Stability of the *Shab* K^+ Channel Conductance in 0 K^+ Solutions: The Role of the Membrane Potential

Froylán Gómez-Lagunas
Facultad de Medicina, Dept. Fisiologia, Universidad Nacional Autónoma de México, Ciudad Universitaria, Apartado Postal 70-250 Mexico D.F. 04510, Mexico

ABSTRACT *Shab* channels are fairly stable with K^+ present on only one side of the membrane. However, on exposure to 0 K^+ solutions on both sides of the membrane, the *Shab* K^+ conductance (G_K) irreversibly drops while the channels are maintained undisturbed at the holding potential. Herein it is reported that the drop of G_K follows first-order kinetics, with a voltage-dependent decay rate *r*. Hyperpolarized potentials drastically inhibit the drop of G_K. The G_K drop at negative potentials cannot be explained by a shift in the voltage dependence of activation. At depolarized potentials, where the channels undergo a slow inactivation process, G_K drops in 0 K^+ with rates slower than those predicted based on the behavior of *r* at negative potentials, endowing the r-V_m relationship with a maximum. Regardless of voltage, *r* is very small compared with the rate of ion permeation. Observations support the hypothesized presence of a stabilizing K^+ site (or sites) located either within the pore itself or in its external vestibule, at an inactivation-sensitive location. It is argued that part of the G_K stabilization achieved at hyperpolarized potentials could be the result of a conformational change in the pore itself.

INTRODUCTION

Potassium ions are known to play important roles in the operation of voltage-dependent K^+ channels (K_v channels). Although it is known that K^+ ions affect the functionality of these proteins (i.e., the stability, selectivity, and the conformational changes referred to as gating), they affect different K_v channels in quite different ways. When the conservation of the signature sequence and the associated selectivity filter of the pore, which contains the only K^+ binding sites visible in crystallographic structures of K^+-selective pores (1–3), are taken into account, this variability of effects is surprising, and it can be said that its mechanistic bases are not yet well understood.

One general observation is that K^+ ions are a necessary cofactor for stability of the functional conformation of the channels (i.e., K^+ ions are needed to keep K channels in a conformation able to conduct permeant ions), although exceptions are known of channels that remain quite stable in the absence of K^+ (see below).

The role of K^+ ions in K channel stability is best observed when the channels are exposed to 0 K^+ solutions on both sides of the membrane: pioneering work by Chandler and Meves (1970) (4) and Almers and Armstrong (1980) (5) using the squid giant axon show that the K^+ current (I_K) and associated gating currents are irreversibly lost on bathing the axon with 0 K^+ (not added) solutions on both sides of the membrane, revealing for the first time the relevant role of K^+ in K_v channel stability. Nonetheless and interestingly, other channels present different responses to K^+ depletion. For example, both $K_v1.3$ and Ca^{2+}-dependent maxi-K channels remain functional after being exposed to 0 K^+ solutions (6,7). Similarly, $K_v2.1$, $K_v1.5$, and delayed rectifier channels of bullfrog neurons remain stable but lose their selectivity, allowing a substantial Na^+ flux in the absence of K^+ (8–11). In contrast, the KcsA channel selectivity filter adopts an anomalous, nonconducting, conformation with low (3 mM) K^+ (12), and the thermal stability of its tetrameric structure is undermined in the absence of either permeant or blocking ions (13).

In the case of *Shaker* B, it has been shown that these channels remain stable in the absence of K^+ on both sides of the membrane as long as they are kept undisturbed (closed) at the holding potential (HP). However, if the channels undergo gating cycles in 0 K^+, evoked with the delivery of activating pulses, the conductance collapses. The extent of collapse depends both on the number of activating pulses and on the [Na^+] in the 0 K^+ solutions. It is thought that the drop of G_K occurs during the closing of the channels, at the end of each activating pulse. The collapse of G_K is completely reversed by very prolonged depolarizations (14,15). During the collapsed (nonconducting) state of G_K, the channels nonetheless continue to exhibit gating currents. In other words, closing a *Shaker* channel in a 0 K^+ solution containing Na^+ will uncouple, in a fully reversible manner, the movement of the voltage sensor from the conduction state of the pore (16,17).

It was shown recently that delayed rectifier *Shab* channels, which share an identical signature sequence with the related *Shaker* B channel, are fairly stable in solutions lacking K^+ on only one side of the membrane (either internal or external), although interestingly, its stability is significantly greater with K^+ present in the external solution. However, if

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Address reprint requests to Froylán Gómez-Lagunas, Facultad de Medicina, Dept. Fisiologia, Universidad Nacional Autónoma de México, Ciudad Universitaria, Apartado Postal 70-250 Mexico D.F. 04510, Mexico. Tel.: 555-623-2263; E-mail: froylangl@yahoo.com.

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the channels are exposed to 0 K^+ (not added) solutions, on both sides of the membrane, G_K collapses. Notably, and in contrast with *Shaker* behavior, the collapse of *Shab* G_K occurs while the channels are kept undisturbed at the HP (HP = −80 mV), and it is irreversible. Furthermore, the extent of collapse is similar for 0 K^+ solutions containing Na^+, NMG^+, or choline$^+$ ions (18). That is, the stabilizing K^+ ion(s) readily dissociate from the apparently closed *Shab* channels when the cell is perfused with 0 K^+ solutions. These observations prompted the hypothesis that there could be a site (or sites) located in the external vestibule of the pore, outside the selectivity filter, where K^+ binding would allosterically maintain G_K stable (18).

To test the previously mentioned hypothesis regarding a possible stabilizing K^+ site located outside the selectivity filter (and therefore outside of the voltage drop across the membrane (19)), and also to test the possibility that the dissociation of the stabilizing K^+ could occur while channels are closed (deactivated) at the HP, the voltage dependence of the *Shab* G_K collapse in 0 K^+ was studied.

Here it is reported that: 1), G_K decay follows a first-order kinetics, with a decay rate that, unexpectedly, presents a strong voltage dependence, such that hyperpolarized potentials dramatically inhibit the G_K drop in 0 K^+; 2), G_K collapse at negative voltages cannot be accounted for by a shift in the voltage dependence of the activation of the channels; but 3), delivery of activating pulses from the stabilizing HP of −140 mV somewhat facilitates the drop of G_K. On the other hand, it is also reported that 4), at depolarized potentials, where the channels undergo a slow inactivation process, the drop of G_K becomes much slower than predicted, based on the trend observed at negative voltages.

These novel observations are consistent with the hypothesized presence of one or more stabilizing K^+ sites located either within the pore itself or in the pore's outer vestibule, at a location affected by the conformational change associated with the slow inactivation of the channels.

MATERIALS AND METHODS

Cell culture and *Shab* channel expression

Insect *Spodoptera frugiperda* Sf9 cells were grown at 27°C in Grace's medium (Gibco, Grand Island, NY). Cells were infected with a multiplicity of infection of 10 with a recombinant baculovirus containing *Shab* (d*Shab* 11) K^+-channel cDNA, as previously reported (18,20). The experiments were conducted 48–72 h after infection of the cells.

Electrophysiological recordings

Macroscopic currents were recorded under whole-cell patch clamp with an Axopatch 1D amplifier (Axon Instruments). Unless otherwise indicated, currents were filtered on line at 5 kHz with the built-in filter of the amplifier and sampled at 100 μs/point, with a Digidata 1322A interface (Axon Instruments, MDS, Sunnyvale, CA). The electrodes were made of borosilicate glass (KIMAX 51) pulled to a 1–1.5 MΩ resistance. Eighty percent of the series resistance was compensated. Unless otherwise indicated the HP was −80 mV. Experiments were carried out at room temperature, as previously reported (18).

Solutions

Solutions referred to herein will be named by their main cation and their location with respect to the membrane, e.g., Na_i.

