**Shab K⁺ channel slow inactivation**

A test for U-type inactivation and a hypothesis regarding K⁺-facilitated inactivation mechanisms

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**Keywords:** Shab, potassium channels, slow inactivation, U-type inactivation, C-type inactivation, *Drosophila*

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**Introduction**

In response to a sustained depolarization, most voltage-gated ion channels open and thereafter inactivate. Inactivation is customarily divided into fast or slow depending on its mechanism and the time scale in which it develops. Slow inactivation is a complex process that develops from the open state over a time course of hundreds of milliseconds to several seconds. The mechanism of slow inactivation is not fully understood and may vary in different channel types.

At first sight slow inactivation may seem too slow to be physiologically relevant; however, it can also develop from closed states and have a slow recovery, producing cumulative inactivation in the time scale of cell signaling. Additionally, when both fast and slow inactivation mechanisms are present they can be coupled.

Among Kv channels, the most extensively studied form of slow inactivation is that present in the *Drosophila* Shaker B channel, termed C-type inactivation by Aldrich’s laboratory because of its dependency on residues present toward the C-terminus domain of the protein. C-inactivation has also been documented in other Kv channels such as HERG and channels of the mammalian Kv1 family.

Shaker C-inactivation develops from the open state with a time constant of ~1–2 sec, in physiological [K⁺]. A hallmark of C-inactivation is its inhibition by external K⁺ (Ko) and tetraethylammonium (TEA o) ions. These, along with other observations, lead to the paradigm that during C-inactivation there is a localized closing or narrowing on the extracellular vestibule of the pore, around Shaker residue 449, a key determinant of the TEA o binding site on the channels. Accordingly, this region of the external vestibule of the pore has come to be referred to as the C-type inactivation gate. K⁺ o and TEA o are thought to inhibit C-type inactivation by sterically hindering the closing of this gate.

Other observations indicate that along with the localized constriction on the external vestibule of the pore, C-inactivation may recruit a larger conformational change that affects the selectivity filter (e.g., refs. 17–21) as well as locations along the S6 segment (e.g., refs. 3, 22–25), in particular the central cavity of the pore. Regarding the latter, and of particular interest to this study, is the recent demonstration that internal TEA (TEAi) inhibits the slow inactivation of Kv2.1 and Shaker channels.

Another protein domain that has been considered to be engaged in slow inactivation gating is the voltage sensor, since inactivation during a long depolarization, is accompanied with a shift of the gating-charge (Q) vs. voltage (V) curve toward hyperpolarized potentials. The term P/C inactivation is used...
which presents an inactivation that preferentially arises from partially activated closed states, follows a U-shaped inactivation curve, and therefore has been termed U-type inactivation.\textsuperscript{32,33} In addition to \textit{Kv}2.1, other \textit{Kv}s including \textit{Kv}1.3 and truncated \textit{Kv}1.5 channels,\textsuperscript{5,34} have also been reported to present U-type inactivation.

In contrast to its inhibitory effect on \textit{C}-inactivation, \textit{K}\textsubscript{o}+ slightly accelerates the open-state inactivation (\textit{OI}) of both \textit{Kv}2.1 and \textit{Kv}1.3 channels, although it slows down, and in a more prominent manner, the dominant closed-state inactivation (\textit{CI}) of \textit{Kv}2.1 channels.\textsuperscript{26,32,34} On the other hand, TEA\textsubscript{o} has no effect either on \textit{OI} or \textit{CI} of \textit{Kv}2.1.\textsuperscript{26} It is noteworthy that, to our knowledge, no mechanism has been proposed to explain the facilitation of inactivation exerted by \textit{K}\textsubscript{o}+ in these channels.\textsuperscript{5}

In contrast to the extensive studies performed to characterize the \textit{Drosophila} Shaker channel inactivation, the inactivation of the related Shab channels\textsuperscript{35,36} has escaped attention. This has occurred in spite of a wealth of evidence indicating significant roles of Shab channels in membrane excitability, for example in action potential repolarization, and repeated spiking in a variety of \textit{Drosophila} muscle and neural cells, as well as in cardiac beat frequency.\textsuperscript{37-43} Clearly, in order to best understand the role of Shab in cell physiology, knowledge of its inactivation gating is required. Additionally, this will undoubtedly increase our understanding of inactivation mechanisms and pore dynamics of \textit{Kv} channels.

Herein, we report the first characterization of the slow inactivation gating of delayed rectifier Shab channels and a critical test for U-type inactivation. Additionally, a novel hypothesis regarding the mechanism of \textit{K}+ facilitated inactivation process is stated.

