Shaker B K^+ conductance in Na^+ solutions lacking K^+ ions: a remarkably stable non-conducting state produced by membrane depolarizations

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1. *Shaker* B K^+ channels, expressed in the insect cell line Sf9, were studied in zero K^+, Na^+ or *N*-methyl-D-glucamine (NMG)-containing solutions. In the absence of K^+ ions on both sides of the membrane, the K^+ conductance collapsed with the delivery of short depolarizing pulses that activated the channels. The collapse of the conductance was fully prevented when the channels were kept closed at a holding potential of −80 mV.
2. The fall in K^+ conductance had the notable characteristic of being strikingly stable. At −80 mV or more negative holding potentials, the conductance never recovered (cells observed for up to 1 h).
3. The extent of collapse of the K^+ conductance depended on the number of depolarizing activating pulses applied in zero K^+ solutions. For moderate to low frequencies of pulsing (1 to 0·002 Hz), the extent of the collapse did not depend on the frequency.
4. K^+, Rb^+, Cs^+ and NH_4^+ added to the external Na^+ solution impeded the fall in K^+ conductance.
5. TEA added to the external, zero K^+, Na^+-containing solution also precluded the fall of the conductance. The protection by TEA paralleled its block of the outward K^+ currents recorded with standard recording solutions.
6. The fall in K^+ conductance was prevented by depolarized holding potentials.
7. The K^+ conductance that was thought to be irreversibly lost at −80 mV or more negative holding potentials was fully recovered, however, after a prolonged (tens of seconds to minutes) change in the holding potential to depolarized values (above −50 mV). Full recovery could be obtained at any time after the former halt of the conductance.

Potassium channels form a highly diverse group, with a large variety of gating mechanisms and kinetics, but having in common the ability to sharply select K^+ over Na^+ ions (Hille, 1992).

In addition to this K^+ selectivity, many kinds of voltage-gated potassium channels are variably modulated by K^+ ions. For example, increasing the external K^+ concentration slows the rate of closing (deactivation) of K^+ channels. This inhibitory effect is thought to be exerted by the binding of K^+ to a site located in the pore of the channels, and it is known as the occupancy hypothesis (Swenson & Armstrong, 1981; Armstrong & Matteson, 1986; Matteson & Swenson, 1986).

Other gating states are also modulated by extracellular K^+ ions. For example, in RCK4 and some mutant *Shaker* channels, after removal of the external K^+, the outward K^+ current vanishes. This effect is reversed by the addition of K^+, and is related to the rate of slow inactivation of the channels (Labarca & MacKinnon, 1992; Pardo *et al.* 1992; López-Barneo, Hoshi, Heinemann & Aldrich, 1993). In contrast, other K^+ channels are tolerant to these changes; with standard high-K^+ internal solutions, *Shaker* B is quite insensitive to changes in the external K^+, and stable outward K^+ currents can be routinely recorded in external solutions that lack K^+ ions (López-Barneo *et al.* 1993; Gómez-Lagunas & Armstrong, 1994; and this study).

Total removal of K^+ ions, i.e. from both the internal and the external solutions, also exerts major effects on some K^+ channels. For example, the conductance of the delayed rectifier K^+ channel of the squid giant axon undergoes a spontaneous and irreversible run-down (Almers & Armstrong, 1980). In contrast, it has recently been shown that, after the total removal of K^+, some mammalian delayed rectifier K^+ channels remain stable and even conduct Na^+ ions (Zhu & Ikeda, 1993; Callahan & Korn, 1994; Korn & Ikeda, 1995).

The results given above indicate that K^+ channels differ greatly in their behaviour in the absence of K^+ ions, and provided the motive for this work.

It is shown here that the *Shaker* B K^+ conductance collapses in Na^+-containing solutions that lack K^+ ions, when the channels go through repeated gating cycles, but it remains unchanged if the channels are kept closed at the holding potential of −80 mV. The fall in conductance is not reversed by simply adding K^+ to the external solution. Moreover, at −80 mV or hyperpolarized holding potentials, the conductance never recovers (cells were observed for up to 1 h). However, the conductance recovers completely after long-lasting (seconds to minutes) depolarizations. The results suggest that gating the channels in zero K^+ leads to a strikingly stable, closed (non-inactivated) conformation of the channels.

METHODS

Cell culture and *Shaker* B channel expression

The insect cell line Sf9, from the army worm caterpillar *Spodoptera frugiperda*, was kept in culture at 27 °C in Graces' media (Gibco BRL). The cells were transfected by infection with the recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus containing the cDNA of *Shaker* B K^+ channels, as previously reported (Klaiber, Williams, Roberts, Papazian, Jan & Miller, 1990; Gómez-Lagunas & Armstrong, 1994). After infection the cells were kept at 27 °C and were used 2 days later for the experiments. Experiments were carried out at room temperature (20–25 °C).

The recombinant virus was kindly provided by Dr C. M. Armstrong (Department of Physiology, University of Pennsylvania, Philadelphia, PA, USA).

Electrophysiological recording

Macroscopic currents were recorded under whole-cell patch clamp (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with an Axopatch-1D connected to a PC 486 computer through a TL2 interface (Axon Instruments, Inc.). The currents were sampled at 100 μs per point, and low-pass filtered at 5 kHz, with the 4-pole Bessel filter built into the amplifier. Except where explicitly indicated, the holding potential was −80 mV. Leak conductance was substracted with a $P/-4$ pulse protocol. Electrodes were pulled from borosilicate glass (KIMAX 51) to a 1·3–2 MΩ resistance and used without any further treatment. The average series resistance was 4·5 ± 0·3 MΩ (range 2·2–9·0 MΩ). About 80% of the series resistance was electronically compensated.

Solutions

Solutions will be named by their main monovalent cation, and will be represented as external–internal, e.g. K_o^+–Na_i^+. Na_i^+ solution was composed of (mM): 90 NaF, 30 NaCl, 2 $MgCl_2$, 10 EGTA and 10 Na-Hepes; pH 7·2. K_i^+ solution was composed of (mM): 90 KF, 30 KCl, 2 $MgCl_2$, 10 EGTA and 10 K-Hepes; pH 7·2. K_o^+ solution (control) was composed of (mM): 100 KCl, 15 NaCl, 10 $CaCl_2$ and 10 Na-Mes; pH 6·4. Na_o^+ solution was composed of (mM): 115 NaCl, 10 $CaCl_2$ and 10 Na-Mes at pH 6·4; or 115 NaCl, 10 $CaCl_2$ and 10 Na-Hepes at pH 7·1. (An external pH of 6·4 is commonly used for Sf9 cells. However, to exclude any possible effect of the external pH, the Na_o^+ solution was assayed at both pH 6·4 and 7·1. No differences were observed, therefore the Na^+ external solution will be referred to as Na_o^+, without any reference to the pH.) NMG_o (*N*-methyl-D-glucamine) solution contained (mM): 115 NMG, 10 $CaCl_2$ and 10 Hepes-TMA (tetramethylammonium); pH 7·1. NMG_i solution contained (mM): 120 NMG, 90 HF, 2 $MgCl_2$, 10 EGTA and 10 Hepes-TMA; pH 7·2.

All other external solutions were prepared from the Na_o^+ solution, by replacing an equiosmolar amount of NaCl with the chloride salt of the test cation. All the chemicals were of analytical grade.

