Pore accessibility during C-type inactivation in *Shaker* K^+ channels

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Abstract ***Shaker*** **K^+ channels inactivate through two distinct molecular mechanisms: N-type, which involves the N-terminal domain and C-type that appears to involve structural modifications at the external mouth of the channel. We have tested pore accessibility of the *Shaker* K^+ channel during C-type inactivation using Ba^{2+} as a probe. We determined that external Ba^{2+} binds to C-type inactivated channels forming an extremely stable complex; i.e. there is Ba^{2+} trapping by C-type inactivated channels. The structural changes *Shaker* channels undergo during C-type inactivation create high energy barriers that hinder Ba^{2+} exit to either the extracellular solution or to the intracellular solution.**

Key words: *Shaker* K^+ channel; Slow inactivation; Pore accessibility; Ba^{2+} block

1. Introduction

In *Shaker* K^+ channels, C-type inactivation involves amino acid residues in the pore (P) region and in the S6 segment [1–5]. Experimental evidence supports the view that C-type inactivation involves structural changes at the outer mouth of the pore compromising cooperative interactions between channel subunits [6,7]. In this regard, Liu et al. [8] proposed that C-type inactivation promotes a local rearrangement and a constriction of the channel at the outer mouth (a local collapse). Starkus et al. [9] have proposed recently an alternative explanation to the physical collapse of the external mouth. Their findings suggest that C-type inactivation results from an alteration of the ion selectivity of the channel. C-type inactivation greatly reduces the permeability of K^+ relative to the permeability of Na^+. During C-type inactivation the channel becomes impermeable to all available ion species. This is because the outer selectivity filter becomes impermeable to K^+, while internal K^+ prevents Na^+ permeation. This 'non-collapse' mechanism is still consistent with the cooperative model [6,7].

In the present work, we have tested pore accessibility using Ba^{2+} as a probe in an attempt to define where the hindrance to the ion passage resides and the extent to which *Shaker* K^+ pore changes its conformation during C-type inactivation. External Ba^{2+} can block open and closed voltage-dependent K^+ channels [10,11]. Armstrong et al. [10] found that Ba^{2+} blocks and stabilizes the closed conformation of the squid axon K^+ channel. More recently, Hurst et al. [11] proposed the existence of two Ba^{2+} binding sites located in the conduction system of the *Shaker* K^+ channel. The deepest site senses 25% of the electrical field.

In this study, we examine the interactions between Ba^{2+} and C-inactivated *Shaker* K^+ channels. We address two specific questions: (1) Is the external Ba^{2+} binding site accessible when the *Shaker* K^+ channels are in the C-inactivated state? (2) If external Ba^{2+} can interact with C-type inactivated *Shaker* K^+ channels, what are the characteristics of such binding?

Part of this work has been presented in abstract form (Basso and Latorre (1997) Biophys. J. 72, A27).

2. Materials and methods

2.1. Oocyte injection

The amino terminal deletion of *Shaker* H4 Δ (6–46) (*Shaker* H4 Δ) was used to prevent fast or N-type inactivation [12]. *Shaker* H4 Δ RNA was in vitro transcribed from DNAs linearized with *Hin*dIII. The synthesis was directed by the T7 promoter in the presence of cap analogue $m^7G(5')ppp(5')G$ using a mMessage mMachine kit (Ambion Inc., Austin, TX). Collagenase (700 units/ml, Gibco BRL, Gaithersburg, MD) treated oocytes (stage V–VI) were injected with 10 ng of RNA in 50 nl of water. Oocytes were maintained at 18°C in amphibian saline solution supplemented with gentamicin (50 mg/ml). Voltage clamp recordings were performed at room temperature (20–25°C) 2 to 5 days following RNA injection.