**Results**

Once open, Shab \textit{K}+ channels undergo a slow inactivation process that develops over seconds.\textsuperscript{36} This is illustrated in Figure 1 which presents potassium currents (\textit{I}\textsubscript{k}) evoked either by a short, 30-ms, depolarization to 0 mV (\textit{Fig. 1A}), or by a long, 12 sec, pulse to the same voltage (\textit{Fig. 1B}), in cells bathed in \textit{Na}+/\textit{K} control solutions (see Materials and Methods). In contrast to \textit{I}\textsubscript{k} evoked with the short pulse, which does not decay, when the membrane is maintained depolarized for 12 sec, \textit{I}\textsubscript{k} gradually decays as channels slowly inactivate from the open state.

![Figure 1](image-url)
The time course of Shab open-state inactivation (OI) is best fitted with the sum of two exponentials than with a simpler single exponential model (p < 0.001). This is illustrated in Figure 1C which shows the fit of the normalized inactivation phase of \( I_k \) in Figure 1B (solid line), with a fast time constant \( \tau_{O.l,n} = 0.84 \) sec (average, 0.6 \pm 0.1 \text{ sec}; see Fig. 1D), with relative amplitude \( A_{O.l,n} = 0.42 \) (average, 0.3 \pm 0.04); and a slow time constant \( \tau_{O.T,n} = 3.3 \) sec (average, 2.1 \pm 0.2 \text{ sec}), with relative amplitude \( A_{O,T,n} = 0.6 \) (average, 0.68 \pm 0.03), plus a constant \( C_{n} = 0.03 \) term (average, 0.05 \pm 0.01, n = 8). The inset shows the residuals of the fit. As commonly found in several types of inactivation, both fast and slow, Figure 1E shows that at fully activating potentials (\( \geq -20 \text{ mV} \)) both time constants are not appreciably voltage-dependent.

Shab \( \tau_{O.l,n} \) has a value similar to the time constant of the Shaker C-type inactivation in equivalent ionic conditions, and faster than that of its mammalian ortholog Kv2.1 channel. Considering that the latter presents a U-type inactivation, our next goal was to determine if Shab presents its defining characteristic, namely a U-shaped inactivation curve.

**Shab inactivation curve: A test for U-type inactivation.** In order to determine if Shab presents U-type inactivation the Shab inactivation curve was analyzed, employing depolarizing pulses of variable duration, using both a modified three-pulse protocol and a standard two-pulse protocol. In the first one, a control pulse was initially applied (\( V_{\text{control}} = +40 \text{ mV}/30 \text{ ms} \)). Thereafter, a pulse (pre-pulse) of variable amplitude and duration was delivered. Finally, a test pulse was applied (\( V_{\text{test}} = V_{\text{control}} \)), and inactivation was evaluated as the ratio of the currents evoked by the last and the first pulse, \( I_{K,\text{test}}/I_{K,\text{control}} \).

The standard protocol lacked the first control pulse, and inactivation was assessed as \( I_{K}(V)/I_{K,\text{max}} \), where \( I_{K,\text{max}} \) is the maximal \( I_k \) measured during the second test pulse that was applied immediately after delivering the first pre-pulse of the indicated amplitude and duration. Both protocols yielded identical results (see below) due to the lack of variation of \( I_k \) in the first pulse of the three-pulse protocol (not shown, but noticeable in Figure 2A right panel).

Figure 2A left panel is a plot of the extent of inactivation (non-inactivated fraction) reached with the delivery of inactivating pulses of increasing duration (0.05, 0.9, 3 and 5 sec, as indicated), applying either the two (closed symbols) or the three-pulse (open symbols) protocols. As expected, very short depolarizations, lasting only 0.05 sec, produce a quite small inactivation at all of the voltages tested. Nonetheless, it is seen that the points of the resulting inactivation curve show a slight hint of a U-shape.

When pulse duration is increased to 0.9 sec the extent of inactivation increases and the points in the curve acquire a marked U-shape, with a well-defined minimum at \( -20 \text{ mV} \). The latter is illustrated by the traces in the right panel which depict two superimposed \( I_k \) obtained with the three-pulse protocol with pre-pulse of either \( -20 \text{ mV}/0.9 \text{ s} \) or \( +20 \text{ mV}/0.9 \text{ s} \), as indicated. Note that as observed in the curve, \( I_{K,\text{test}} \) at \( +40 \text{ mV} \) following the \( +20 \text{ mV} \) pre-pulse is notably larger than \( I_{K,\text{test}} \) following the \( -20 \text{ mV} \) pre-pulse. Finally, and importantly: observe that because of the slow kinetics of inactivation (Fig. 1), a 0.9 sec pulse is clearly not sufficient long to approach steady-state conditions.

When pulse duration is further increased to 3 and 5 sec, and thus steady-state inactivation is progressively approached, two facts are observed: first, the extent of inactivation increases, and second, and more interesting, the marked U-shape of the points observed with the shorter 0.9 sec pulses, becomes less evident (Fig. 2A).