Internal solutions contained (in mM):
K_i: 30 KCl, 90 KF, 2 $MgCl_2$, 10 EGTA-K, 10 Hepes-K
Na_i: 30 NaCl, 90 NaF, 2 $MgCl_2$, 10 EGTA-Na, 10 Hepes-Na
External solutions contained (in mM):
Na_o: 145 NaCl, 10 $CaCl_2$, 10 Hepes-Na
K_o: 100 KCl, 45 NaCl, 10 $CaCl_2$, 10 HEPES-Na
XK_o: X KCL, (145 − X) NaCl, 10 $CaCl_2$, 10 HEPES-Na. where X stands for the [K^+].

The pH of all solutions was 7.2.

Complete exchange of the bath solution occurred within 3–4 s as evaluated by the change in the equilibrium potential of K^+ (data not shown), which is less than 10% of the smallest G_K decay time constant reported herein; thus, no correction was applied to the reported times in 0 K^+.

The G_K drop in 0 K^+ follows first-order kinetics (Fig. 1, see Results). Thus, the decay constant r of the process does not depend on [K^+] in initial control solutions (i.e., r does not depend on the initial amount of functional, available-to-conduct, AK channels harboring stabilizing K^+ ions in them). Therefore, curves showing the G_K drop as a function of time spent in 0 K^+ were obtained from experiments with XK_o/Na_i initial control solutions having a variable potassium concentration X (mM), that depended on the number of channels (size of I_K) present in the corresponding cells.

Data Analysis

Results are expressed as the mean ± SE of the indicated number of experiments. When necessary, the *t*-test was used to evaluate statistical significance. Curves were fitted with the SigmaPlot version 8.0 (SPPSS) Marquardt-Levenberg algorithm.

RESULTS

On exposure to 0 K^+ (not added) solutions on both sides of the membrane, *Shab* channels cease to conduct K^+. The irreversible collapse of the *Shab* conductance (G_K) is illustrated in Fig. 1 *A*, which presents a control I_K (*left panel, Before*) evoked by a 0-mV/30-ms activating pulse in K_o/Na_i solutions (see Methods). After checking of I_K stability (not shown), the cell was superfused with the 0 K^+, Na_o solution (Na_o/Na_i) for 5 min, with the channels kept undisturbed (closed, see below) at the HP of −80 mV (as indicated by the *arrow*). Finally, the cell was returned to the control K_o solution and, ~30 s later, the channels' ability to conduct K^+ was tested with the delivery of an activating pulse (*right panel*). There was a substantial reduction of I_K, which demonstrates that, on exposure to 0 K^+ solutions, the majority of the channels lose their ability to conduct ions.

To learn more about the nature of the physical process underlying the drop of G_K, the kinetics of the collapse was studied. The results in Fig. 1 *B* report G_K decay as a function of the time spent in 0 K^+, from experiments such as those depicted in Fig. 1 *A*. The G_K drop at time t is measured by the

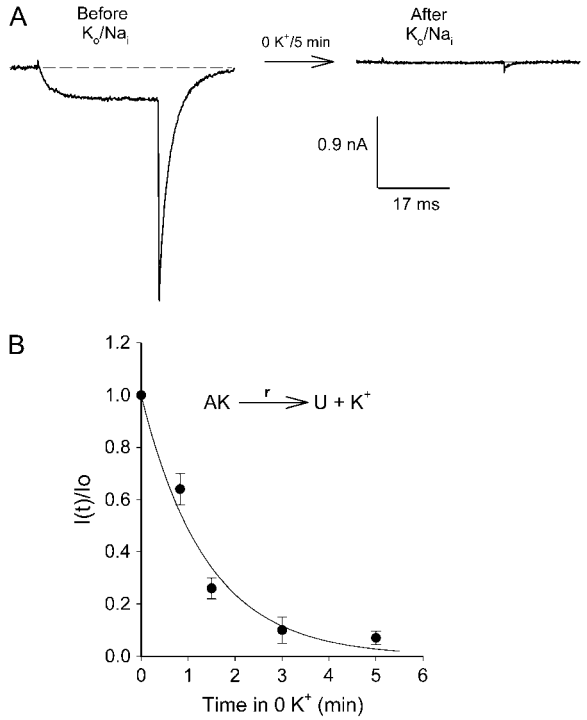

FIGURE 1 Collapse of *Shab* K^+ conductance in 0 K^+ solutions. (*A*) Control I_K evoked by a 0-mV/30-ms test pulse applied from the HP of −80 mV, with the cell in K_o/Na_i (see Methods) solutions (*left panel*). Thereafter, the cell was immersed 5 min in the 0 K^+, Na_o solution (Na_o/Na_i), keeping the membrane potential constant at −80 mV, as indicated by the arrow. Finally, the cell was superfused with the K_o solution, and the state of channels was tested with the delivery of a test pulse (*right panel*). (*B*) I_K decay as a function of the time spent in 0 K^+, as in *A*. $I(t)/I_o$ is the ratio of the I_K left after t minutes in 0 K^+, $I(t)$, to the control current I_o. Currents were isochronally measured at the end of the test pulse. The line is the fit of the points with the function $I(t)/I_o = \exp(-rt)$, with $r(-80\text{ mV}) = 0.79\text{ min}^{-1}$. The points are the mean ± SE of at least four experiments at each time.

ratio $I(t)/I_0$, where $I(t)$ is the K^+ current remaining after t minutes in 0 K^+, and I_0 is the control current. Note that G_K decay follows an exponential time course (*line through the points*), which indicates that, during exposure to 0 K^+, G_K undergoes a first-order decay reaction as the channels proceed from an initial available-to-conduct state, with K^+ bound to them (AK), to a final unable-to-conduct state (U), reached once the stabilizing K^+ leaves the channels, with a decay rate r of 0.72 min^{-1} at the HP of −80 mV, as indicated by the kinetic scheme in the figure. The first-order kinetics of the collapse of G_K indicates that r does not depend on the [K^+] in the initial control solutions (see Methods). It is pertinent to point out that after the dissociation of the stabilizing K^+, a conformational change must take place that sinks the channels into the final state U. This intermediate step is not shown in the kinetic scheme of the figure (see Discussion).

The results in Fig. 1 demonstrate that the stabilizing K^+ (i.e., the K^+ ion(s) that maintain(s) the channels able to conduct) is located at a site(s) from which it is easily removed by just washing the cell with the Na_o solution despite the negative membrane potential that should attract positive K^+ ions. This suggests that the stabilizing K^+ site(s) could be located in a superficial location, such as the external vestibule of the pore, outside the selectivity filter, and the voltage drop across the membrane, as previously proposed (see Introduction). The latter implies that G_K decay should not be affected by the membrane potential. To test this possibility the drop of G_K in 0 K^+ was studied at several membrane potentials.