2.2. Electrophysiology

Macroscopic currents were recorded using two-microelectrode voltage clamp (Dagan Corporation, Minneapolis, MN). The external solution was perfused manually. Bath solution was (mM): 140 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.6 (294 mOsm). In the experiments to measure Ba^{2+} block, the desired [Ba^{2+}] were attained by replacing NaCl with isotonic $BaCl_2$.

To demonstrate Ba^{2+} binding to the C-type inactivated channels, channels were inactivated applying a 2-min depolarizing pulse, usually to 0 mV, from a holding potential of −100 mV. The membrane was kept in contact with the Ba^{2+} containing solutions for variable periods of time when measuring Ba^{2+} association to the C-type inactivated channels. After the Ba^{2+} period, the oocyte was perfused with a Ba^{2+}-free solution for the periods indicated in the figures and text. Membrane was then repolarized to −100 mV and the outward current amplitude was determined at the end of a 15-ms test pulse to 0 mV repeated in most experiments once every 5 s. The current amplitude, $I(t)$, of sequential pulses was fitted to a single exponential term.

To study the open state blockage by external Ba^{2+}, we tested the inhibition by Ba^{2+} of the non-inactivated fraction of the current. The Ba^{2+} inhibition data for the individual components were fit by least squares minimization to

$$\text{Inhibition} = 1-(I_{Ba}/I_o) = [Ba]^N/([Ba]^N + K_d^N) \quad (1)$$

where I_{Ba} is the current not blocked in presence of Ba^{2+}, I_o is the current evoked by a depolarizing test pulse as described above in the

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absence of Ba^{2+}, K_d is the apparent dissociation constant and N is the Hill coefficient.

Curve fitting and the kinetic models was performed using either Microsoft Excel's solver (version 7.0) or Nfit (The university of Texas, Galveston, TX).

3. Results

In *Shaker* H4 Δ K^+ channels, establishment of the C-inactivated state takes several seconds and inactivation is always incomplete (e.g. [13]). Upon depolarization to 0 mV the time course of the inactivation is well described by a function of the form $I(t) = I(0)[P+(1-P)\exp(-t/\tau_i)]$, where the time constant $\tau_i = 4.0 \pm 0.3$ s ($n = 7$; mean ± S.D.) and the pedestal P amounts to 12.6 ± 1.7% of the maximal current (inset Fig. 1A).

Fig. 1A shows that channel recovery from inactivation has two components. The early component was faster than our testing pulses frequency, and the second phase was fitted to an exponential function with time constant of 1.5 s. The time course of the recovery from C-type inactivation is the same whether the membrane is kept depolarized from a few seconds or several minutes (7 min in the case shown in Fig. 1A). This finding argues against the possibility that the inactivated state reached by *Shaker* H4 Δ channels after a very long depolarization is different to the C-type inactivated state.

3.1. Existence of a C-type inactivated state

The question of whether external Ba^{2+} can bind to the C-type inactivated state was addressed first. The membrane was depolarized to 0 mV for a period of 2 min to ensure that channel inactivation had reached equilibrium. Then, keeping the membrane at 0 mV, Ba^{2+} was added to the external solution during 5 min to a final concentration of 20 mM (t_{Ba}; Fig. 1B, top). During this time Ba^{2+} binds to the open and, possibly, to the inactivated state. Ba^{2+} was then removed, and the membrane kept at 0 mV for the next 5 min. During this time the Ba^{2+} bound to the open state is expected to be released (see below). The membrane was now repolarized to −100 mV and the fraction of channels blocked was tested with depolarizing pulses to 0 mV given at 5 s intervals. In the absence of Ba^{2+}, all channels leave the inactivated state during the first 5 s after repolarization (Fig. 1A). However, in the presence of Ba^{2+} we found that the fractional current measured on the first pulse after repolarization was only 0.42 (Fig. 1B). This result implies that Ba^{2+} was not released from an appreciable fraction of the C-type inactivated channels during the 5-min period the membrane was washed with a Ba^{2+}-free solution. During the succeeding 5-s periods at −100 mV, 30% of the Ba^{2+} complex dissociated very fast, and the remaining was released with a time constant of ca. 120 s.