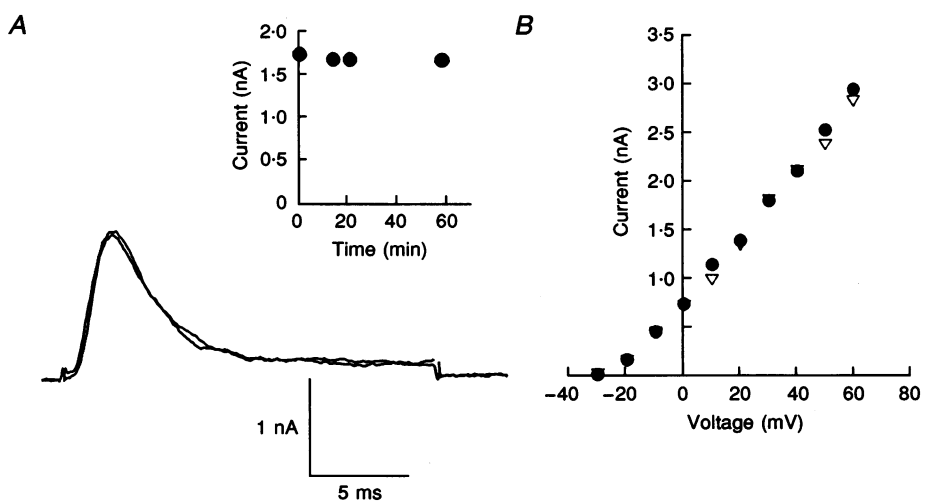

Figure 1. K^+ currents in zero and low external K^+

A, K^+ currents elicited by 30 ms pulses to +20 mV in Na_o^+–K_i^+ (see Methods). One of the currents was recorded shortly after the establishment of the whole-cell configuration, and the other 1 h later. The inset shows the peak current at +20 mV as a function of the time of recording in Na_o^+–K_i^+. *B*, peak current as a function of the voltage, with zero (●) and 2·5 mM K^+ (▽) in the external Na_o^+ solution. The currents were elicited by 30 ms pulses to the indicated voltages, delivered at a rate of one pulse every 10 s. Holding potential (V_h), −80 mV.

In some experiments the N-type inactivation was removed by adding the proteolytic enzyme papain (Sigma) to the Na_i^+ solution at a concentration of 0·1 mg ml^{-1}, as previously reported (Gómez-Lagunas & Armstrong, 1995).

Total perfusion of the external solution was accomplished in a maximum of 20 s. In all cases, however, the solution was perfused for 45 s before the delivery of the first voltage pulse. A total of ninety-three cells were observed for this study.

RESULTS

Shaker B K^+ currents in the absence of extracellular K^+

Some K^+ channels give stable outward currents only when there are permeant ions in the external solution (see Introduction). Therefore in order to study the behaviour of *Shaker*B channels in the absence of K^+ ions on both sides of the membrane, it is first convenient to look briefly at how the channels respond both to the absence and to small millimolar additions of K^+ to the external solution. Figure 1*A* shows that in the absence of extracellular K^+ (not added), the *Shaker* B K^+ conductance is stable. The figure compares two K^+ currents at +20 mV recorded in Na_o^+–K_i^+ solutions (see Methods), one taken shortly after the establishment of the whole-cell configuration, and the other 1 h later. The currents superimpose well. The inset shows the peak current at +20 mV as a function of the time of recording. The size of the current was constant for more than 1 h.

The amplitude of the outward K^+ currents is insensitive to relatively small changes in the external K^+ concentration, when the frequency of pulsing is low enough to allow a complete recovery from inactivation (Gómez-Lagunas & Armstrong, 1994). This is shown in Fig. 1*B*, which compares

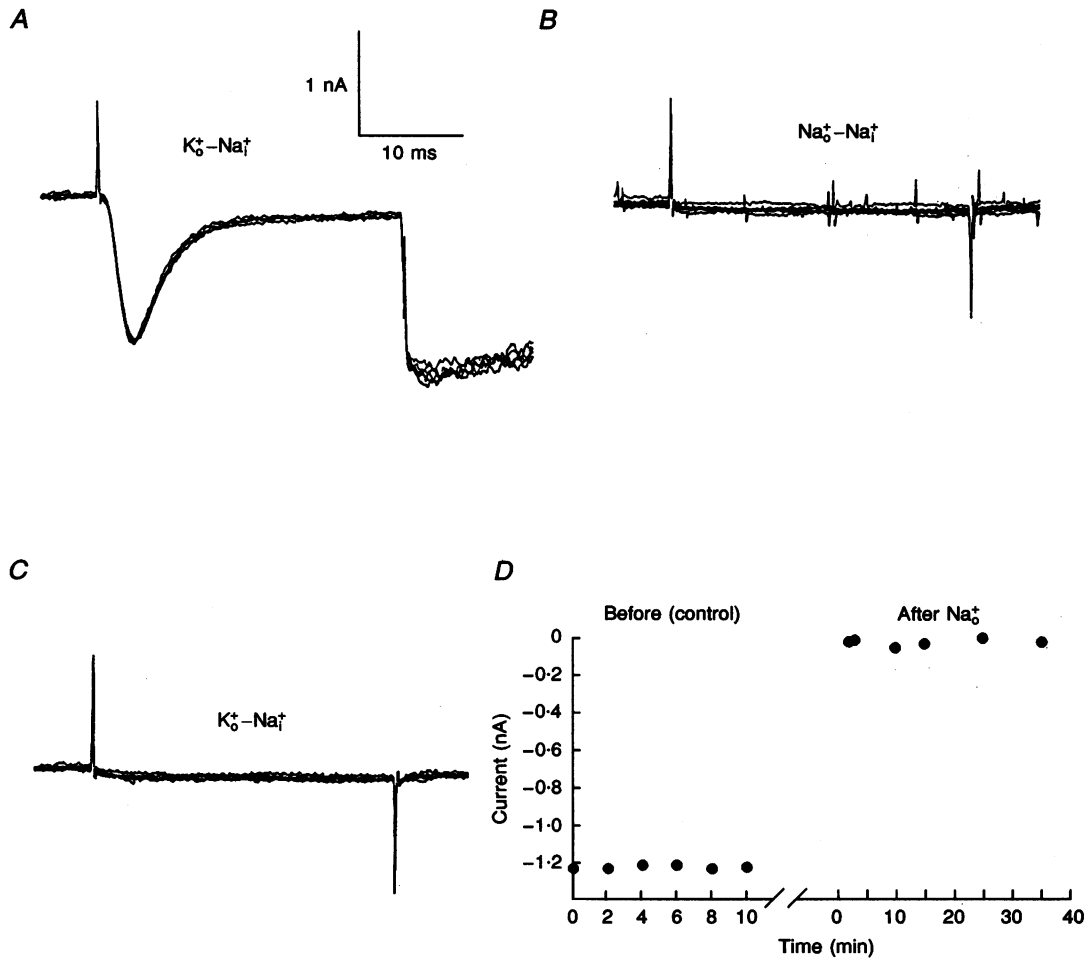

Figure 2. Delivery of ten depolarizing pulses in zero K^+, Na^+ solutions collapses the *Shaker* K^+ conductance