Fig. 2 compares the extent of the G_K drop obtained at the HP of −80 mV, as in Fig. 1 *A*, with that obtained at the HP of −140 mV during a 5-min exposure of the cells to 0 K^+ solutions. The left panel in Fig. 2 *A* shows a control I_K, evoked by a −10-mV/30-ms test pulse applied from the usual HP of −80 mV in K_o/Na_i solutions (*Before*). Thereafter, the HP was shifted to −140 mV, and 15 s later, the cell was superfused with the, 0 K^+, Na_o solution (Na_o/Na_i) for 5 min, with the HP maintained at −140 mV (not shown, indicated by the *arrow*). Then, the cell was returned to the control K_o solution, the HP was shifted back to −80 mV, and the state of the channels was tested, as in the control. The size

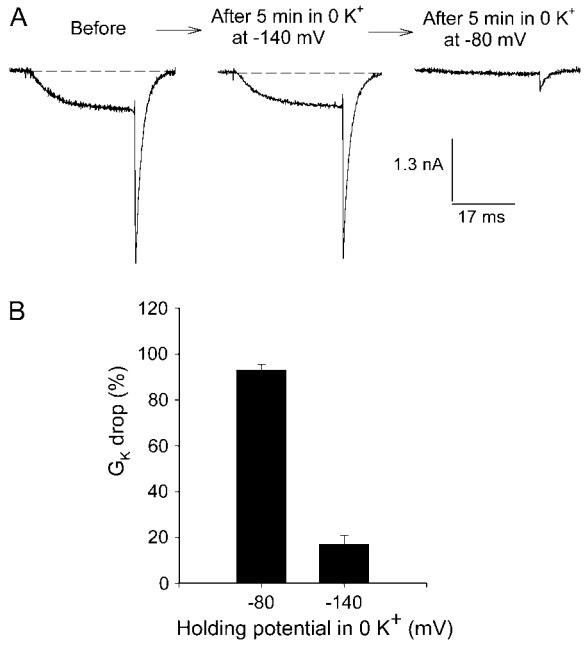

FIGURE 2 Inhibition of G_K collapse at −140 mV. (*A, left panel*) control I_K evoked by a −10-mV/30-ms pulse applied from the HP of −80 mV in K_o/Na_i solutions. Thereafter, the HP was shifted to −140 mV, and 15 s later the cell was superfused for 5 min with the Na_o solution (Na_o/Na_i, not shown, indicated by the *arrow*). Then, the cell was returned to the K_o solution, the HP was shifted back to −80 mV, and 30 s later I_K in the middle panel was recorded, as in the control. There was only a scant decrement of I_K. Next, the cell was immersed again for 5 min in the Na_o solution, this time keeping the HP at −80 mV (indicated by the *arrow*). Finally, with the cell back in K_o the trace in the right panel was recorded. (*B*) G_K drop (%) after a 5-min exposure to 0 K^+ (Na_o/Na_i) solutions with the HP either at −140 (17 ± 4%, $n = 5$) or at −80 mV (93 ± 2.5%, $n = 4$), as indicated.

of I_K in the middle panel demonstrates that the G_K drop was surprisingly small at the HP of −140 mV.

Subsequently, and after the trace in the middle panel had been recorded, to better compare the HP effect on G_K stability, the cell was immersed again in 0 K^+ (Na_o/Na_i) solutions for 5 min, but this time with the HP at −80 mV, after which, with the cell back in the K_o solution, the activity of the channels was tested. The trace in the right panel shows that, as expected, there was a nearly complete G_K drop (~90%). The effect of the voltage on the drop of G_K is best observed in Fig. 2 *B*, which presents the average drop after a 5-min exposure to 0 K^+ with the HP at either −80 mV (93 ± 2.5%, $n = 4$) or −140 mV (17 ± 4%, $n = 5$), as indicated. Clearly, the hyperpolarized membrane potential drastically inhibits the collapse of G_K in 0 K^+.

Fig. 3 *A* presents G_K drop as a function of HP and of time spent in 0 K^+ solutions, from experiments as in Fig. 1 *B*. Note that regardless of the HP, G_K decay follows an exponential time course, as expected, and the rate of decay becomes progressively and significantly slower as the HP becomes more negative. Thus, as shown previously, at −140 mV the channels become quite stable in 0 K^+. G_K decay is so slow that, within the time span considered, the fall of the current approaches the linear variation of an exponential function at times smaller than its corresponding time constant.

The above results demonstrate that the rate of decay, *r*, is voltage dependent. This is best observed in Fig. 3 *B*, which presents a plot of *r* against the HP during channel exposure to 0 K^+ solutions. Note that *r* varies in a nonlinear fashion with the voltage. As a first approximation (see below and Discussion), the points were fitted with an exponential function plus an offset (line through the points): $r_n(V) = 0.01 + 86\exp(0.06V)$, where the subscript n stands for negative voltages ($V \leq -80$ mV). It is interesting that this equation has the monoexponential form of the *G-V* relationship of voltage-dependent channels at voltages where the probability of opening is low (e.g., 21,22), plus an offset. However, until we know more regarding the factors involved in G_K stabilization, we should be cautious in affording a physical interpretation to its parameters. The exception is the small offset (0.01 min^{-1}), which has a clear physical meaning: it suggests that, even when hyperpolarized potentials confer a striking stability to the channels, no matter how negative the potential could be there would always be an exponential, although quite slow, G_K decay in 0 K^+.

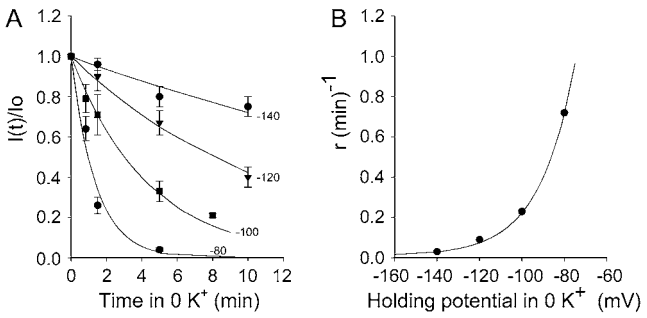

FIGURE 3 Collapse of G_K as a function of the membrane potential during channel exposure to 0 K^+ solutions. (*A*) Fraction of conducting channels ($I(t)/I_o$) as a function of the time spent in 0 K^+ (Na_o/Na_i) solutions at the indicated HPs. The lines are the fit of the points with exponential functions, as in Fig. 1 *B*. (*B*) Decay rate constant *r* as a function of the membrane potential *V*; *r* was obtained from the curves in *A*. The line is fit to the points with the equation $r_n(V) = 0.01 + 86\exp(0.06V)$. Subscript n stands for negative potentials.

The observations in Figs. 2 and 3 demonstrate that, unexpectedly, the collapse of G_K in 0 K^+ is markedly voltage dependent. This finding suggests the following possibilities regarding the mechanism for G_K drop: 1), that at the negative potentials studied, the stabilizing K^+ could leave the channels during their rare stochastic openings, which are expected to occur more frequently at −80 than at −140 mV (as suggested by the form of $r_n(V)$); 2), that the voltage dependence of the activation of the channels could be significantly shifted to the left in 0 K^+, so that the channels would be open at negative potentials, with more of them being open at −80 mV than at more negative potentials; or 3), that the channels are not tightly closed by the cytoplasmic activation gate at −80 mV (i.e., that the closed conformation of the activation gate could allow the leakage of K^+ ions), and that closing becomes tighter at more negative potentials (e.g., 23,24). It is pertinent to point out that these possibilities share the common characteristic of considering that the huge stabilization afforded by hyperpolarized potentials arises from a reduced exit of the stabilizing K^+ ions, from either the open or the closed state of the channels. The experiments that follow were conducted to distinguish among these possibilities.

To explore the possibility that channel activation could be significantly shifted toward hyperpolarized potentials in 0 K^+ solutions, the conductance-versus-voltage relationship of the channels was studied at several [K^+] across the membrane. Fig. 4 *A* shows I_K obtained in two cells expressing different numbers of channels. Currents were activated by 100-ms pulses from −70 to +50 mV, applied every 5 s in 10-mV increments from the HP of −90 mV in either 100 mM K_o^+/Na_i or 2 mM K_o^+/Na_i solutions, as indicated. For clarity, the figure shows the current at every other pulse beginning at −60 mV. The traces indicate that the activation of the channels is not significantly shifted to negative potentials when [K^+] is decreased. The latter is best seen in Fig. 4 *B*, which presents the average G_K-V_m relationship at several [K^+] across the membrane. Note that, interestingly, despite the critical role of K^+ in G_K stability, the G_K-V_m relationship is not importantly affected by the [K^+] across the membrane (see legend to Fig. 4).