The U-shape completely disappears when the pulse duration is significantly increased (Fig. 2B, left panel) to 20 sec (\( \sim10 \) times the slowest inactivation time constant, Fig. 1 and see below), yielding near steady-state conditions, under which the inactivation curve follows the classical \( h_p \) Boltzmann equation. This is also noted in the inset, which compares the curves obtained with 3 sec (duration \( \geq \tau_{O.l,n} \)) and 20 sec pre-pulses (duration \( >\tau_{O.l,n} \)). The same \( h_p \) Boltzmann-curve is obtained both with \( N_a \) or 100 mM \( K_p \) solutions.44 The right panel serves to illustrate \( I_k \) behavior with 20 sec pre-pulses, by showing two superimposed \( I_k \) evoked with the 3-pulse protocol applying either \( -20 \text{ or } +20 \text{ mV} \). Notice that the corresponding \( I_{K,\text{test}} \) at the final test pulse of \( +40 \text{ mV} \), have a size difference that is hardly distinguishable from the noise, as clearly seen with the expanded scale of the inset (indicated by the arrow).

In contrast to U-type curves, which present a sizable positive slope at positive potentials, the Boltzmann equation, which fits Shab inactivation under near steady-state conditions, approaches 0 when \( V > > V_{1/2} \). This is illustrated in Figure 2C, which plot the chord slope obtained with the average relative currents following pre-pulses of \( -20 \text{ and } +20 \text{ mV} \) \( ([I_k(-20 \text{ mV})-I_{K,\text{ref}}(-20 \text{ mV})]/40) \) of the indicated duration, as in Figure 2A and B. The magnitude of the slope provides a measurement of departure from a Boltzmann relation, in other words it serves to indicate how marked is the U-shape character of the corresponding curve. Note that as pre-pulse duration increases, approaching near steady-state conditions, the slope falls steeply toward 0, as contained in the Boltzmann equation. The open circle departs from the general tendency of the remaining points (closed circles). This point corresponds to 0.05 sec pre-pulses, too short to produce a significant inactivation (Fig. 2A).

It is clear that the U-shape of the inactivation curves as in Figure 2A are due to kinetic processes, which are the result of being far from steady-state conditions (see ref. 45 and Discussion). After rejecting U-inactivation, as the type of inactivation present in Shab channels, our next goal was to determine whether Shab presents the characteristic features of C-type inactivation.

**Shab inactivation is not C-type: Effects of TEA\(_p\) and K\(_p^*\) on OI.** In order to determine if inactivation follows a C-type mechanism, the effects of TEA\(_p\) and K\(_p^*\) on open state inactivation (OI) were determined by applying 7 sec pulses, that open and inactivate the channels, to cells bathed in either TEA or K\(_p^*\)-containing external solutions. Figure 3A presents the outcome of adding TEA to the external solution. The traces in the left panel compare representative \( I_k \) recorded either in the reference Na\(_p^*\) solution (larger trace, labeled Na) or with 15 mM TEA\(_p\) (smaller trace, labeled TEA) solution that inhibits 60% of channels.
Figure 2. Inactivation curve under far and near steady-state conditions. (A) Left panel, inactivation promoted with short depolarizing pulses, as indicated. Inactivation was assessed with either a three (closed symbols) or a two pulse protocol (open symbols) (see text). Right panel, superimposed $I_K$, obtained with the three-pulse protocol, applying a $-20$ or a $+20$ mV pre-pulse of $0.9$ sec duration, as indicated. The inset shows $I_K$ evoked by the final test pulse in an expanded scale. (B) Left panel, $h_\infty$ inactivation curves (data from ref. 44). The curve was obtained applying 20-sec pre-pulses of the indicated amplitude in $\text{Na}_o$ solutions. The line is the fit of the points with the Boltzmann equation $h_\infty = 1/(1+\exp[(zF/RT)(V_m-V_{1/2})])$, with parameters $V_{1/2} = -56.3$ mV, $z = 4.6$. Inset: inactivation after either 3- (open circles) or 20-sec pulses (closed circles). Right panel, superimposed $I_K$ recorded as in (A) right panel, but applying either $-20$ or $+20$ mV pulses of 20 sec duration, as indicated. The inset shows $I_K$ evoked by the final, $+40$ mV, test pulse (arrow) in an expanded scale. (C) Inactivation curve chord slope, between $-20$ and $+20$ mV, as a function of pre-pulse duration. Points are mean ± SEM of at least four experiments.
In order to facilitate its comparison, $I_k$ with TEA$_o$ was scaled to the peak $I_k$ in 0 TEA$_o$ (middle trace). As observed in Na$_o$ solutions (Fig. 1), OI in the presence of TEA$_o$ also follows a two exponential time course (right panel), but it is seen that in this condition, Shab OI becomes noticeably faster. This is quantified in the histogram on the right panel, which compares inactivation time constants in Na$_o$, with those obtained with 15 mM TEA$_o$. Notice that TEA$_o$ significantly decreases (~44%) both the fast ($p = 0.02$, $n = 4$) and the slow ($p = 0.023$, $n = 4$) OI time constants, without significantly changing their relative amplitudes.