Fig. 1C shows that increasing the test pulse frequency after Ba^{2+} removal greatly increases the rate of recovery. The time constants for recovery are the same regardless of whether Ba^{2+} was loaded into closed (hatched bars) or into C-type inactivated (open bars) channels. Therefore, when the membrane is repolarized, Ba^{2+}-bound C-type inactivated channels change to Ba^{2+}-bound closed channels. The values for the

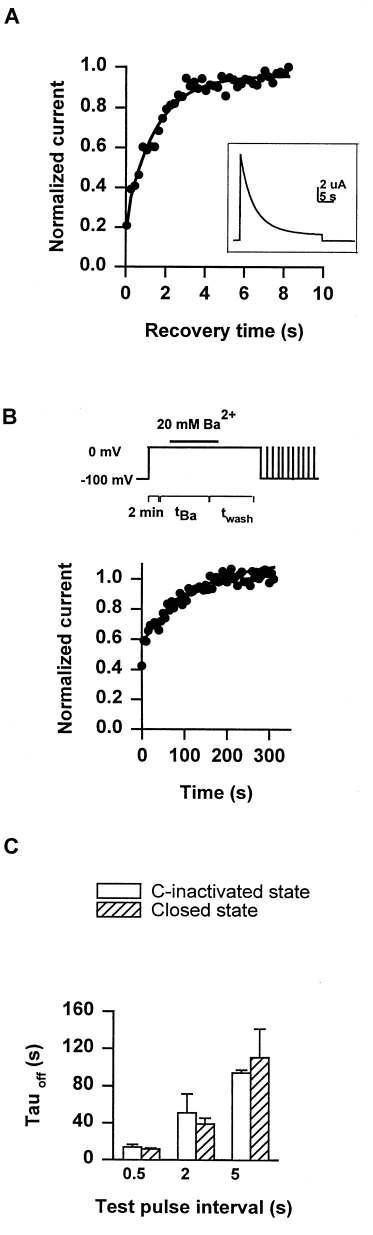

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Fig. 1. Recovery from inactivation and Ba^{2+} interaction with C-type inactivated channels. A: Recovery from inactivation, after keeping the channels for 7 min at 0 mV. The recovery was tested from a holding of −90 mV, with test pulses of 8 ms at 0 mV, once every 200 ms. The slow component of the current recovery was fitted to an exponential with a time constants $\tau = 1.5$ s. Notice that there is also a fast component that was not well solved under the present conditions. Inset: C-type inactivation of *Shaker* H4 Δ during a depolarization to 0 mV for 20 s. Holding potential was −100 mV. B: Ba^{2+} can reach a binding site in C-type inactivated channels. Top: Experimental protocol. Bottom: Symbols show the current evoked at the end of each test pulse. From the extrapolation of the single exponential to $t = 0$ (solid line) the fraction of unblocked channels was 0.62. C: Ba^{2+} exits the channel from the same site when loaded into the C-type inactivated channels or into closed channels. Ba^{2+} was loaded into C-type inactivated channels at 0 mV for 5 min. After that period Ba^{2+} was removed and the membrane kept for 5 min at 0 mV after Ba^{2+} washout. The membrane was repolarized to −100 mV and the exit of barium from C-type inactivated channel was tested with test pulse intervals of 0.5, 2 or 5 s. The data were fitted to a single exponential with time constants of 13.8 ± 2.8, 51.3 ± 21 and 94.0 ± 3, respectively ($n = 4$). Ba^{2+} was loaded into the closed channels for 5 min at −100 mV and the exit was tested at the same frequencies. The time constants obtained were 11.6 ± 1.1, 39.3 ± 6, 110 ± 31 ($n = 4$).

time constants we obtained at 0.2 Hz test pulse frequency are in good agreement with the results obtained by Hurst et al. [11].