Incidentally, it is also pertinent to note that *Shab* channels conduct K^+ well even with only 2 mM K^+ in the recording solutions. Moreover, as mentioned above, the G_K-V_m relationship in 2 mM K^+ (*solid circles*) is not much different

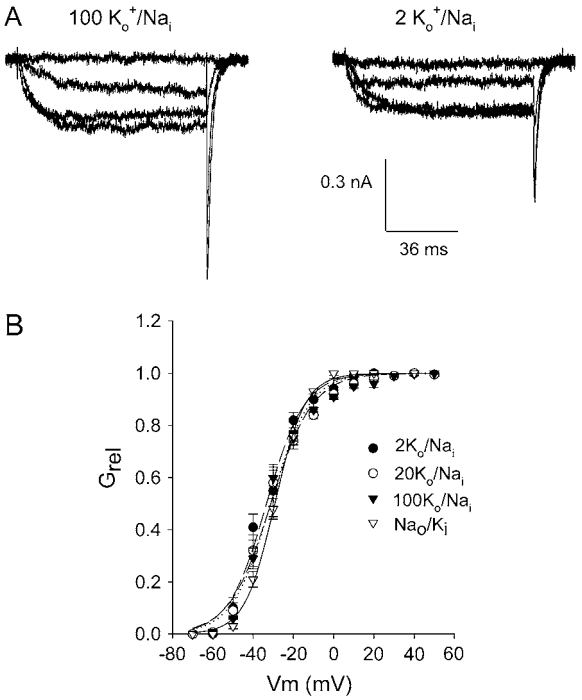

FIGURE 4 Conductance-versus-voltage relationship of *Shab* channels as a function of the K^+ distribution across the membrane. (*A*) I_K evoked by 100-ms pulses from −60 to 0 mV applied in 20-mV increments from the HP of −80 mV, in either 100 mM K_o^+/Na_i or 2 mM K_o^+/Na_i solutions, as indicated (see text). (*B*) G_K versus pulse potential. G_K was obtained from the relative, isochronal, tail currents at −80 mV $I_{tail}(V)/I_{max}$, where $I_{tail}(V)$ is the amplitude of the tail current at the end of the pulse potential V, and I_{max} is the maximal amplitude of the tail currents. V was varied from −70 to +50 mV in 10-mV steps. In the experiments in Na_o/K_i, the tail currents were measured at a repolarization potential of −60 mV. The points are the mean ± SE of at least four experiments at each [K^+]. The lines are fit to the points with Boltzmann functions, as indicated: $2K_o/Na_i$: $V_{½} = -33.3$ mV, $z = 2.7$; $20K_o/Na_i$: $V_{½} = -31.2$ mV, $z = 2.4$; $100K_o/Na_i$: $V_{½} = -31.2$ mV, $z = 2.6$; Na_o/K_i: $V_{½} = -29.3$, $z = 3.3$.

from that obtained in high, 100 mM, K^+ conditions (*solid triangles*). The latter is in contrast with the behavior of KcsA channels, where the pore adopts a nonconducting, collapsed, conformation in the presence of 3 mM K^+ (12) (see Discussion).

The results in Fig. 4 suggest that the drop of G_K at −80 mV, as well as the protection afforded by hyperpolarized potentials, cannot be explained by a significant shift of the voltage dependence of activation of the channels toward negative potentials in the absence of K^+. Accordingly, the next question is whether the explanation is that channels are not tightly closed at −80 mV (i.e., if the closed conformation of the cytoplasmic activation gate allows leakage of the critical K^+ ions toward the internal solution), and whether channels close tighter at hyperpolarized membrane potentials? If this were the case, it would be expected that any manipulation that facilitates K^+ exit from the channels would also enhance the drop of G_K. Therefore, to gain some insight regarding this point, the effect of repeated activation of the channels in 0 K^+ solutions, from the protecting HP of −140 mV, was studied.

Fig. 5 reports the effect of the delivery of 10 short activating, 0-mV/5-ms, pulses (referred to as pulsing) applied from the HP of −140 mV at the rate of 1 Hz during a total 1.5-min exposure of the cells to 0 K^+ solutions. The 5-ms pulse duration was chosen because a significant percentage of the channels would be opened by each pulse, whereas, on the other hand, the total time spent at 0 mV (50 ms) would alone account for 0.05% of the total time at −140 mV in 0 K^+. Finally, the delivery of one pulse every second from −140 mV allows a full recovery from inactivation (in Na_o/K_i solutions F. Gomez-Lagunas and D. Balleza, unpublished observations, and see below). Therefore, the experimental conditions guarantee that whatever the observed reduction of G_K may be, it could not be interpreted as the result of the time spent at a potential different from the stabilizing −140 mV.

Fig. 5 *A* (*left panel*) is a control I_K evoked by a 0-mV/30-ms pulse applied from −80 mV in K_o/Na_i (*Before*). Thereafter, the HP was changed to −140 mV, and 15 s later

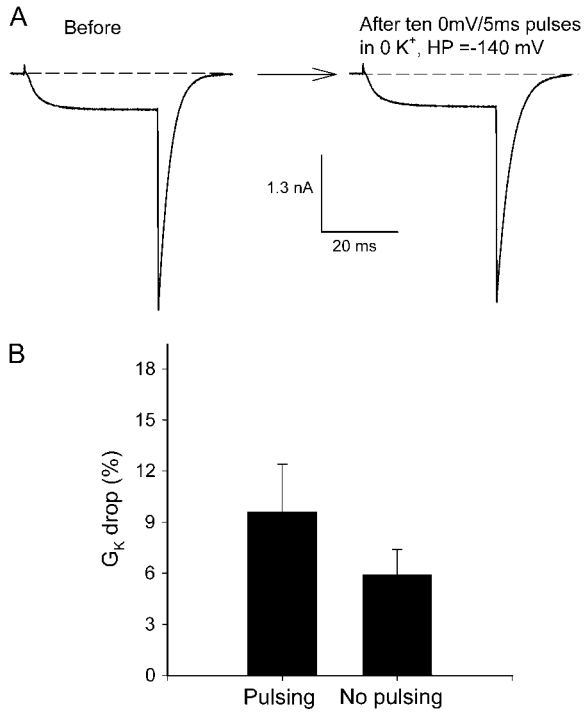

FIGURE 5 Drop of G_K with the delivery of activating pulses from the stabilizing HP of −140 mV. (*A*) Control I_K elicited by a 0-mV/30-ms pulse from the HP of −80 mV in K_o/Na_i (*left panel, Before*). Thereafter, the HP was shifted to −140 mV, and 15 s later the cell was superfused with the Na_o solution, and 10 0-mV/5-ms pulses were applied at the rate of 1 Hz (indicated by the *arrow*). Finally, after 1.5 min in Na_o/Na_i, the cell was returned to the control K_o solution, the HP was shifted back to −80 mV, and I_K was recorded as in the control (*right panel*). (*B*) G_K drop after a 1.5-min exposure to 0 K^+ solutions at the HP of −140 mV, obtained either without pulsing (*left bar*, 5.9 ± 1.5%, $n = 8$) or with the delivery of 10 0-mV/5-ms pulses (*right bar*, 9.6 ± 2.8%, $n = 7$), as in *A* (see text).

the cell was superfused with the Na_o solution, and 10 0-mV/5-ms pulses were applied at 1 Hz (not shown, indicated by the *arrow*). Finally, after 1.5 min in 0 K^+, the cell was returned to the K_o solution, the HP was shifted back to −80 mV, and the current in the right panel was recorded, as in the control. There was only a scant reduction of I_K.