TEA$_o$ facilitation of Shab inactivation is in clear contrast to its opposite, inhibitory, effect on C-inactivation,$^6$-$^9$ and to its lack of effect on the inactivation of the Kv2.1 channel.$^{26}$

Next we investigated the role of K$^+$ on Shab OI. Figure 3B left panel compares $I_k$ evoked by a +20 mV/7 sec pulse with the cell bathed in 100 K$^+$ (smaller $I_k$, labeled K) against $I_k$ recorded in the same cell bathed in 0 K$^+$ solution (trace labeled Na). $I_k$ in 100 K$^+$ is much smaller than in 0 K$^+$, because with 100 mM K$^+$ the pulse voltage is near the K$^+$ equilibrium potential. Therefore, to compare their kinetics the trace in 100 K$^+$ was scaled to peak $I_k$ in 0 K$^+$ (middle trace). Note that, in contrast to its known inhibitory effect on C-type inactivation, K$^+$ clearly speeds Shab OI. Quantitation is reported in the right panel which shows that K$^+$ significantly decreases (~30%) both the fast ($p = 0.027$) and the slow ($p = 0.019$) time constants. This speeding-up occurred without a significant change in the relative amplitudes of their corresponding OI phases (see figure legend), as seen with TEA$_o$.

TEA$_o$ blocks $I_k$ in 100 K$^+$ as effectively as in 0 K$^+$ (not shown), therefore we wondered whether the individual effects of 15 mM TEA$_o$ and 100 mM K$^+$ could be additive. The superimposed traces in Figure 3C illustrate that, unexpectedly, they are not (the same result was observed in at least two other cells, see Discussion). The above observations are summarized in Figure 3D which, for clarity, presents the plot of the least squares curves that fit OI, in the indicated conditions.
The former observations show that Shab inactivation presents properties opposite to those characteristic of the C-type inactivation mechanism. Also, it is important to point out that: the facilitation exerted by TEA\(_o\) and K\(_o\) does not by itself suggest an inactivation mechanism involving a closing or significant narrowing of the extracellular entry of the pore.

Our next goal was to determine if Shab presented closed state inactivation (CI), and whether the pore had to open for K\(^+\) and TEA to be able to affect inactivation.

**Inactivation from closed states.** We determined the extent and kinetics of closed-state inactivation by assessing the cumulative inactivation produced by short 20-ms repeated pulses of either 0 or +20 mV, which activate the channels without allowing the development of significant OI, applied at a rate that precluded significant recovery from inactivation between consecutive pulses (1.3 \(\times\) 10\(^{-4}\) Hz).

**Figure 4A** illustrates \(I_{K}\) obtained with the above protocol, in the reference Na\(^+\)/K solutions. For clarity only the first \(I_{K}\) recorded are shown. Note that, although none of the currents presents a noticeable OI, \(I_{K}\) decreased with each consecutive pulse, therefore indicating the presence of closed state inactivation (CI\(_{Na}\)). This is best seen in **Figure 4B** which presents the average relative \(I_{K}\) as a function of the number of pulses.

In order to determine CI kinetics, the points in **Figure 4B** were plotted as the average relative \(I_{K}\) vs. time (**Fig. 4C**). CI\(_{Na}\) follows a single exponential time course (solid line), with time constant \(\tau_{CI,Na}=0.61\) sec, plus a constant term \(C_{Na}=0.39\), which accounts for the fraction of channels that do not manage to inactivate during their transit along closed states, at this voltage. Note that, interestingly, \(\tau_{CI,Na}\) at \(-80\) mV is basically identical to the fast OI\(_{Na}\) time constant (**Fig. 1**), which accounts for \(-30\%\) of OI\(_{Na}\). This is easily observed in **Figure 4C** which for comparison also presents the curve that describes OI\(_{Na}\) time course (dashed line).

The former observations show that, in contrast to Shaker which mainly inactivates from the open state, Shab inactivates from both open and closed states, but in contrast to its ortholog Kv2.1, Shab channels do not preferentially inactivate from closed states.