A, control, inward K^+ currents evoked by 30 ms pulses to +20 mV delivered at a rate of one pulse every 2 min over a period of 10 min, in K_o^+–Na_i^+. The inward slow tails mark the end of the pulse. *B*, currents evoked by ten 30 ms pulses to +20 mV delivered at a rate of 1 pulse s^{-1} in Na_o^+–Na_i^+. *C*, currents elicited by 30 ms pulses to +20 mV in K_o^+–Na_i^+, after the ten pulses in Na_o^+–Na_i^+. The first pulse was applied 45 s after the start of the perfusion with the K_o^+ solution; the next pulses were delivered at varying intervals over the next 35 min. *D*, peak current at +20 mV as a function of the time of recording in K_o^+–Na_i^+ before (left), and after (right) the ten pulses in Na_o^+. V_h, −80 mV.

the peak current *vs.* voltage curve (I–V) in 0 (Na_o^+) and 2·5 mM external K^+. The channels were activated by 30 ms pulses of the indicated amplitude, delivered at a rate of one pulse every 10 s, from the holding potential (V_h) of −80 mV. The amplitude of the currents is the same (for the effect of K^+ at other frequencies, see the detailed study of Baukrowitz & Yellen, 1995).

The results in Fig. 1 show that *Shaker* B channels differ from other K^+ channels that require millimolar [K^+] in the external solution, in order to yield stable currents that do not fall into a slow inactivated state (see Introduction). In those cases, the addition of millimolar K^+ to the extracellular solution increases the outward current, despite the reduction of the driving force for K^+ (Pardo *et al.* 1992; López-Barneo *et al.* 1993). This effect is more pronounced in those channels that have relatively fast kinetics of slow inactivation. In fact, addition of K^+ to NMG external solutions has been reported to produce a slight increase (less than 20%) of the peak outward current through *Shaker* B channels (López-Barneo *et al.* 1993). This effect is probably due to the increased rate of C-type inactivation in NMG compared with that in Na^+ (see Fig. 1 of López-Barneo *et al.* 1993), and it is not observed when K^+ ions are added to Na^+ external solutions and the frequency of pulsing is low (Fig. 1*B*).

The *Shaker* B K^+ conductance collapses in the absence of K^+ ions when the membrane is briefly depolarized

With the aim of looking at the behaviour of the channels in the absence of K^+ ions on both sides of the membrane, the currents were recorded with a pipette filling solution in which K^+ ions were replaced by Na^+ (Na_i^+), and the current was monitored in both K^+ (K_o^+) and Na^+ (Na_o^+) external solutions.

Figure 2*A* shows inward K^+ currents recorded with the Na^+ internal solution (Na_i^+) and 100 mM K^+ in the external solution (K_o^+–Na_i^+), evoked by 30 ms pulses to +20 mV delivered at a rate of one pulse every 2 min, from a V_h of −80 mV. The currents show the normal kinetics of *Shaker* B. The inward slow tail currents mark the end of the pulse. These tails are caused by the reopening of the channels from the fast or N-type inactivated state, as the channels recover from inactivation (Demo & Yellen, 1991; Ruppersberg, Frank, Pongs & Stocker, 1991). Note that the currents recorded over a period of 10 min are totally stable. In fact, under these conditions the currents are stable for hours (not shown, but apparent from the results below).

Once the stability of the current was established, the cell was changed (see Methods) to a Na^+ external solution (Na_o^+–Na_i^+) and ten 30 ms pulses to +20 mV were applied from the V_h of −80 mV, at a rate of one pulse per second (i.e. covering a period of only 10 s) with the Na_o^+ solution flowing. The traces in Fig. 2*B* show that, in the absence of K^+, *Shaker* B does not conduct sodium (the calculated Nernst potential for Na^+ is +2 mV); the spikes in the traces were due to the perfusion with Na_o^+ solution.

Finally, immediately after the delivery of the ten pulses, the cell was brought back to the K_o^+–Na_i^+ solution. Forty-five seconds after the start of the perfusion, 30 ms pulses to +20 mV were applied from −80 mV, at varying intervals over a period of 35 min, in an attempt to record again the current through the channels. The traces in Fig. 2*C* show that the current experienced a dramatic reduction (98%) compared with the control in Fig. 2*A*. More importantly, over the next 35 min there was no sign of recovery. This last

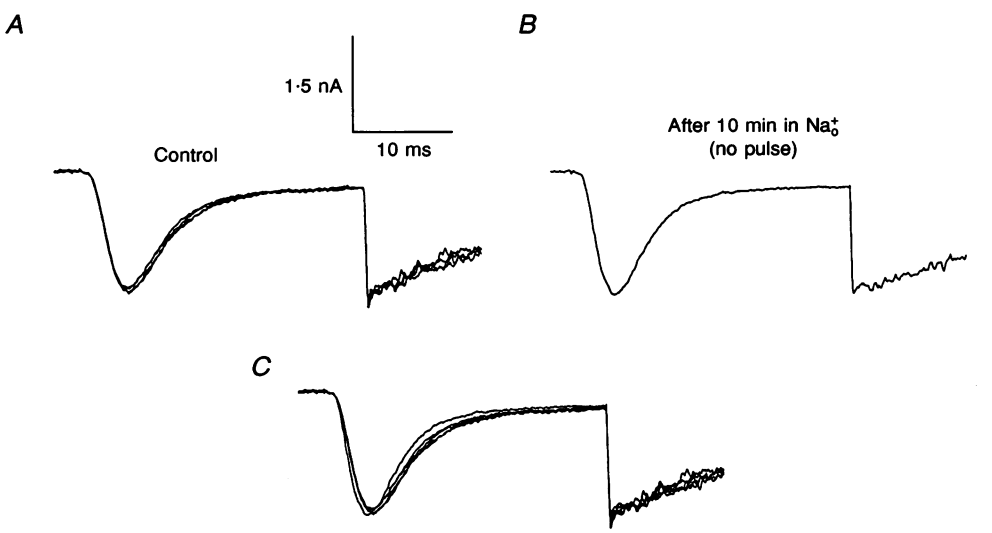

Figure 3. Keeping the channels closed in zero K^+, Na^+ solutions fully prevents the collapse of the conductance

A, control K^+ currents evoked by 30 ms test pulses to +20 mV delivered at a rate of one pulse every 2 min, over a period of 10 min, in K_o^+–Na_i^+. *B*, K^+ current evoked by a test pulse in the K_o^+–Na_i^+ control solutions, applied 45 s after the start of the perfusion with the K_o^+ solution. The cell had previously been immersed in the test Na_o^+–Na_i^+ solutions for 10 min, with the membrane potential constant at −80 mV (not shown). *C*, the traces in *A* and *B* are displayed together.

feature is better seen in Fig. 2*D*, where the peak current at +20 mV, in K_o^+–Na_i^+, is plotted before (Fig. 2*A*) and after (Fig. 2*C*) the ten pulses in Na_o^+–Na_i^+.

The lack of any recovery over the 35 min shown in Fig. 2*D* cannot be easily conceived to be the result of inactivation (the current did not recover at all in any of the ninety-three cells used for this study; most of them were observed for at least 30 min, and ten for 1 h). Moreover, changing the V_h to a hyperpolarized potential (−130 mV) did not increase the current (not shown).

In summary, the K^+ conductance, stable in the absence of only either external or internal K^+ (Figs 1 and 2*A*), was drastically reduced in Na_o^+–Na_i^+ with the delivery of ten depolarizing pulses.