The latter result is best appreciated in Fig. 5 *B*, which presents the average G_K drop obtained from experiments as in *A*. Notice that the difference in the extent of drop that occurs either just passively (i.e., without pulses, *left bar,* 5.9 ± 1.5%, $n = 8$) or with the added effect of the repeated pulses (*right bar,* 9.6 ± 2.8%, $n = 7$) is small (not statistically different, $p > 0.05$) but nevertheless noticeable; moreover, if the pulse duration is increased to 30 ms to allow the full activation of the channels on each pulse, then the G_K drop increases ~6% over the value obtained with the shorter pulses (G_K drop = 15.2 ± 1.8%, $n = 5$; not shown). The above observations indicate that the activation of the channels somewhat facilitates the drop of G_K (by comparison, at −80 mV, without pulsing, G_K drops ~74% after 1.5 min in 0 K^+). Thus, it seems that part of the stabilizing effect of hyperpolarized HPs arises from a reduced exit of K^+ ions from the channels. The latter could be produced either by a tighter closing of the activation gate or by a reduction of the probability of opening as the membrane becomes hyperpolarized or both (see Discussion). Finally, it is pertinent to mention that these results also show that the behavior of *Shab* is different from that of *Shaker* channels whose G_K collapses in a reversible manner only with delivery of activating pulses in 0 K^+ such that a single pulse produces an ~40% drop of G_K.

Drop of G_K at depolarized potentials

The variation of the decay rate, r, with voltage (Fig. 4) predicts that G_K should decay rapidly at depolarized potentials. Specifically, at 0 mV we would expect $r(0) = 86\ min^{-1}$; thus, after a brief 50-s exposure of the cell to 0 K^+ solutions, G_K should drop completely. Fig. 6 presents a test of this prediction. The figure shows 2 I_K recorded in K_o/Na_i solutions; the current in the left panel is a control I_K (*Before*), whereas the right panel shows the I_K left after the cell has been bathed in 0 K^+ (Na_o/Na_i) solutions for 50 s, with the HP = 0 mV (not shown, indicated by the *arrow*). Clearly, there still remains a substantial fraction of the control I_K (~50%). Therefore, G_K decay at 0 mV is much smaller than its predicted drop, based on the trend observed at negative potentials.

A possible explanation for the above discrepancy is that whereas within the range of negative potentials, at which the r-V_m relationship was studied (Fig. 3), the drop of G_K likely occurs while the channels are closed (Figs. 4 and 5, and see Discussion), at 0 mV the G_K drop (i.e., the dissociation of the stabilizing K^+) probably occurs while the channels are inactivated. On inactivation the K^+ conductance drops.

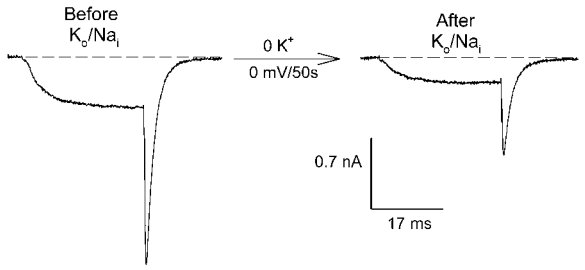

FIGURE 6 G_K drop after a 50-s exposure of the channels to 0 K^+ at 0 mV. I_K evoked by a −10-mV/30-ms test pulse applied from −80 mV, in K_o/Na_i (*Before*). Thereafter, the HP was shifted to 0 mV, and 15 s later the cell was superfused for 50 s with the Na_o solution. Finally, the cell was superfused back with K_o, HP was shifted back to −80 mV, and 30 s later the channels were activated by a test pulse (*right panel*).

Moreover, the typical, C-type, slow inactivation is thought to involve a partial closure of the extracellular portion of the pore (see Discussion). Therefore, in the case of *Shab*, it would be expected that the exit of the critical K^+ ions would be either halted or delayed if channels were slowly inactivated during the exposure to 0 K^+ solutions at 0 mV.

Therefore, to gain insight into this point, Fig. 7 presents the most basic characteristics of the slow inactivation of *Shab* channels, together with a test to best assess whether G_K actually collapses in 0 K^+ once the channels have been inactivated. Fig. 7 *A* (*left panel*) presents a reference I_K evoked by a short 0-mV/30-ms pulse under standard recording conditions (Na_o/K_i solutions). As expected, there is no inactivation during the pulse, but if its duration is increased to 7 s, then a slow inactivation of the channels becomes apparent (*right panel*). Preliminary observations indicate that, in contrast to the C-type inactivation of *Shaker* channels, external K^+ does not slow the rate of inactivation of *Shab* (work in progress, not shown).

Thus, to better understand the role of the HP in G_K stability in 0 K^+, the steady-state inactivation of the channels was studied as a function of $[K^+_o]$. Fig. 7 *B* shows that with standard $[K^+]$ inside the cell (K_i), the parameters of the steady-state inactivation curve (hereafter also referred to as h_∞) change only slightly on removal of the external K^+ (Na_o/K_i (*solid circles, solid line*): $V_{½} = -56.3$ mV, $z = 4.5$; $100K^+_o/Na_i$ (*open circles, dashed line*): $V_{½} = -53$ mV, $z = 5.0$). Therefore, at the HP of 0 mV the channels are inactivated at steady state regardless of the external $[K^+]$. The latter suggests that the G_K drop at 0 mV (Fig. 6) probably occurred while the channels were at the indicated level of steady-state inactivation.

This possibility is further examined with the experiments in Fig. 7 *C* that compare the percentage reduction of G_K obtained when the HP is switched from −80 to 0 mV either 15 s (as commonly done, as in Figs. 3–6) or 1 min before the cell immersion in the Na_o solution (undoubtedly sufficient time to ensure steady-state conditions). The bars in the figure demonstrate that, regardless of the time at which the HP was