3.2. Barium is trapped by C-type inactivated channels

To measure the dissociation of Ba^{2+} from the C-type inactivated channels we applied 20 mM external Ba^{2+} for a period of 5 min keeping the membrane at 0 mV. After this period, the cell was perfused with a Ba^{2+}-free solution for different time periods (t_{wash}) before repolarizing to −100 mV (Fig. 1B, top). Fig. 2A shows that the normalized current measured right after repolarization is virtually the same for all the t_{wash} tested. Therefore, Ba^{2+} binding to the C-inactivated channels at 0 mV in the time span tested is essentially irreversible.

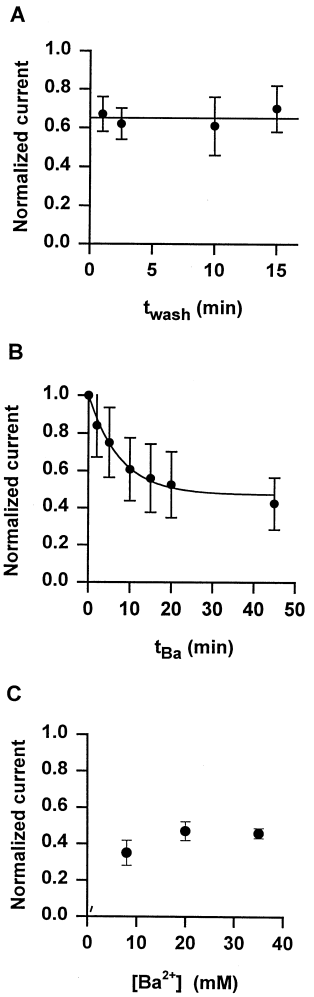

Fig. 2. Rates of dissociation and formation of the C-type inactivated channel-Ba^{2+} complex. A: Binding of Ba^{2+} to the C-type inactivated state is essentially irreversible. The fraction of channels unblocked after different washed out times (t_{wash}) is shown (I at $t_{wash}/I_{(control)}$). B: Rate of formation of the C-inactivated Ba^{2+} complex. The fraction of unblocked current, I at $t_{Ba}/I_{(control)}$, measured with a test pulse after repolarization as function of the time in the presence of external Ba^{2+} is shown. The kinetic was fitted to a single exponential (Eq. 2) with a time constant of $\tau_{on} = 7.65 \pm 2.0$ min and a pedestal $C = 0.47 \pm 0.05$ ($n = 3$). C: The pedestal C is independent of [Ba]. The onset kinetic for [Ba] = 8, 20 and 35 mM was determined, and fitted to a single exponential to obtain the pedestal. The values were 0.35 ± 0.06, 0.47 ± 0.05 and 0.46 ± 0.03 for the [Ba] of 8, 20 and 35 mM, respectively ($n = 3$ or 4).