Is the fall in conductance the result of a spontaneous run-down? In order to address this question, the channels were exposed to the Na_o^+–Na_i^+ solutions without applying any pulse, i.e. with the membrane potential kept constant at −80 mV. Furthermore, the time of exposure to the Na^+ solutions was lengthened to 10 min as in the control, instead of only 10 s (as in Fig. 2*B*). The results of a representative experiment are shown in Fig. 3.

Figure 3*A* shows five control K^+ currents in K_o^+–Na_i^+, evoked by 30 ms pulses to +20 mV delivered every 2 min. Having checked the stability of the currents, the cell was superfused with the Na_o^+ solution for 10 min, keeping the membrane potential constant at −80 mV (not shown). Finally, the cell was brought back to K_o^+–Na_i^+ and, 45 s later, a 30 ms pulse to +20 mV was applied to test the state of the channels. Figure 3*B* demonstrates that the channels were now as able to conduct K^+ as they were during the control. In Fig. 3*C*, the currents from Fig. 3*A* and *B* are displayed together. The currents are basically identical. The same result was observed in all the cells that were tested in this way; the ratio of the peak currents (I/I_o) before (I_o) and after (I) the exposure to zero K^+ was 0·998 ± 0·01 ($n = 4$).

Therefore, placing the channels in zero K^+, Na^+ solutions, for at least 10 min, does not affect their ability to conduct K^+ as long as the channels remain closed. The fall in K^+ conductance (Fig. 2) is the result of the repeated depolarizations in zero K^+, and this is studied further below.

Figure 4*A* shows K^+ currents before (C) and after (A) the application of a single 30 ms pulse to +20 mV in Na_o^+–Na_i^+. The K^+ conductance was reduced, although not as much as

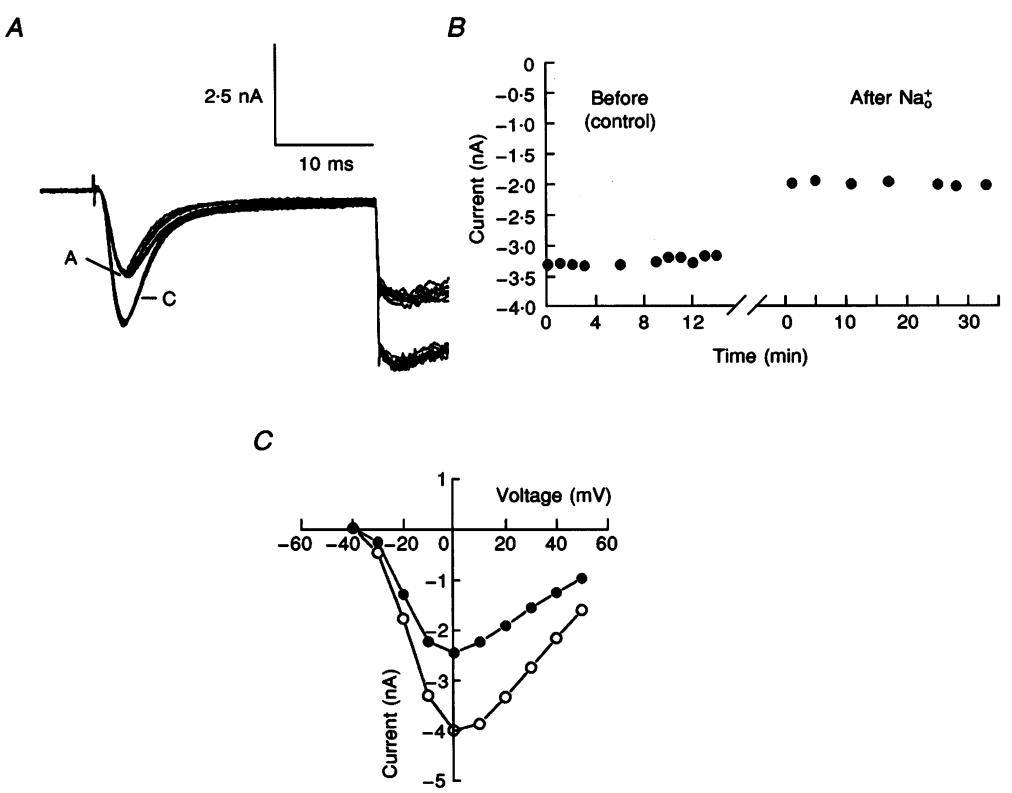

Figure 4. Delivery of only one 30 ms depolarizing pulse in zero K^+ solutions reduces the K^+ conductance

A, superposed K^+ currents at +20 mV recorded in K_o^+–Na_i^+ before (C; control) and after (A) the delivery of one 30 ms pulse to +20 mV in Na_o^+–Na_i^+ (not shown). *B*, peak current at +20 mV as a function of the time of recording, before (left) and after (right) the delivery of the single pulse in zero K^+. The average amplitude of the currents after the pulse in zero K^+ is 39% of the ones in the control. *C*, *I–V* relationship in the control (○), and after the pulse in Na_o^+ (●). V_h, −80 mV.

in Fig. 2, and again the currents showed no sign of either recovery or further decrement over the next 40 min. This is seen best in Fig. 4*B* where the peak current at +20 mV is plotted against the time of the recording, before and after the pulse in zero K^+. Figure 4*C* shows the *I–V* relationship of both the control and the remaining currents in Fig. 4*A*. In both cases the channels start to activate between −40 and −30 mV, and the current peaks at 0 mV, i.e. the reduction of the current was not due to a shift in the voltage dependence of the channels.

Figures 2 and 4 indicate that the effect of repeated depolarizations is cumulative. This is illustrated further in Fig. 5*A*, which shows the extent of reduction of the K^+ conductance as a function of the number of depolarizing pulses. Except for $n = 15$ pulses, which were applied only at 1 Hz, all the other pulses were applied both evenly spaced over 10 s (i.e. 10 pulses at 1 pulse s^{-1}, etc.), and over 10 min (i.e. 10 pulses at 1 pulse min^{-1}, etc.), i.e. the effect of the same number of pulses applied both at moderate and low frequency is compared. It is clearly seen that the extent of reduction of the K^+ conductance depends on the number of pulses, but not on the frequency of pulsing, in the range of moderate to low frequencies tested (1 to 0·002 Hz).

The above results suggest that the collapse of the K^+ conductance is not the result of inactivation, as it is well known that inactivation is frequency dependent.

The amplitude of the test pulses (+20 mV) was chosen because this voltage fully activates the channels and elicits currents of a convenient amplitude. Figure 5*B* illustrates the fall in conductance as a function of the amplitude of the pulses. In order to have a reference, the steady-state activation of the channels, in K_o^+–Na_i^+, was also plotted. This was evaluated as the relative amplitude (I/I_{max}) of the inward deactivation tails at −80 mV, following the activation of the channels by 30 ms pulses of the indicated amplitude, in a cell where the N-type inactivation had been previously removed by the addition of the proteolytic enzyme papain to the internal Na_i^+ solution (not shown; see Gómez-Lagunas & Armstrong, 1995). The extent of reduction of the K^+ conductance has a voltage dependence parallel to that of the activation of the channels. This suggests that it is the activation of the channels, or an associated gating step (and not just the membrane depolarization), that depresses the conductance in the absence of K^+.