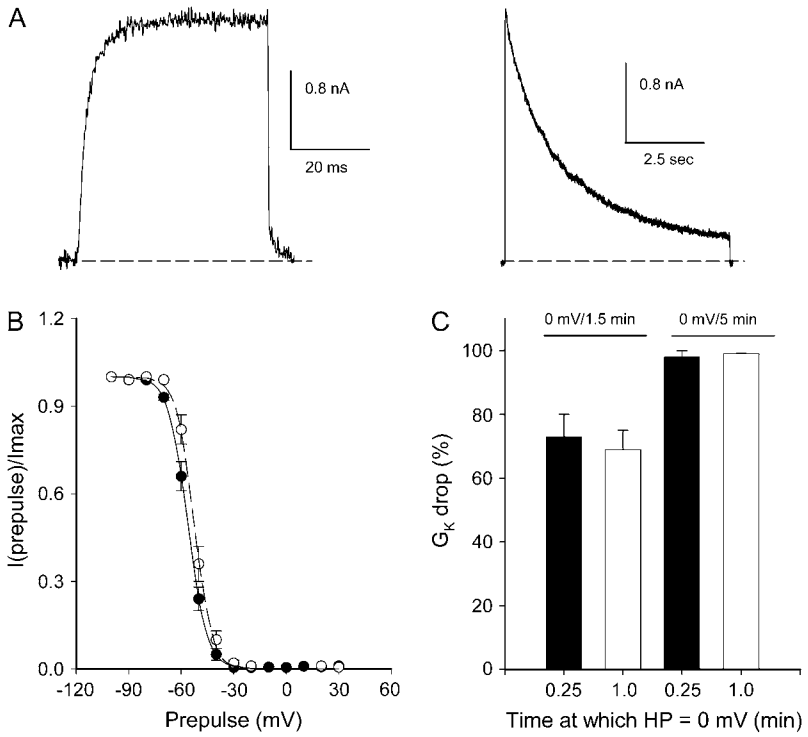

FIGURE 7 Channel inactivation reduces the rate of drop of G_K in 0 K^+. (*A*) I_k evoked by either a short 30-ms (left panel) or a long 7-s pulse (*right panel*) to 0 mV from the HP of −80 mV, in Na_o/K_i solutions. (*B*) Steady-state inactivation (h_∞) as a function of the membrane potential. Inactivation was measured by the standard two-pulse method. The membrane was first depolarized for 20 s to the indicated prepulse potential, each prepulse was followed by a test pulse to 0 mV, with the cell in either Na_o/K_i (*solid circles, solid line*) or 100 K_o^+/K_i (*open circles, slashed line*) solutions. I(prepulse)/I_{max}, is the ratio of I_K evoked by the test pulse that followed the indicated prepulse potential, I(prepulse), to the maximal I_K, I_{max}, evoked by the test pulse. The points are the mean ± SE of $n = 4$ experiments in each condition. The lines are the fit of the points with the Boltzmann equation: $I(\text{prepulse})/I_{max} = 1/(1 + \exp((zF/RT)(V_m - V_{½})))$, where F, R, and T have their usual meaning. In Na_o/K_i: $V_{½} = -56.3$ mV, $z = 4.6$; $100K_o/K_i$: $V_{½} = -53$ mV, $z = 5.0$. HP = −80 mV. (*C*) Collapse of G_K from inactivated channels. G_K drop after either a 1.5- (*left bars*) or a 5-min (*right bars*) exposure to 0 K (Na_o/Na_i) solutions at 0 mV, as a function of time (0.25 or 1 min) at which the HP was shifted from −80 to 0 mV before the perfusion of the Na_o solution, as indicated (see text).

shifted, there are no significant differences ($p > 0.05$) in the extent of G_K drop attained after either a 50-s (*solid bars*) or a 5-min (*open bars*) exposure to 0 K^+, as indicated. Thus, changing the HP 50 s before going to 0 K^+ allows the channels to reach their steady-state level of inactivation.

The above observations, along with the trend observed at negative potentials, indicate that the development of inactivation greatly slows the rate of decay of G_K (see Discussion). The latter point is further tested below.

If the development of inactivation at depolarized voltages actually slows the decay of G_K, then, according to the h_∞ curves, its decay should be faster at intermediate potentials (e.g., −50 mV), at which not all of the channels are inactivated, than at more depolarized potentials (e.g., at −30 or 0 mV) at which all channels should be inactivated at steady state. Fig. 8 *A* tests this prediction by comparing the time course of the drop of G_K at 0 mV with that obtained at the HPs of either −50 (*left panel*) or −30 mV (*right panel*). Note that G_K decay is clearly faster at −50 ($r(-50) = 1.78$ min^{-1}) than at 0 mV ($r(0) = 0.9$ min^{-1}). On the other hand, the decay rate at 0 mV is comparable to, although quantitatively slightly slower than, that at −30 mV ($r(-30) = 1.36$ min^{-1}). These results are in qualitative agreement with the hypothesis proposing that inactivation slows the decay of G_K. The difference in decay rates at 0 and at −30 mV can be accounted for by means of the hypothesis that there is a small shift of the h_∞ curve (compared with that in Na_o/K_i) with 0 K^+ on both sides of the membrane (see Discussion).

The plot of r versus V_m in the entire voltage range studied is presented in Fig. 8 *B*. The dashed line is the exponential fit of the points at negative potentials ($r_n(V)$, Fig. 3 *B*). Clearly, this trend is no longer followed at depolarized voltages (see Discussion). Finally, to best compare the voltage dependence of the decay rate r with the steady-state gating of the channels, normalized r values (r/r_{max}) (*squares*) were plotted together with the steady-state inactivation and activation (*solid circles*) points in Na_o/K_i solutions of Figs. 4 *B* and 7 *B*, respectively. It is observed that the apparent reduction of r at depolarized voltages exhibits a qualitative relation with the inactivation state of the channels and that, on the other hand, the inhibition of the drop of G_K exerted by hyperpolarized potentials maintains no obvious relation with the voltage dependence of the activation of the channels.

DISCUSSION

On exposure to 0 K^+ solutions on both sides of the membrane, the *Shab* G_K undergoes an irreversible collapse (18). The G_K drop occurs while the channels are kept undisturbed at the test HP. An important finding of this work is the demonstration that the collapse of G_K follows first-order decay kinetics with a decay rate r that is a complex function of the membrane potential.

Even when the drop of G_K has a marked voltage dependence, the stabilizing K^+ ions readily dissociate from the channels when the membrane is perfused with 0 K^+ solutions, regardless of the membrane potential, and therefore

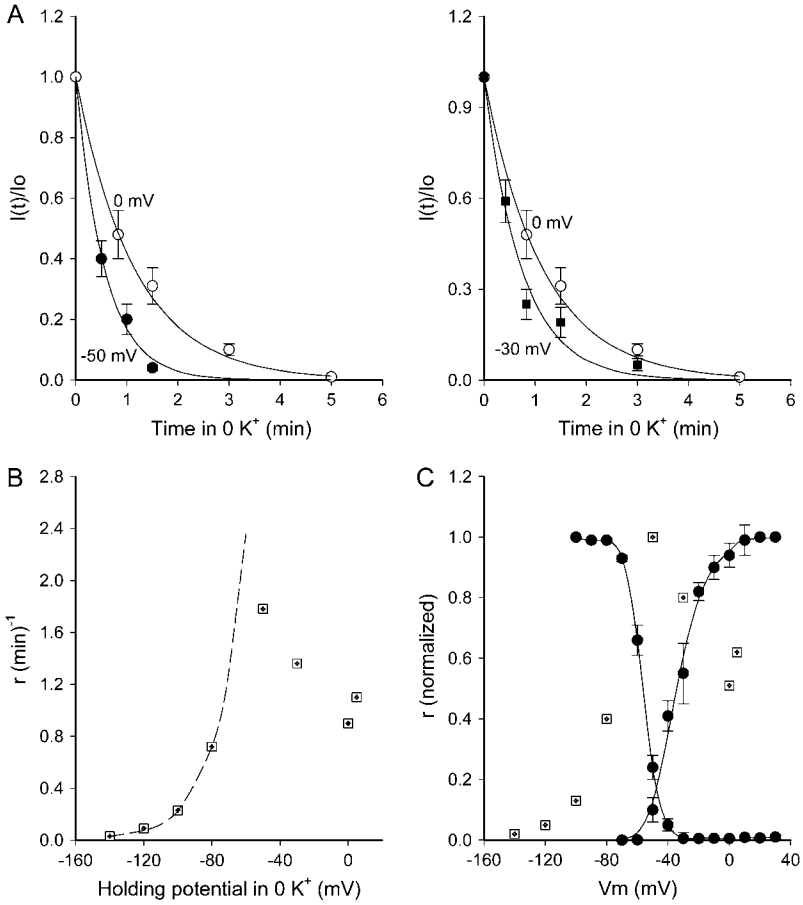

FIGURE 8 Collapse of G_K at depolarized membrane potentials ($V_m > -80$ mV). (*A*) Fraction of conducting channels ($I(t)/I_o$) as a function of the time spent in 0 K^+ (Na_o/Na_i) solutions at the indicated HPs. The lines are the fit of the points with exponential functions, as in Fig. 3 *A*, with rate constants: $r(-50) = 1.78$ min^{-1}, $r(-30) = 1.36$ min^{-1}, and $r(0) = 0.9$ min^{-1}. (*C*) *r* versus HP in the whole range of voltages studied. The dashed line is the plot of $r_n(V)$ (Fig. 3 *B*). (*D*) Comparison of the voltage dependence of *r* (*squares*) with the activation (G_{rel}), and the steady-state inactivation (h_∞) gating (*solid circles*). The values of *r* were normalized with their maximum experimental value ($r(-50)$). G_{rel} and h_∞ are the relationships in 0 K_o^+ solutions (Na_o/K_i) of Figs. 4 *B* and 7 *B*, respectively.

regardless of the associated gating state of the channels, and so the drop of G_K cannot be totally halted within the whole range of potentials that were studied.