Next we investigated whether channels have to open for its inactivation to be modulated by K\(^+\) and TEA\(_o\). **Figure 4D** shows the plot of the average relative \(I_{K}\) vs. time, obtained with the above protocol in cells bathed in 100 K\(^+\) (closed circles). \(I_{K}\) falls along an exponential time course (solid line), as channels inactivate from closed states, with time constant \(\tau_{CI,K}=0.4\) sec, plus a constant term \(C_{K}=0.2\). Importantly, CI\(_K\) is significantly faster and therefore more complete (p < 0.01), than CI\(_{Na}\). The latter is visually appreciated in the figure which, for a reference, also presents the time course of CI\(_{Na}\) (dotted line). Finally, notice that \(\tau_{CI,K}\) at \(-80\) mV is basically equal to \(\tau_{CI,TEA}\) (dashed line), as previously also observed in Na\(^+\) (**Fig. 4C**).

The role of TEA\(_o\) on closed state inactivation is presented in **Figure 4E**, which shows CI in cells bathed in 15 mM TEA\(_o\) (closed circles). CI\(_{TEA}\) follows an exponential time course (solid line through the points) with time constant \(\tau_{CI,TEA}=0.5\) sec, plus a constant term \(C_{TEA}=0.19\). Notice that CI\(_{TEA}\) is faster, and hence more complete, than CI in the absence of TEA\(_o\) (CI\(_{Na}\), dotted line, p < 0.01). Also, notice that, as previously found with both Na\(^+\) and 100 K\(^+\) solutions, \(\tau_{CI,TEA}\) is basically identical to \(\tau_{CI,TEA}^{o}\) (dashed line).

**Figure 4F** compares CI in the ionic solutions tested. It is easily appreciated that CI\(_{TEA}\) and CI\(_K\) are indistinguishable (**Fig. 4F**), and faster and more complete than CI\(_{Na}\). These data shows that, at the tested concentrations, K\(^+\) and TEA\(_o\) exert similar effects on both open (**Fig. 3**) and closed state inactivation.

The above observations indicate that TEA\(_o\) and K\(^+\) facilitate Shab inactivation, regardless of the state of the activation gate. This suggest the hypothesis that Shab reaches the same inactivated conformation from either open or closed states, and further indicates that Shab inactivation does not obey a C-type inactivation mechanism.

Interestingly, and related to the latter, recent observations point to the involvement of the central cavity in the slow inactivation gating of several Kv channels, exhibiting both C- and non-C-type inactivation mechanisms. Therefore, we decided to test the effect of adding an intracellular pore blocker that binds to the central cavity on Shab inactivation.

**Inactivation inhibition by the intracellular pore blocker quinidine.** It has been reported that inactivation properties may change upon establishing the inside-out configuration of the patch-clamp technique.27,46 Therefore, we decided to use quinidine (Qd), as this molecule added to the extracellular solution rapidly equilibrates across the membrane blocking the Shab open pore from the intracellular side of the membrane,47 allowing tests of Qd effect on inactivation in the whole-cell recording configuration.

In order to evaluate the effect of Qd on Shab inactivation, a 0 mV/7 sec pulse was applied to channels bathed in a control 5 K\(^+\) solution.39 Thereafter, Qd was added at either 0.03 or 0.1 mM concentrations in the 5 K\(^+\) solution, and once it equilibrated across the membrane (not shown), its effect on inactivation was tested.

**Figure 5A** shows two superimposed \(I_{K}\) recorded before (larger \(I_{K}\)) and after the addition of 0.1 mM Qd (smaller \(I_{K}\)). Qd blocked Shab, markedly reducing \(I_{K}\) amplitude and, more interesting for the present discussion, slowing inactivation when compared with control \(I_{K}\). This is best appreciated in the right panel which presents \(I_{K}\) with Qd normalized to control peak \(I_{K}\). In these superimposed traces, Qd inhibition of inactivation is easily appreciated by comparing \(I_{K}\) size at pulse-end in control vs. in Qd-blocked channels.