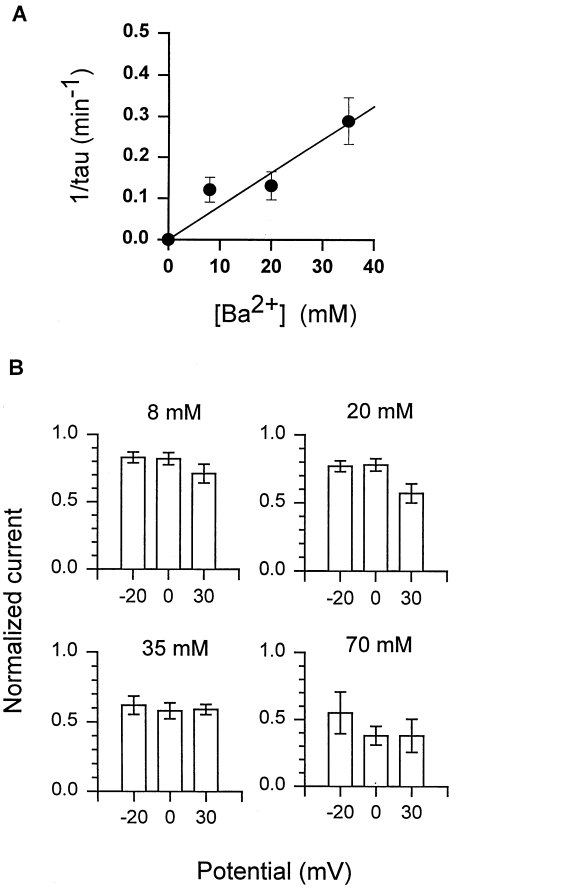

Fig. 3. Barium binding to C-type inactivated channels follows a bimolecular reaction and is voltage independent. A: $[Ba^{2+}]$ dependence of the reciprocal of the blocking time constant ($1/\tau_{on}$). $1/\tau_{on}$ was estimated from the fitting of the onset kinetics to a single exponential. The $1/\tau_{on}$ v/s [Ba] data were fitted to a linear equation (Eq. 3) with $k_{off} = 0$. The slope corresponds to the second order association rate constant, $k_{on} = 0.14$ M^{-1} s^{-1}. B: Fraction of unblocked channels is voltage independent. Experiments were done at 8, 20, 35 and 70 mM Ba^{2+}. $t_{Ba} = 15$ min, and $t_{wash} = 5$ min.

3.3. Kinetics of Ba^{2+} binding to C-type inactivated channels

The rate of Ba^{2+} binding to the C-inactivated state was measured by exposing the inactivated channels to Ba^{2+} for various periods of time (t_{Ba}) using the experimental protocol shown in Fig. 1B, top. The fraction of unblocked channels was tested after a 5-min period[1] in the absence of Ba^{2+}. Fig. 2B shows the Ba^{2+} entrance to C-type inactivated channels. The current decay induced by external Ba^{2+} was fit to a single exponential term of the form:

$$I_{fractional} = (1-C)\exp^{(-t/\tau_{on})} + C. \quad (2)$$

The result shown in Fig. 2B is unexpected, since if the reaction of Ba^{2+} with the inactivated state is essentially irreversible, the residual current C should be negligible at very long times (i.e.

[1] A 5-min period for the Ba^{2+} washout was chosen because it allowed complete removal of the divalent cation from the external compartment, in particular when high (70 mM) Ba^{2+} concentrations were used.

at $t = \infty$ all channels should be in the inactivated channel Ba^{2+} conformation). Our results indicate that despite the fact that the time constant τ_{on} gets shorter as the $[Ba^{2+}]$ is increased (Fig. 3A) the value of C remains about the same (~ 0.47) (Fig. 2C).

Ba^{2+} interaction with closed *Shaker* H4 Δ channels can be accounted for by a model of two sequential binding sites [11]. Barium forms first a 'labile' closed Ba^{+2} complex that forms and dissociates fast. This labile complex can be transformed slowly into a 'stable' complex, which dissociates with a time constant of minutes. To explain the residual current, C, it can be assumed that the inactivated Ba^{2+} complexes can be transformed upon repolarization into either labile or stable closed Ba^{2+} complexes. Upon repolarization all those channels that undergo a transition from the inactivated Ba^{2+} state to the labile closed Ba^{2+} state will dissociate instantaneously. Therefore, the residual current C remaining at very long times is a function of the distribution of Ba^{2+} into labile and stable complexes (Fig. 2B and C). Since the inactivated Ba^{2+} complex is very stable, we expect that at long times, i.e. $t \gg \tau_{on}$, the fractional current vs. time curves to reach the same C in the whole $[Ba^{2+}]$ range tested.