The observations presented in this article agree with the hypothesized presence of one or more stabilizing K^+ sites located either within the pore itself or, as previously proposed, in the external vestibule of the pore, at a site that probably senses the conformational change associated with the slow inactivation of the channels (see below).

A comparison of the decay rate *r* with the rate of ion permeation through ion channels

Based on the trend observed at negative voltages, it was predicted that at 0 mV, the decay rate, *r*, should be equal to 86 min^{-1} (i.e., $r(0) = 1.4$ s^{-1}) and thus should produce a 50% drop of G_K after only ~0.5 s in 0 K^+. Despite this expected rapid drop of G_K, the predicted value of $r(0)$ (and even more so its observed, smaller, value) is very small compared with the rate of K^+ permeation through the pore of K^+ channels (~10^7 s^{-1}). Regarding the latter rate, it is thought that electrostatic repulsion among permeant ions is an important factor for achieving this elevated throughput rate of ions in multi-ion pores. Thus, it is expected that, in the absence of electrostatic repulsion forces, the last ion that leaves K^+ channels, in 0 K^+ solutions, would do so more slowly. In agreement with this view of permeation, by measuring the rate of entry into the C-type inactivated state, Baukrowitz and Yellen (25) estimated that the rate of exit of the last K^+ ion from the *Shaker* channel is 6.7×10^3 s^{-1}. This rate remains much larger than the expected (and therefore the observed) rate of decay $r(0)$ of *Shab* G_K.

This previous discussion suggests that the rate-limiting step in G_K collapse could be the conformational change that must follow the exit of the stabilizing K^+, rendering the channels unable to conduct. Therefore, it appears likely that, regardless of the voltage, the magnitude of *r* may reflect (approach) the rate of this conformational change. It would be interesting to determine whether the preceding K^+ dissociation step attains a value similar to that reported in the study of the *Shaker*.

Collapse of G_K at depolarized membrane potentials ($V_m > -80$ mV)

At depolarized potentials G_K also collapses in 0 K^+. The collapse occurs at all the depolarized voltages tested and stands in contrast with the behavior of *Shaker* channels in

which depolarized HPs (≥ -40 mV) preclude the drop of G_K (14).

In particular, for potentials above -50 mV, observations suggest that the stabilizing K^+ dissociates either from channels dwelling in the slow inactivated state or from the fraction of channels that may not be inactivated at steady state. Whatever the case, based on the trend observed at negative potentials, it appears that the development of inactivation greatly slows the decay of G_K.

Although the slow inactivation of *Shab* has not been studied in detail, it is well accepted that the extensively studied slow, C-type, inactivation of *Shaker* channels involves a constriction of the extracellular portion of the pore (26–28). However, and of importance to this work, this constriction is not sufficiently narrow to completely impede the movement of ions between the pore and the extracellular solution, as demonstrated by the ability of external Ba^{2+} to block the pore of the C-type inactivated *Shaker* channels (29).

Similarly, in $K_v1.3$ channels, addition of K^+ ions to the external solution increases the rate of recovery from inactivation even if the addition is made after the channels have been inactivated. This suggests that K^+ might be able to gain access to the pore of slowly inactivated channels (30).

Taking into account the previously mentioned examples and the results of this work, it appears reasonable to set forth the hypothesis that the slow inactivation of *Shab* channels does not completely block communication between the pore and the external solution.

Whatever the case, the results of this work qualitatively agree with the view that development of inactivation, at depolarized potentials, slows the G_K drop. Therefore, the variation of r with the membrane potential in the entire range of voltages studied was fitted with an equation that considered that: 1), the experimental value of $r(0)$ is a measure of the rate of decay of G_K from inactivated channels; and that 2), the voltage dependence of r at depolarized potentials is conferred by the voltage dependence of the probability of the channels being inactivated; and finally that 3), at hyperpolarized voltages, $r(V)$ is given by the empirically founded relation $r_n(V)$ (Fig. 3 *B*) times the probability of the channels not being inactivated, h_∞, as follows: $r(V) = r_n(V) \times h_\infty + r(0) \times (1 - h_\infty)$.

Fig. 9 presents the fit of r in the entire range of voltages studied with the above equation $r(V)$. The figure shows the plot of the equation with the h_∞ curve obtained with 0 K^+ (Na_o/K_i, Fig. 7 *B*) in the external solution ($V_{½} = -56$ mV, $z = 4.6$) (*dotted line*), together with the fit of the points obtained considering the parameters of the h_∞ relation as adjustable parameters (*solid line*). A visual evaluation shows that even with the parameters of h_∞ in Na_o/K_i the $r(V)$ equation affords a reasonably good fit of the points. The fit was much better when the parameters of h_∞ were adjusted (in this case $V_{½} = -59$ mV, $z = 3.0$). Note that these parameters are close to those of the h_∞ curve in Na_o/K_i.

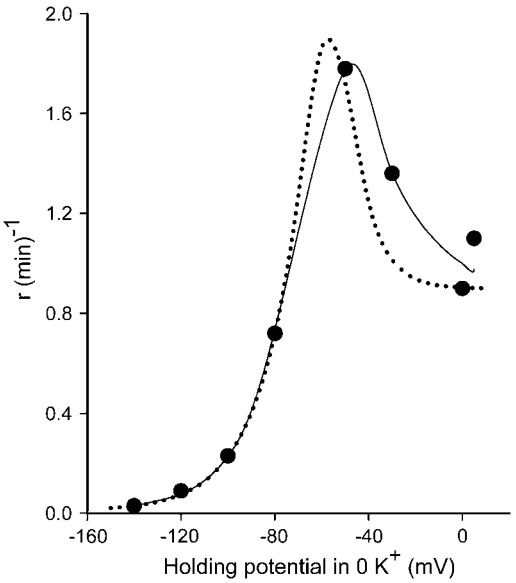

FIGURE 9 Voltage dependence of the decay rate constant r in the entire range of voltages studied. The points in the plot are the experimental values of r of Fig. 8 *C*. The lines are the plot of the equation $r(V) = r_n(V) \times h_\infty + r(0) \times (1 - h_\infty)$, where $r(0s) = 0.90\ min^{-1}$, $r_n(V) = 0.01 + 86exp(0.06V)$ is the variation of r at negative potentials obtained in Fig. 3 *B*; and h_∞ is the steady-state inactivation relation (see text). The dotted line is the plot of the equation with the parameters of h_∞ in Na_o/K_i of Fig. 7. The solid line is the fit of the points obtained considering the parameters of h_∞ as adjustable parameters ($V_{½} = -59$ mV, $z = 3.0$).

Their difference can be explained by hypothesizing that, as the internal [K^+] decreases, the h_∞ curve, in the corresponding Na_o/K_i solutions, should become less steep and slightly shifted toward the left along the voltage axis. Finally, the deviation of the fitted curve (*solid line*) to the points at the more depolarized potentials could likely arise from the contribution that the fraction of channels that may not be inactivated at steady sate (and that therefore collapse from the open state) do to the value of r.

Inhibition of the collapse of G_K by hyperpolarized membrane potentials ($V_m < -80$ mV)

On exposure to 0 K^+, G_K drops spontaneously while the HP is kept constant at -80 mV. An important result of this work lies in the finding that hyperpolarized potentials drastically inhibit the drop of G_K. The possibility that this phenomenon could be caused by a shift of the voltage-dependent activation of the channels toward negative potentials in 0 K^+ was shown to be unlikely with the demonstration that the G_K-V_m curve is quite insensitive to the [K^+] across the membrane.

