topological location of the conformational change is different in the two states.

About the relation $I_1 < I_2$

In the wild-type (WT) channels after pulsing with Ba^{2+} it is observed that $I_1 < I_2$ (Figs. 2 and 5), and that I_1 has a slightly longer time to peak than I_2, at +20 mV ($\Delta t_{peak} = 0.8 \pm 0.02$ ms, $n = 22$).

After removal of the N-type inactivation, and with a less positive pulse, $V_p = 0$ mV, to test the state of the channels, the slower activation of I_1, compared to I_2, is easily observed (Fig. 7). Thus, in the WT Shaker, the slower activation of I_1 is not so evident; Δt_{peak} is small, because of the magnitude of the pulse used throughout the work to test the state of the channels and because of the fast inactivation, which causes I_1 to reach a smaller amplitude than that of I_2. In fact if, after pulsing with Ba^{2+} in 0 K^+, the WT channels are activated with a $V_p = 0$ mV, instead of +20 mV, then the time to peak of I_1 is notoriously lengthened compared to I_2 ($\Delta t_{peak} = 1.7 \pm 0.3$ ms, $n = 4$) (not shown).

This pattern ($I_1 < I_2$; $\Delta t_{peak} > 0$) is not observed with other protecting ions (Gómez-Lagunas, 1997); therefore it must arise from a characteristic interaction of Ba^{2+} with the channels. Besides, it is known that after the channels are loaded with Ba^{2+} the apparent rate of activation decreases, as Ba^{2+} dissociates from them (see figure 1 of Harris et al., 1998), and that the rate of Ba^{2+} dissociation depends on the membrane potential (Armstrong et al., 1982; Neyton and Miller, 1988a; Harris et al., 1998). Therefore, the simplest interpretation is that the differences between I_1 and I_2 are determined by the rate of exit of the protecting Ba^{2+} from the pore of the channels.

Na^+ conduction in zero K^+

It has been reported that in 0 K^+ *Shaker* Δ4-46 conducts Na^+ transiently, before falling into the nonconducting conformation studied here (Ogielska and Aldrich, 1998; Melishchuk et al., 1998). The traces in 0 K^+ in Fig. 1 *C* show no sign of a time-dependent current; this could be due to the presence of the N-inactivation, which could end conduction before the Na^+ current becomes detectable, or to the slight differences in the solutions employed in these studies. Indeed in a few cells a small time-dependent current in the first pulse applied in 0 K^+ (not shown) has been observed, but even in those cells treated with papain, a time-dependent current that could indicate Na^+ permeation through the channels has not been consistently observed.

Na^+ currents are also observed in C-inactivated *Shaker* Δ6-46 (Starkus et al., 1997), but this state and the nonconducting (noninactivated) state studied here are clearly different. Moreover, it seems that during C-inactivation the channels cannot fall into the nonconducting state described here, as suggested by the intrinsic voltage dependence of the K^+ conductance drop (Fig. 3 *B*; see also Gómez-Lagunas, 1997; Melishchuk et al., 1998).

Ba^{2+} protection and Ba^{2+} block

Considering that Ba^{2+} block is measured in the presence of K^+, which in turn affects the binding of Ba^{2+} to the pore of the channels (Armstrong et al., 1982; Neyton and Miller, 1988a,b; Hurst et al., 1995; Harris et al., 1998), it is not surprising that the known features of Ba^{2+} block could not be directly translated into those of Ba^{2+} protection in zero K^+.

Nevertheless, it is important to point out that the biggest difference between protection and block is in the voltage dependence of internal Ba^{2+} action. Block is dependent on the pulse and not on the holding potential (e.g., Armstrong et al., 1982; Slesinger et al., 1993; Lopez et al., 1994), whereas Ba^{2+} protection has the opposite dependence. It remains to be determined if this difference is simply due to the absence of K^+ in the protection experiments, or if it comes from a characteristic feature of the conductance drop (see below).

The HP dependence of internal Ba^{2+} protection

Negative HPs speed recovery from inactivation, populate closed states located farther from the open state, and reduce internal Ba^{2+} (and K^+) protection in 0 K^+. Considering that

Ba^{2+} still protects after the abolishment of the N-type inactivation, the effect of the HP cannot be explained by the need for a simultaneous interaction of Ba^{2+} and the fast inactivation gate with the channels for Ba^{2+} protection to occur.

One explanation could be that the Ba^{2+} (and K^+) ions are pulled out of the channels, back into the internal solution, by the hyperpolarized HPs, thus reducing their effectiveness.

Another possibility is that Ba_i^{2+} (and K_i^+) could protect by binding in an internally located site that does not sense the voltage drop across the membrane (so explaining the lack of effect of the V_p), a site different from that where external Ba^{2+} protects, and the HP dependence of Ba_i^{2+} (and K_i^+) protection could be related to the voltage dependence of the closing reaction; protection would be less likely when the channels dwell in closed states located farther from the open state. The latter possibility would be in agreement with the observations of Armstrong and coworkers, which indicate that the collapse of the conductance is more likely to occur at intermediate closed states than at states located farther from the open state (Melishchuk et al., 1998). Further experiments are needed to distinguish among these possibilities.

It seems that, depending on how they close, *Shaker* channels can operate in two modes of gating. In one of them, the conducting mode, the channels are able to open and conduct K^+ as soon as the membrane is depolarized; in the other one, the nonconducting mode, the channels are unable to conduct K^+ until the membrane remains depolarized for prolonged periods (Gómez-Lagunas, 1997; Melishchuk et al., 1998). Recent observations by Armstrong's group have shown that during the nonconducting mode the gating charge movement is different from that occurring during the conducting mode (Melishchuk et al., 1998). Passage from the conducting to the nonconducting mode occurs when the channels close without K^+ (Gómez-Lagunas, 1997; Melishchuk et al., 1998). Ba^{2+} is able to act like a K^+ ion, keeping the channels in the conducting mode of gating.

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