As inactivation is coupled to the activation of the channels, the role of inactivation was next explored by pulsing in Na^+ (as in Fig. 2), but this time from a depolarized holding potential, to keep the channels inactivated. This was first done with the wild-type *Shaker* B channel that has both an N- and a C-type inactivation (Hoshi, Zagotta & Aldrich, 1991). The results of these experiments are shown in Fig. 6.

Figure 6*A* shows a control current in K_o^+–Na_i^+, evoked by a test pulse from a V_h of −80 mV. Next, the V_h was changed

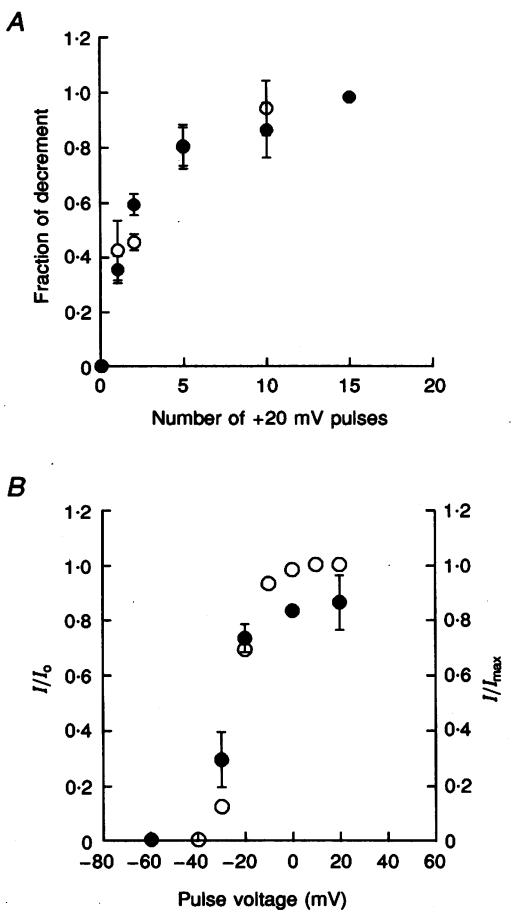

Figure 5. Reduction of the K^+ conductance as a function of the number and amplitude of the pulses delivered in zero K^+

A, current reduction after the delivery of the indicated number of 30 ms pulses to +20 mV in Na_o^+–Na_i^+ (as in Fig. 2), for pulses evenly applied over 10 s (●), and 10 min (○). The only exception is the point for fifteen pulses that were only tested at 1 Hz. The points are the means ± S.E.M. of at least four experiments. *B*, K^+ conductance reduction as a function of the voltage during the test pulses. The left vertical axis shows the ratio of the current at +20 mV in K_o^+–Na_i^+, before (I_o) and after (I) the delivery of ten 30 ms pulses of the indicated amplitude in Na_o^+–K_i^+ (●). The points are the means ± S.E.M. of at least three experiments. The right vertical axis shows the steady-state activation of the channels, determined as the relative amplitude of the inward, deactivation, tail currents at −80 mV in K_o^+–Na_i^+, following 30 ms pulses of the indicated amplitude (○), in a cell where the N-type inactivation of the channels had been previously removed by the addition of papain (0·1 mg ml^{-1}) to the Na_i^+ solution (not shown). The amplitude of the tails was measured 900 μs after the end of the activating pulse. V_h, −80 mV.

to −20 mV, and 1 min later a test pulse was applied to check the state of the channels (Fig. 6*B*). Only the leak current is present (in this case the *P*/−4 protocol was not used). Afterwards, the cell was changed to the Na_o^+–Na_i^+ solutions, with V_h held constant at −20 mV, and ten test pulses were delivered (without the *P*/−4 protocol) at a rate of 1 pulse s^{-1} (not shown). After that, the cell was brought back to the control solutions. Then V_h was changed back to −80 mV, and 1 min later a test pulse was delivered to determine the state of the channels. Figure 6*C* shows that the conductance did not collapse. In Fig. 6*D*, the currents in Fig. 6*A* and *C* are plotted together. The current was not reduced at all. The same results were observed when the channels were inactivated in the Na_o^+–Na_i^+ solutions, before the delivery of the test pulses (not shown).

The above observation could indicate that the conductance is protected either by the inactivation of the channels or by a depolarized V_h. To discriminate between these possibilities, other experiments were conducted from a variety of holding potentials. The results are presented in Fig. 6*E*, which shows the ratio of the current at +20 mV (I/I_o), before (I_o) and after (I) the delivery of ten test pulses in Na^+, from the indicated V_h. Even a slightly depolarized V_h (−50 mV) fully protects against the effect of pulsing in zero K^+. Although it is not possible to evaluate the steady-state inactivation of the channels without permeant ions, the results in Fig. 6*E* suggest that it is the depolarized V_h and not the associated inactivation that protects the K^+ conductance.

The above conclusion could seem surprising at first, given that it is through the delivery of depolarizations (short