The above observation is quantitatively presented in **Figure 5B**, which illustrates the extent of inactivation reached at the end of the inactivating pulse, as in **Fig. 5A** (see figure legend). Note that in the absence of Qd the extent of inactivation (92.6 \(\pm\) 4%, \(n = 5\)) is significantly larger than that observed in channels bathed in either 0.03 mM (81.6 \(\pm\) 4%, \(n = 5\); p = 0.0318), or 0.1 mM Qd solutions (66.8 \(\pm\) 5%, \(n = 5\); p = 0.0011). Although further work is needed to determine the mechanism by which Qd hinders inactivation, this observation points to the participation of the central cavity of the pore in the slow inactivation gating of Shab channels.
Figure 4. Closed state inactivation. (A) I, evoked by four consecutive 0 mV/20 ms pulses, applied at 1.3 x 10^-4 Hz (Na/K). (B) Average relative I, as a function of the number of applied pulses, as in (A). (C) Points in (B) plotted as Relative I, vs. time (ClNa, closed circles). The solid line is the fit of the points with the equation: I/Io = Aexp(-t/τ) + C, where I is I, at time t; Io is I, at t = 0; τClNa = 0.61 sec, AClNa = 0.61, CClNa = 0.39. The dotted line is the curve that fits ClNa time course. (D) Cl time course with 100 K⁺ (closed circles, ClK). The solid line is the least squares fit of the points with τClK = 0.4 sec, AClK = 0.8 and CClK = 0.19. Dashed line: OI Na time course; dotted line: CI K time course. (E) Cl time course in the indicated conditions. HP = -80 mV. The points are the mean ± SEM of at least four experiments on each condition.
A kinetic model of Shab inactivation. The observations reported in this study were synthesized in a kinetic model (Fig. 6A). The model is an extension of a kinetic scheme developed to explain the effect of the anti-inflammatory drug celecoxib on Shab channels. The model consists of five closed (Co to C4) and a single open state (O). Inactivation (I) develops from the open state, but with a very slow rate, approaching near steady-state conditions. On the other hand, when channels inactivate from closed states during the activating pulse and, because pulse duration is relatively short, they will not have sufficient time to inactivate fully from the open state, yielding the U-shaped curve.

In addition to OI, Shab also undergoes closed state inactivation. CI develops with a time constant equal to that of the fast OI component (~0.6 sec). Considering that the latter accounts for ~30% of $I_K$ decay during a pulse, and that ~40% of channels do not undergo CI upon delivery of a moderate, 0 mV, pulse from -80 mV, we conclude that although Shab has significant CI it does not have the so called preferential closed state inactivation seen in other Kv whose inactivation is also facilitated by $K_o^{-0.5}$.

This demonstrates that $K_o$ facilitation is a property not strictly associated with inactivation processes that preferentially develop from closed states. Additionally, as Shab presents significant CI, our observations also show that the term U-type inactivation is inadequate to denote inactivation process significantly arising from pre-open closed states.

Importantly, we demonstrate that the inactivation curve presents an apparent U-shape when pulse durations are too short to approach steady-state conditions. In contrast, when the inactivating pulse durations are much longer than the slowest inactivation time constant, which allows the channels to approach near steady-state conditions, the inactivation curve ceases to be U-shaped, and it is fitted by the classical $h_o$ Boltzmann curve.

The U-shape of the inactivation curves obtained when pulse durations are too short (duration ≤ slowest inactivation time constant) are the result of kinetic processes arising from the relative values of activation and inactivation rates. At more depolarized voltages channel activation is faster, and since inactivation is voltage-independent, and can be reached from both open and closed states (see below), delivering pulses too short to approach steady-state inactivation causes the fewer of these channels to inactivate from closed states during the activating pulse and, because pulse duration is relatively short, they will not have sufficient time to inactivate fully from the open state, yielding the U-shaped curve.

On the other hand, as steady-state conditions are approached channels have enough time to reach a time-invariant extent of inactivation, both from closed and open states, and the U-shape of the curve vanishes. Based on this, and on the extensive discussion of Yue and coworkers, we consider that only in limiting conditions, such as when inactivation arises only from closed states, would a U-shaped curve be observed, near steady-state conditions. On the other hand, when channels inactivate from the open state, but with a very slow rate, approaching near steady-state conditions may be technically difficult to achieve, but this

### Discussion

Shab channels present a slow inactivation process that develops from the open state with two voltage-independent time constants. In the reference $Na_v$ solution the slower constant has a magnitude (~2 sec) similar to that of Shaker C-type inactivation, and much faster than that of OI of the related Kv2.1 channels, under comparable recording conditions.

The observations reported in this study were synthesized in a kinetic model (Fig. 6A). The model is an extension of a kinetic scheme developed to explain the effect of the anti-inflammatory drug celecoxib on Shab channels. The model consists of five closed (Co to C4) and a single open state (O). Inactivation (I) develops from the open state, but with a very slow rate, approaching near steady-state conditions. On the other hand, when channels inactivate from closed states during the activating pulse and, because pulse duration is relatively short, they will not have sufficient time to inactivate fully from the open state, yielding the U-shaped curve.

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The U-shape of the inactivation curves obtained when pulse durations are too short (duration ≤ slowest inactivation time constant) are the result of kinetic processes arising from the relative values of activation and inactivation rates. At more depolarized voltages channel activation is faster, and since inactivation is voltage-independent, and can be reached from both open and closed states (see below), delivering pulses too short to approach steady-state inactivation causes the fewer of these channels to inactivate from closed states during the activating pulse and, because pulse duration is relatively short, they will not have sufficient time to inactivate fully from the open state, yielding the U-shaped curve.