3.4. Ba^{2+} binds to C-type inactivated Shaker H4 Δ in a bimolecular reaction

A simple and testable model of the mode of action of Ba^{2+} on C-type inactivated channels is direct binding to the inactivated state, I, to form the complex IBa

$$\mathrm{I} \underset{k_{off}}{\overset{k_{on}[Ba^{2+}]}{\rightleftharpoons}} \mathrm{IBa}. \quad (\mathrm{R}-1)$$

This model predicts that the current decay in the presence of external Ba^{2+} is described by a single exponential with

$$\tau_{on} = 1/(k_{on}[Ba^{2+}] + k_{off}). \quad (3)$$

However, since the inactivated channel-Ba^{2+} complex is extremely stable, $\tau_{on} \approx 1/k_{on}[Ba^{2+}]$. Fig. 3A shows that, in agreement with the prediction of the simple model, the reciprocal of τ_{on} is directly proportional to the Ba^{2+} concentration. The above result suggests that Ba^{2+} binds *directly* albeit very slowly to C-type *Shaker* H4 Δ channels. The rate constant for the bimolecular reaction described in the scheme is $k_{on} = 0.14\ M^{-1}\ s^{-1}$.

Another reasonable model could involve Ba^{2+} binding to the open state, O, and a subsequent transformation of the open Ba^{2+} complex, OBa to IBa according to the reactions

$$\mathrm{I} \rightleftarrows \mathrm{O},$$

$$\mathrm{O} + \mathrm{Ba} \rightleftarrows \mathrm{OBa}, \quad (\mathrm{R}-2)$$

$$\mathrm{OBa} \rightleftarrows \mathrm{IBa}.$$

The results shown in the inset of Fig. 1A demonstrate that the first step has a relaxation time of 4 s and an equilibrium constant of 0.12. Since the overall process has a time constant of minutes this step can be assumed to be in equilibrium. The second step, Ba^{2+} binding to the open state, has not been studied and we characterized it taking advantage of the fact the C-type inactivation *Shaker* H4 Δ channel is incomplete. In this case, external Ba^{2+} was added after C-type inactivation reached equilibrium and we obtained the Ba^{2+} blockade characteristics from the decrease of the steady-state current ($\approx 12\%$). Blockade of open channels reached steady state in less than 10 s. We tested the inhibition at different Ba^{2+} concentrations to obtain an apparent dissociation constant. The best fitting to the Hill equation (Eq. 1)) was obtained with a K_d^N at 0 mV of $0.098 \pm 0.004\ mM^N$ and a Hill coefficient (N) of 0.57 ± 0.03 (data not shown).

In conclusion, the second step is also in equilibrium and at a $[Ba^{2+}] > 1$ mM is completely displace toward the formation of OBa. Therefore, the model predicts that if the IBa state is reached via the OBa state, $1/\tau_{on}$ of Fig. 3A has to be *independent* of $[Ba^{2+}]$. The data shown in Fig. 3A contradict this prediction of the kinetic scheme R-2 and are additional evidence that the Ba^{2+} site in the C-type inactivated state can be accessed directly.

3.5. Voltage and Ba^{2+} concentration dependence of Ba^{2+} binding to the C-inactivated state

To measure the rate of C-inactivated Ba^{2+} complex formation as a function of Ba^{2+} concentration and voltage we used the protocol showed in Fig. 1B, top, with a 15-min external Ba^{2+} treatment. We induced C-type inactivation with applied voltages of −20, 0, and 30 mV (Fig. 3B). Fig. 3B shows that there is no enhancement on the rate of complex formation with depolarization as examined for each $[Ba^{2+}]$.