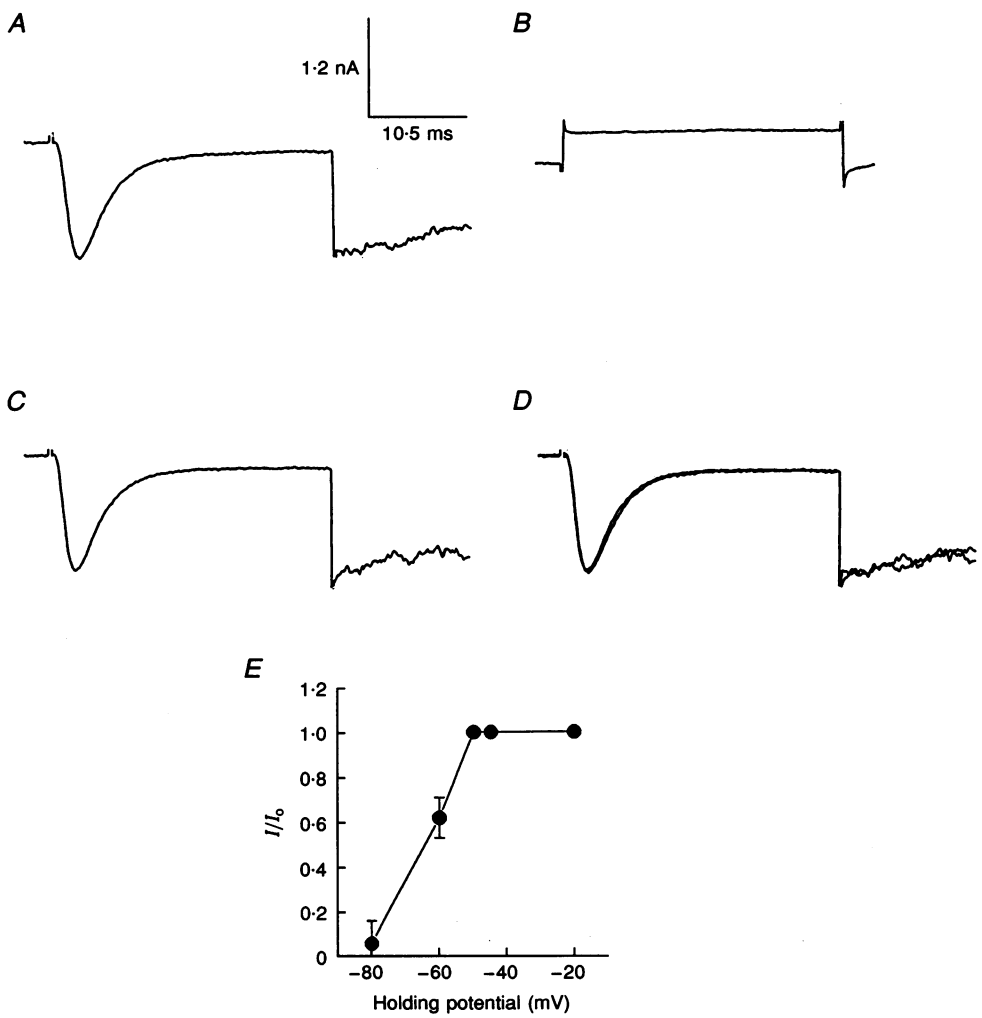

Figure 6. K^+ conductance reduction as a function of the holding potential

A, control current at +20 mV in K_o^+–Na_i^+. V_h, −80 mV. *B*, current evoked by a 30 ms pulse to +20 mV from a V_h of −20 mV, in K_o^+–Na_i^+. There is no time-dependent current because all the channels are inactivated. The leak current was not substracted. *C*, current at +20 mV in K_o^+–Na_i^+, recorded after the delivery of ten 30 ms pulses to +20 mV from −20 mV in Na_o^+–Na_i^+ (not shown). V_h, −80 mV. *D*, the currents in *A* and *C* are displayed together. *E*, ratio of the currents at +20 mV before (I_o) and after (I) the delivery of ten 30 ms pulses to +20 mV in Na_o^+–Na_i^+ from the indicated holding potentials, determined as illustrated in *A*–*D*.

depolarizations from −80 mV) that the channels become unable to conduct. However, the states that the channels can go through with short depolarizations are different from those with sustained depolarizations (a change in the V_h). For example, the last condition is expected to slow or even impede the closing of the channels, whether they are inactivated or not, given that recovery from both forms of inactivation ends when the channels are finally closed.

This interpretation is in accordance with the lack of effect of the frequency of pulsing (Fig. 5*A*). If inactivation (either N-type, C-type or both) was protecting, then the collapse of the K^+ conductance should depend on the frequency of pulsing, in a way that would reflect the time course of recovery from inactivation. However, regardless of the mechanism, the experiments in Fig. 6 further suggest that inactivation (either N- or C-type) is not the cause of the reduction of the K^+ conductance.

At this point it was of interest to know which other conditions could also prevent the fall in conductance: the next section deals with this problem.

Monovalent cations that either permeate or block prevent the fall in conductance

In order to determine the ion content of the external solution needed to protect the conductance, K^+, Rb^+, Cs^+ and NH_4^+ were added to the Na_o^+ solution, and the fall in the conductance was assayed as in Fig. 2. The results are shown in Fig. 7*A*, where the ratio of the currents at +20 mV, before (I_o) and after (I) the delivery of ten test pulses, is plotted against the concentration of the cations. The reduction of the current decreases as the concentration of the cations increases, following a Langmuir adsorption isotherm. This is best seen in the inset of Fig. 7*A*, which shows the double reciprocal plot of the main figure. Notably, the affinity constant (K_d) is basically the same for all the ions (K^+, 2·85 mM; Cs^+, 2·22 mM; NH_4^+, 3·50 mM). Rb^+ was tested only at 10 mM.

As the equal potency with which the ions protect does not correlate with the selectivity of the channels, it could be that the site to which the cations bind is located towards the external side of the pore.

It is pertinent to point out that the potency with which the cations in Fig. 7*A* protect is different from that with which they affect C-inactivation (López-Barneo *et al.* 1993), thus although the ionic conditions were not totally equivalent, this suggests that the two effects might not be related.

It was of interest to see if other bigger ions, known to interact with the external mouth of the channels, could also protect the conductance. Figure 7*B* shows the effect of adding TEA to the external solution; the figure also shows the block by TEA at 0 mV, assayed in a cell bathed in

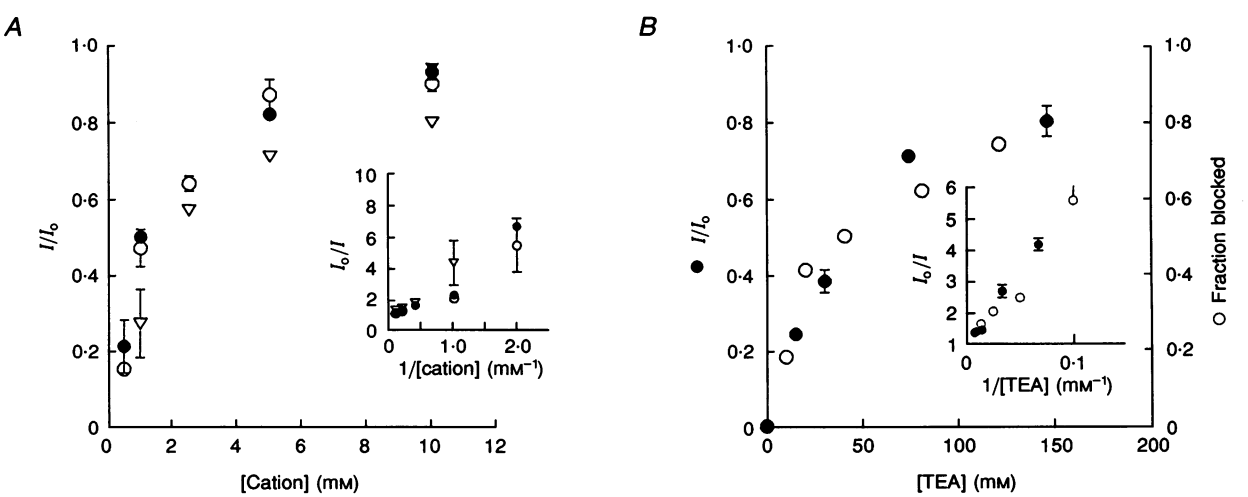

Figure 7. Monovalent cations that either permeate or block protect the K^+ conductance

A, ratio of the currents at +20 mV before (I_o) and after (I) the delivery of ten test pulses in Na_o^+–Na_i^+, determined as in Fig. 2, with the chloride salt of K^+ (●), Rb^+ (▼), Cs^+ (○) or NH_4^+ (▽) added at the indicated concentrations to the Na_o^+ solution (see Methods). The points are the means ± S.E.M. of at least three experiments. The inset shows the double reciprocal plot of the main figure. The points for each ion were fitted with a least-squares line with: K^+, K_d = 2·85 mM, r = 0·96; Cs^+, K_d = 2·22 mM, r = 0·95; NH_4^+, K_d = 3·50 mM, r = 0·98. Rb^+ was tested only at 10 mM. *B*, ratio of the currents at +20 mV before (I_o) and after (I) the delivery of ten test pulses in Na_o^+–Na_i^+, with the indicated [TEA] added to the Na_o^+ solution (see Methods). The fraction of the channels blocked by TEA added to the Na_o^+ solution (Na_o^+–K_i^+) was determined as $1 - (I/I_o)$, where I is the peak current at the indicated $[TEA]_o$ and I_o is the current in the control at 0 mV. The inset shows the double reciprocal plot of the main figure. The points were fitted with a least-squares line, that yielded a K_d of 49 mM for the TEA protection of the conductance (r = 0·95), and a K_d of 44 mM for TEA block (r = 0·98). V_h, −80 mV.