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by itself should not vindicate the identification of a particular inactivation as U-type.

Shab inactivation is faster in the presence of either external K\textsuperscript{+} or TEA. Additionally, we found that when both ions are present, their individual effects are not additive, despite the fact that TEA\textsubscript{o} blocks I\textsubscript{K} in 100 mM K\textsuperscript{+} as effectively as in Na\textsubscript{o}. A possible explanation of these observations is that the individual effect of the ions may have reached saturation at the concentrations tested, and that TEA\textsubscript{o} and K\textsuperscript{+} speed inactivation by binding to different sites. TEA\textsubscript{o} on its blocking site in the external vestibule of the pore,\textsuperscript{14} and K\textsuperscript{+} within the selectivity filter.\textsuperscript{31} This proposal is in agreement with the observation that K\textsubscript{o} decreases the OI rate of Kv2.1 channels.\textsuperscript{26,32}

As mentioned before, TEA\textsubscript{o} significantly speeds both OI and CI of Shab, and in contrast it does not affect at all the inactivation of the Shab mammalian ortholog Kv2.1.\textsuperscript{26} A possible explanation for this difference is that Shab presents a cysteine at the Shaker position 449, a key determinant of the external TEA binding site of Kv channels,\textsuperscript{9,14,15} while Kv2.1 presents a tyrosine (Y), at the equivalent position. Tyrosine residues yield a high affinity TEA\textsubscript{o} blocking-site,\textsuperscript{14} where, interestingly, TEA\textsubscript{o} binds without affecting inactivation.\textsuperscript{9} This is thought to occur because the Y-binding site seems to hold TEA in a relatively external position, outside the voltage drop across the membrane, as inferred from the voltage-independent TEA\textsubscript{o} block - age of pore.\textsuperscript{9} In other words, it seems that TEA\textsubscript{o} needs to bind deep enough to sense the voltage drop across the membrane, as it does in Shab (data not shown), to either down or upregu - late inactivation, as it does in for example Shaker\textsuperscript{6} and Shab, respectively.

External K\textsuperscript{+} significantly speeds Shab OI while in Kv2.1 the same effect is observed although it is less pronounced.\textsuperscript{26,32} This

Figure 6. A kinetic model of Shab inactivation. (A) Kinetic model of Shab inactivation. C, I and O have their standard meaning (see Materials and Methods and Table 1). (B) Comparison of the model predictions with the voltage-dependent activation of Shab channels. The left panel shows good agreement between the model and the data for short activating pulses from -50 to +50 mV. The G-V curve, plotted as a fractional activated current in the right panel is also well explained by the model. (C) Model prediction of slow inactivation. For long activating pulses, the model also provides a reasonable good fit of inactivation time course, although it predicts a somewhat larger remaining current at pulse end (Vm = 0 mV). (D) Inactivation curve. For long inactivating pulses (12 sec, black trace) the model predicts a monotonically increasing inactivation at depolarized potentials, as contained in the Boltzmann curve, while for shorter pulses (5 sec) a U-shaped inactivation is expected as a consequence of the channel not having reached steady-state during the pulse.
An opposite effect of Qd is observed in C-type inactivating Kv1.4 channels, in which Qd speeds inactivation through an allosteric effect.\(^{35,36}\) Although further work is needed to fully characterize Qd role on Shab inactivation, its effect appears to be similar to the inhibitory effect of internal TEA on Shaker inactivation and on Kv2.1 OI,\(^{26,27}\) and hence suggests that involvement of the central cavity may be a common theme in different slow inactivation mechanisms.

**Comparison with C-type inactivation.** Shab inactivation (OI/CI) is accelerated by K\(^+\) and TEA\(^-\). This feature stands in contrast to the characteristic inhibitory effect that these ions exert on C-inactivation,\(^{6-9}\) which is thought to arise from a sterically impediment of a local narrowing of the extracellular entry of the pore that accompanies inactivation,\(^{9,10,12}\) referred to as the closing of the C-inactivation gate.\(^{5,10,16}\) In the case of Shab, as well as those Kvs whose inactivation is facilitated by TEA\(_o\) and K\(^+\), the up-modulation effect of these ions is not easily reconciled with a mechanism presenting the C-inactivation narrowing of the extracellular entry of the pore, nor a general significant constriction of the selectivity filter.