4. Discussion

4.1. C-type inactivation involves a cooperative conformational change in the outer region of Shaker K^+ channels

It is clear at present that C-type inactivation involves the concerted participation of all four subunits of voltage-dependent K^+ channels [6,7] a process that is modulated by external K^+ [4,14,15]. The C-inactivated state binds Cd^{2+} several thousand-fold better than either the open or the closed state when the tyrosine 449 was replaced by cysteine [16]. This observation is also consistent with a cooperative mechanism. The 449 residues form part of the outer pore region in *Shaker* K^+ channels. Since the ability to bind Cd^{2+} changes during C-type inactivation, the spatial distribution of the residues in position 449 should change. This, added to the fact that only external TEA is able to modify C-type inactivation increasing the inactivation time constant [4,5,17], are strong indications that C-type inactivation involves a structural modification of the external mouth of voltage-dependent *Shaker* K^+ channels. Liu et al. [8] proposed that C-type inactivation involves a structural constriction of the external mouth leading to a long term closure of the ion-conducting pathway ('collapse hypothesis'). However, Molina et al. [5] suggested that the molecular rearrangement that takes place during C-type inactivation is restricted to a local site in the pore. Starkus et al. [9] showed that during C-type inactivation, the ion selectivity of *Shaker* channels is greatly reduced. Inactivated channels are electrically silent because internal K^+ ions prevent Na^+ ions from permeating through the channel. They proposed that during C-type inactivation the selectivity filter, located near the external mouth, changes its properties ('non-collapse hypothesis').

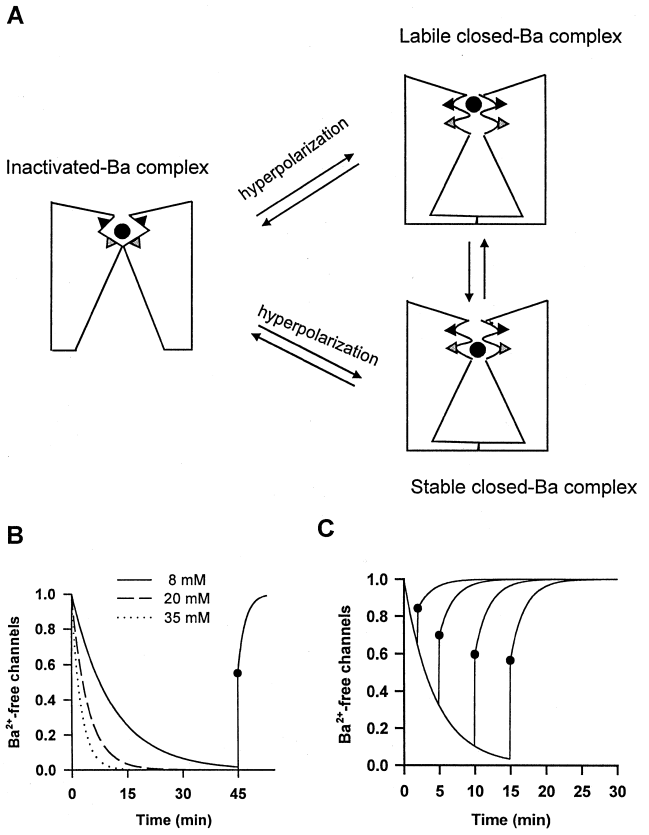

Fig. 4. Diagram illustrating the conformational changes that the pore of *Shaker* H4 Δ K^+ channels undergoes when entering the C-inactivated state and the predictions of the model. A: The cartoon shows a Ba^{2+} (●) bound to its site in the C-type inactivated state (inactivated Ba^{2+} complex at 0 mV). Upon repolarization to −100 mV, Ba^{2+} is redistributed between two sites present in the closed channel: a labile site, from where Ba^{2+} is released very fast, and a stable site, from where Ba^{2+} is released slowly. In our experiment we can only discriminate the exit of Ba^{2+} from the stable site. B and C: Model fitting the main properties of the interaction of Ba^{2+} with C-type inactivated channels. B: The entrance kinetics to the C-type inactivated state and the exit after repolarization at three different [Ba]. The entrance kinetics were simulated with a single exponential function with $\tau = k_{on}$ [Ba] where $k_{on} = 0.14$ M^{-1} s^{-1}. When $t = 45$ min, the membrane is repolarized to −100 mV, and the exit of Ba^{2+} follow two phases: 55% of the current recovered very fast and 45% slowly, following a single exponential with a $\tau = 120$ s. C: The entrance kinetic of Ba^{2+} into C-type inactivated channels ([Ba] = 20 mM), and exit after repolarization at different times. The fraction of current (with respect to the total current) that recovered very fast is shown with a solid circle. The curve described by the solid circles follows very closely that shown in Fig. 2.