Na_o^+–K_i^+. TEA protects, and it does this with a concentration dependence that parallels its block of the outward K^+ currents, recorded in Na_o^+–K_i^+. The inset of Fig. 7*B* shows the double reciprocal plot of the points in the figure. The K_d for the protection of TEA (49 mM) was practically identical to that for the block of the channels (44 mM). These values are in good agreement with those reported for the TEA block of *Shaker* B channels in other expression systems, under similar conditions (e.g. Choi, Aldrich & Yellen, 1991).

The above results suggest that external TEA remains able to block *Shaker* B channels in the absence of K^+ ions, and that in doing this it also inhibits the fall in conductance. This characteristic is another difference between *Shaker* B and those K^+ channels that conduct Na^+, where TEA ceases to block in the absence of K^+ ions (Ikeda & Korn, 1995).

Regarding the effect of the ions of the internal solution, addition of 20 mM K^+ to the Na_i^+ solution fully prevents the channels from falling into the non-conducting state. Lower concentrations do not protect completely (not shown).

Finally, it is pertinent to indicate that the fall in conductance was also observed in NMG solutions (NMG_o–NMG_i; see Methods), but the extent of reduction, $36 \pm 5\%$ ($n = 4$), brought about by ten test pulses, was smaller than that in Na^+ solutions (Fig. 5*A*). Nonetheless, delivery of extra

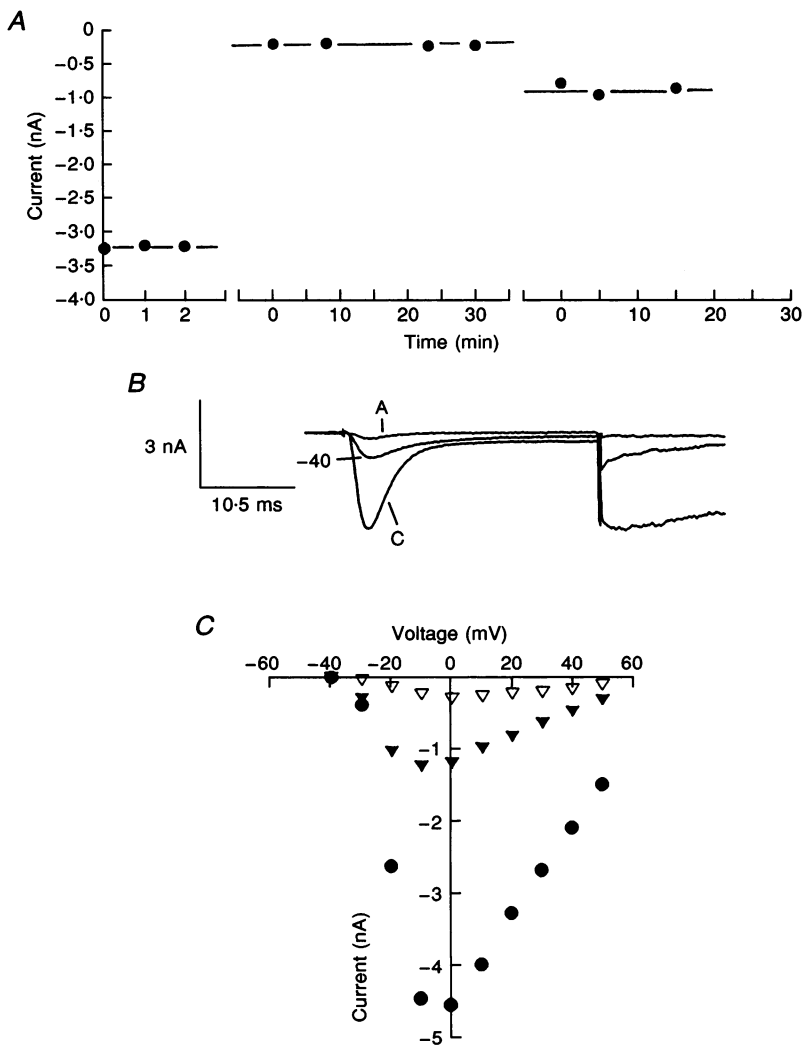

Figure 8. Long-lasting depolarizations allow the recovery of the K^+ conductance

A, peak K^+ currents at +20 mV as a function of the time of recording in K_o^+–Na_i^+, before (left-hand section) and after (middle section) the delivery of ten 30 ms pulses to +20 mV from a V_h of −80 mV in Na_o^+ –Na_i^+, as in Fig. 2. The current fell from −3·3 to −0·24 nA, and did not recover during the next 30 min. Afterwards, the V_h was changed to −40 mV for 1 min, and after another minute with the V_h back at −80 mV (not shown), 30 ms pulses to +20 mV were delivered; the results are illustrated in the right-hand section. The current increased 3·8 times (−0·91 nA). *B*, K^+ currents at +20 mV before (C), after pulsing in zero K^+ (A) and after the 1 min change of V_h to −40 mV (−40). *C*, *I–V* relationship of the currents in *B*.

depolarizations (~25) also completely eliminated the conductance (not shown). Therefore, the collapse of the conductance is not a specific effect of Na^+ carried out in the absence of K^+.

The above results indicate that the binding of a pertinent, permeant or blocking, monovalent cation is necessary to avoid the fall in conductance (some monovalent cations that block from the inside may not work, e.g. Na^+ blocks K^+ channels from the inside (Marty, 1983) and does not protect).

Having explored the conditions that protect, the next question was whether there was a way to restore the conductance of channels that had previously lost it. The next section deals with this problem.

The K^+ conductance is fully recovered by long-lasting (seconds to minutes) depolarizations

Gating the channels in zero K^+ causes a seemingly irreversible fall in conductance (Figs 2–4). However, after having observed that a depolarized V_h protects (Fig. 6), it was thought that perhaps exposing the channels, whose conductance had been previously collapsed, to long-lasting depolarizations could restore their ability to conduct. Figure 8 shows the result of exposing non-conductive channels to a prolonged depolarization.