Another characteristic of C-inactivation is its dependency on the residues present at the Shaker positions 449 and 463. As mentioned before, Shab presents a cysteine while Shaker has a threonine at position 499. It is known that the mutation T449C on Shaker preserves the hallmarks of C-inactivation.\(^{11}\) As it does on OI, K\(^+\) also facilitates Shab inactivation, but in contrast it inhibits the dominant closed state inactivation of Kv2.1.\(^{26}\) Whatever the cause of this difference may be, it suggests that the relative weights of CI/OI of these channels may not simply be due to their different relative values of inactivation and activation rate constants, but to a qualitative difference on its corresponding CI; even though further work is needed to clarify this point, the differential effects of K\(^+\) and TEA on OI/CI of Kv2.1 suggest that inactivation of the latter may be a more complex process than that operating on Shab, as it is also suggested by the regulation of Kv2.1 inactivation by modulatory subunits.\(^{26}\)

**Role of the central cavity on Shab inactivation.** Quinidine is a lipophilic cation that blocks Shab IK\(_c\),\(^{47}\) upon binding to the central cavity of the pore.\(^{47,52-54}\) Here we show that Qd block hinders Shab inactivation.

Although Qd competes with pore K\(^+\) ions, mutually destabilizing their binding,\(^{47}\) and this effect by itself should be expected to slow down inactivation, the observation that Qd inhibits OI in the presence of only 5 mM K\(^+\), a [K\(^+\)] rather low to significantly speed inactivation, suggest that Qd inhibition of inactivation is not the result of a reduced K\(^+\) occupancy of the pore. Thus, it appears that Qd binding to the central cavity somehow hinders the Shab inactivation conformational change.

**Table 1. Kinetic model rate constants**

| Rate constants in s\(^{-1}\) @ 0 mV | Charge (in e\(_q\)) |
|-------------------------------------|---------------------|
| k\(_o\) = 700                       | 0.7                 |
| k\(_1\) = 50                        | 0.2                 |
| q = 0.5                             | 0.1                 |
| q’ = 0.1                            | 0.6                 |
| p = 0.05                            | 0.1                 |
| p’ = 0.06                           | 0.2                 |
| s\(_2\) = 35                        | 0                   |
| s\(_2\)' = 0.3                      | 0                   |
| k = 240                             | 0.5                 |
| k\(_1\)' = 130                      | 0.5                 |
| s\(_1\) = 0.03                       | 0.1                 |
| s\(_1\)' = 0.1                      | 0.1                 |
| s\(_2\) = 3                          | 0                   |
| s\(_2\)' = 0                        | 0                   |

**Comparisons with C-type inactivation.** Shab inactivation (OI/CI) is accelerated by K\(^+\) and TEA\(^-\). This feature stands in contrast to the characteristic inhibitory effect that these ions exert on C-inactivation,\(^{6-9}\) which is thought to arise from a sterically impediment of a local narrowing of the extracellular entry of the pore that accompanies inactivation,\(^{9,10,12}\) referred to as the closing of the C-inactivation gate.\(^{5,10,16}\) In the case of Shab, as well as those Kvs whose inactivation is facilitated by TEA\(_o\) and K\(^+\), the up-modulation effect of these ions is not easily reconciled with a mechanism presenting the C-inactivation narrowing of the extracellular entry of the pore, nor a general significant constriction of the selectivity filter.

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0 mV (inactivated). In other words, this indicates that inactivation does not impede the leak of the stabilizing K⁺ ion(s) from the Shab pore toward the external solution.⁴⁴

Taken together, the above observations cannot be reconciled, in an obvious manner, with an inactivation mechanism involving a significant narrowing or closing of the extracellular portion of the pore, as it is considered to occur in C-inactivation. Hence, we propose that when Shab inactivates there is only a rearrangement of the pore, affecting the central cavity, which somehow halts K⁺ conduction. The latter suggests that K⁺, and TEA, may speed inactivation by allosterically favoring the internally located conformational change of the pore. Moreover, we hypothesize that similar mechanisms may operate in other channels whose slow inactivation is facilitated by external K⁺, such as Kv2.1 and Kv1.3.

Materials and Methods

Cell culture and channel expression. SP² cells grown at 27°C in Grace’s media (Gibco) were infected, at a multiplicity of infection of ~10, with a recombinant baculovirus containing Shab K⁺-channel cDNA (dShab 11) as reported.³⁷ Experiments were conducted 48 h after the infection.

Electrophysiological recordings. Currents were recorded under whole-cell patch-clamp with an Axopatch 1D amplifier (Axon Instruments). The currents were filtered online and sampled at frequencies set to fulfill the Nyquist criteria, with a pClamp 7 using a Digidata 1200 interface (Axon Instruments). Curves were compared with the extra-sum-of-squares F-test using GraphPad Prism version 5.00 (GraphPad Software, www.graphpad.com). Significance level was set to 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This study was performed in partial fulfillment of the requirements of the Doctorate degree in Biomedical Sciences of E.C. at the UNAM. The authors thank J.M. Hernandez for cell culture. This work was supported by DGAPA_UNAM grant IN229712 and CONACYT grant 153504.

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