4.2. External Ba^{2+} can reach the C-inactivated state

Our results strongly suggest that Ba^{2+} can reach its binding site when *Shaker* H4 Δ K^+ channels are in the C-type inactivated state. When the membrane is depolarized the channels are in two predominant states, open or C-type inactivated. Therefore, we can postulate two alternative kinetic models for Ba^{2+} interaction with the channel. Ba^{2+} can bind directly to the C-type inactivated state (R-1) or can bind to the open state and then, with Ba^{2+} in the conduction pathway, undergoes a transition to an inactivated state (R-2). Levy and Deutsch [18] gave evidence that the pore of Kv1.3 channels is accessible to K^+ when channels are C-type inactivated. Their results are consistent with a model in which K^+ can bind and unbind slowly from a channel in the inactivated state. It is tempting to suggest, therefore, that Ba^{2+} being so similar in radius to potassium, follows the same transport route as K^+ when the channel is in the C-type inactivated state. Our results (Fig. 3A) indicated that Ba^{2+} can bind directly to the C-type inactivated state. The association rate constant for this process is 0.14 M^{-1} s^{-1}. Thus, Ba^{2+} must surmount an energy barrier of 16 kcal/mol to reach the site when the channel is in the C-type inactivated state. Once Ba^{2+} surmounts the energy barrier and reaches its site in the C-type inactivated state, it is trapped in the channel in a very stable complex.

4.3. Explaining the results with a simple model

An economical model able to explain the present results is depicted in Fig. 4A. The molecular changes that take place during C-type inactivation makes difficult the entrance of Ba^{2+} into the *Shaker* K^+ channel pore. However, the conformational change of the external mouth of the channel is not enough to completely hinder the accessibility to the Ba^{2+} site. The model shown in Fig. 4A explicitly assumes that there is a single binding site for Ba^{2+} in the C-type inactivated channel (see Fig. 3A). During repolarization the equilibrium is displaced to the right and labile closed and stable closed Ba^{2+} complexes are formed. Barium leaves the fraction of Ba^{2+} closed labile complexes very fast and the relaxation of the current amplitude that follows is originated from the fraction of stable closed Ba^{2+} complexes. The model predicts that inhibition must be complete and that the time constants become shorter as the [Ba^{2+}] is increased (Fig. 4B). Upon repolarization, the model predicts that Ba^{2+} exit has a very fast and a slower kinetic component. Therefore, the predicted kinetic for Ba^{2+} entrance to the site is described by an exponential plus an 'apparent' residual current (Fig. 4C).

The stability of the Ba-inactivated state complex speaks against the possibility that inactivated channels allow ions to move freely toward the internal site and that restriction to the passage of ions is confined only to the external mouth. The extreme stability of the Ba-inactivated state complex can only be explained if the movement of Ba^{2+} toward the cytoplasmic side is also hindered. It is possible that as proposed by Starkus et al. [9], internal K^+ in the same way it prevents Na^+ ions from permeating, does not allow Ba^{2+} to exit toward the cytoplasmic side of the channel.

5. Addendum

After this paper was submitted for publication, Harris et al. (Biophys. J. 74:1808–1820, 1998) published observations giving further support to the existence of a Ba-blocked inactivated state in which Ba^{2+} is trapped between the activation and the C-type inactivation gates. Also, Kiss and Korn (Biophys. J. 74:1840–1849, 1998) proposed that C-type inactivation is the result of a constriction *at* the selectivity filter. This constriction is hindered when the filter is occupied by K^+. In view of these new observations, Ba^{2+} in the C-type inactivated channel should reside between the selectivity filter and the activation gate.

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