On the other hand, the possibility that the behavior of G_K at negative potentials could be caused by a voltage-dependent leakage of K^+ ions toward the internal solution through the closed activation gate also seems unlikely

because 1), the facilitation that channel activation exerts on the drop of G_K is small; and 2), in the case of *Shaker*, it has been reported that the leak of ions that occurs through the closed activation gate is quite small and not voltage dependent (24).

Finally, the observation that channel activation from −140 mV facilitates the drop of G_K indicates that the voltage-dependent scant openings that take place at negative voltages may certainly contribute both to the passive drop of G_K and to the stabilization afforded by hyperpolarized potentials. How big is this contribution? Because a 5-ms pulse seems to drop ~1% of G_K, and a sixfold longer pulse of 30 ms should drop ~1.5% of G_K, and also because of the low probability of opening of K_v channels at negative potentials (20), it seems reasonable to conclude, in the absence of further information, that the scant openings at negative potentials may not explain the full extent of drop of G_K that is observed at negative potentials.

With the previous arguments in mind, the following hypothesis can be set forth: 1), G_K collapse at negative potentials likely occurs more frequently while the channels are closed than during their scant stochastic openings (although during the latter the drop of G_K is facilitated). Thus, 2), part of the inhibition of the G_K drop that occurs as the membrane is hyperpolarized may be caused by a voltage-dependent retention of the stabilizing K^+ ions in the channels, as the probability of opening becomes much smaller. Finally, 3), as the membrane potential is hyperpolarized, channels may adopt conformations, most likely conformations adopted by the selectivity filter itself, that are progressively more stable in the absence of K^+ (for example, the selectivity filter could accommodate Na^+ ions, thus gaining stability in 0 K^+) (ongoing research agrees with this proposal, F. Gómez-Lagunas, L. D. Islas, and M. Ambriz-Rivas, unpublished observations).

The latter hypothesis is rendered plausible by the presence of K^+ channels that are stable in 0 K^+ (see Introduction) and also by both experimental observations as well as molecular dynamics simulations of 2TM K^+ channels demonstrating that the pore of, at least some, K^+ channels is flexible enough to be even endowed with gating abilities (e.g., 31–35).

Which of the K^+ sites (S1-S4) that comprise the selectivity filter may adopt a conformation (or geometry) that allows the channels to withstand the removal of K^+ at hyperpolarized potentials? Although this question is not easy to answer, our previous work with *Shaker* channels, whose conductance collapses in 0 K^+ with voltage dependence reminiscent of that occurring in *Shab* (see below, 16), may offer a clue: The collapse of G_k is inhibited by scorpion toxins whose block of the pore is destabilized by external K^+ ions (36). The toxins present a conserved lysine, located at position 27 of charybdotoxin, that is thought to interact with the most external K^+ site of the selectivity filter (SF) (37,38). That is, it appears that the proper occupancy of only the most external K^+ site of the SF is needed to prevent the collapse of the *Shaker* G_K. Therefore, it can be speculated that only the first K^+ site of the *Shab* SF would need to undergo a suitable change for the conductance to be stable at hyperpolarized potentials.

The voltage dependence of r at negative potentials may proceed from a number of factors such as voltage-dependent transitions of the channels as they redistribute among closed states, reorientation of the ions within the pore, and the previously mentioned hypothetical conformational change that stabilizes the conductance. Regarding the possible reorientation of ions within the pore, it has been shown that this can contribute to the voltage-dependent gating of some channels, such as ClC-0 channels, in which it produces an apparent gating charge of ~1 (39). Further work is required to understand the marked voltage dependence of the drop of the *Shab* G_K at negative potentials.

Comparison with other K^+ channels

K^+ removal exerts variable effects on different K^+ channels. It has been proposed that the latter is the consequence of differences in the K^+ affinity of the pore of the different channels (e.g., 7,40,41). However, the variable responses that the channels exhibit on K^+ removal seem too complex to be explained solely by differences in its K^+ affinity, as the following comparison suggests.

Shab behavior on exposure to 0 K^+ differs in many aspects from that of *Shaker* B; for example: 1), *Shaker* channels remain stable if they are kept undisturbed at the HP during exposure to 0 K^+, whereas under the same conditions *Shab* G_K undergoes an exponential decay; 2), the *Shaker* G_K collapses only when the channels are gated by the delivery of activating pulses; 3), in *Shaker* the collapse of G_K is fully reversible, whereas in *Shab* it is irreversible; 4), depolarized HPs impede the *Shaker* G_K drop, whereas at these potentials the *Shab* G_K falls even faster than at negative potentials; and 5) it appears that in *Shaker* the collapse of G_K occurs during the deactivation of the channels (14–18,36,42), whereas in *Shab*, G_K decays in 0 K^+ regardless of the gating state of the channels (although inactivation appears to slow the rate of decay, whereas the delivery of activating pulses facilitates it).

Therefore, it would be expected that mutations abolishing the slow inactivation of *Shab* would speed the decay of G_K at depolarized potentials. In contrast, in the case of *Shaker* it has been shown that mutations at position 449, located in the extracellular vestibule of the pore, that prevent C-type inactivation, as T449Y and T449V (27), also prevent the collapse of G_K (16).

Shab channels present a cysteine at the *Shaker* 449 position, and *Shab* slow inactivation presents certain properties that differ from those that are characteristic of the typical C-type *Shaker* inactivation (work in progress). Thus, it would be interesting to determine whether some of the differences in the collapse of the conductance of these

channels could be accounted for by the different residues that they present at this critical position.

Another residue that might play a role in the collapse of *Shab* G_K is a cysteine located at position 463, in the S6 helix, because *Shaker* channels present an alanine at this position, and the mutation A463C speeds the rate of C-type inactivation, and it appears that this prevents the collapse of G_K (43; see also Discussion of Ambriz-Rivas et al. (18)).

Finally, it is important to mention that Armstrong's laboratory shows that the extent of reduction of the *Shaker* G_K produced by each pulse applied in 0 K^+ is greater when the membrane is repolarized to intermediate potentials, at pulse end. The latter was explained by means of the hypothesis that closing to intermediate closed states facilitates G_K collapse (16). This behavior is reminiscent of the observation that the rate of decay r of *Shab* is bigger at intermediate HPs (-30 and -50 mV), although in this case this maximum is not related to the deactivation gating of the channels.

Further work is required to understand the molecular and mechanistic bases of the differential response of *Shab* and *Shaker* to K^+ removal, although, at this point, their differences appear too complex to be explained solely by a difference in K^+ affinity for its respective pores.

Regarding the comparative behavior of KcsA and *Shab* channels under low- or 0 K^+ conditions, it has been reported that the pore of KcsA adopts a nonconducting, collapsed, reversible conformation in 3 mM K^+ (12), whereas it was shown herein that *Shab* channels conduct well with only 2 mM K_o^+ in the recording solutions. Further work is needed to determine whether this particular difference is the result of a differential K^+ affinity for these pores. On the other hand, in agreement with the stabilizing effect of K^+ on *Shab* G_K, it has been shown that the absence of K^+ undermines the thermal stability of the KcsA tetramer (13).

Finally, the behavior of *Shab* on exposure to 0 K^+ solutions seems to be similar to that reported in the pioneering studies of Armstrong's laboratory with the squid K^+ channel. This similarity lies in that in the latter the stabilizing K^+ also leaves the channels, whereas they are closed at the HP, and in that the G_K drop is also irreversible (5,44). It would be interesting to determine whether the decay of the squid K^+ channel activity presents a voltage dependence similar to the one reported in this work.

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