Figure 8*A* (left-hand section) shows the size of the peak current at +20 mV as a function of the time of recording in the control solutions. The current had an initial amplitude of −3·3 nA. Then, after the delivery of ten 30 ms pulses to +20 mV from −80 mV in Na_o^+–Na_i^+ (not shown), the current dropped to −0·24 nA, corresponding to a 93% reduction (middle section). As always, there was no sign of recovery over the next 30 min. Afterwards, the V_h was changed for 1 min to −40 mV (not shown), and after another minute with the V_h back at −80 mV, 30 ms pulses to +20 mV were applied to test the state of the channels. The results presented in the right-hand section of Fig. 8*A* indicate that, surprisingly, there was a 3·8-fold increment of the current (−0·91 nA). Note that the current remains constant at its

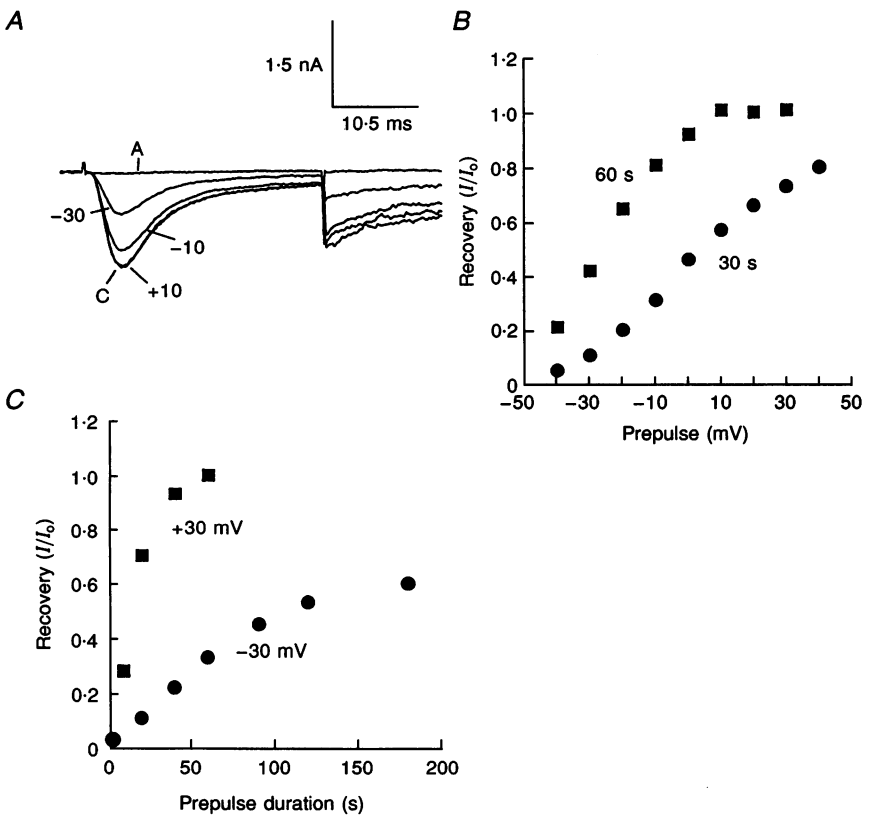

Figure 9. K^+ conductance recovery as a function of the voltage

A, K^+ currents evoked by a 30 ms pulse to +20 mV from a V_h of −80 mV, in K_o^+–Na_i^+, recorded in the following order. Firstly, a control current was recorded (C). Then, after the delivery of fifteen test pulses in Na_o^+–Na_i^+ (not shown), the current labelled A was recorded. Note the 100% reduction of the current. Afterwards, the V_h was changed for 1 min to −30 mV, and after another minute with the V_h back at −80 mV, the current labelled −30 was recorded. Note the clear recovery. Then the same procedure was repeated but the V_h was changed to −10 mV (trace labelled −10). Finally, the V_h was changed for 1 min to +10 mV. The trace labelled +10 shows that there was 100% recovery. *B*, ratio of the currents in the control (I_o), before the delivery of the fifteen test pulses in zero K^+, and after a 30 or 60 s change of the V_h to the indicated voltages (I). The changes in the V_h were made as in *A*. *C*, time course of recovery from the non-conducting state at −30 and +30 mV.

new level. Figure 8*B* shows the current at +20 mV in the control (C), after pulsing in zero K^+ (A), and after the 1 min depolarization to −40 mV (−40). Figure 8*C* shows an *I–V* relationship of each of the three stages. These results demonstrate that the conductance can be recovered by long-lasting depolarizations.

This behaviour also rules out inactivation (either N- or C-type) as the cause of the drop in the K^+ conductance. Recovery from inactivation requires the membrane potential to be at resting or hyperpolarized potentials, but never depolarized.

Figure 9*A* illustrates the extent of recovery at three voltages. The trace labelled C shows a control current at +20 mV. Then fifteen pulses (1 pulse s^{-1}) were delivered in zero K^+ (not shown); this caused a 100% reduction of the current, as shown by the trace labelled A (after). Then V_h was changed for 1 min to −30 mV, and after another minute with the V_h back at −80 mV, the trace labelled −30 was recorded. Note the clear increment of the current. Afterwards, the same procedure was repeated but the V_h was changed to −10 mV. The trace labelled −10 shows that the current experienced a further increment, but was still smaller than that of the control. Finally, the V_h was changed for 1 min to +10 mV, and after another minute with the V_h back at −80 mV, the trace labelled +10 was recorded. A 100% recovery was then reached.

Figure 9*B* shows the extent of recovery as a function of the voltage for prepulses of 30 and 60 s. Note that (a) even with small depolarizations, such as −40 mV, some recovery is observed if the potential is left there for at least tens of seconds, and (b) even with strong depolarizations (above 0 mV), it takes tens of seconds to reach a 100% recovery.

Figure 9*C* shows the time course of recovery at −30 and +30 mV. At −30 mV, even after 180 s, the recovery is incomplete. At +30 mV recovery follows a single exponential time course, with a time constant of 19 s, and a maximum recovery of 100%. It is always possible to obtain a complete recovery at any time after the pulse-evoked fall in conductance (not shown).

Thus recovery from the non-conductive state is a slow voltage-dependent process that, depending on the magnitude of the depolarization, requires from tens of seconds to

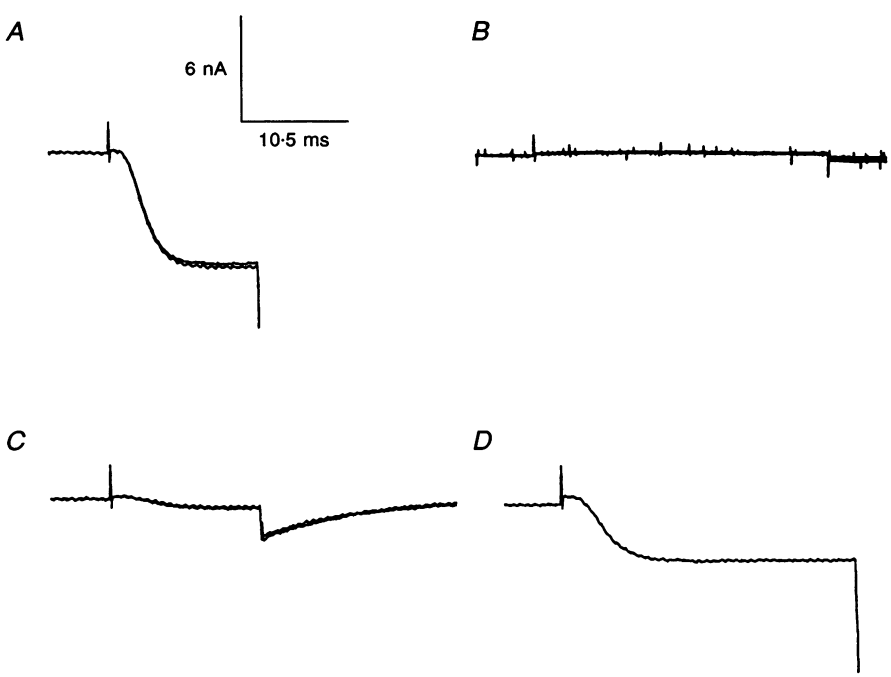

Figure 10. Fall–recovery cycle of the K^+ conductance in channels with N-type inactivation removed

A, control currents elicited by a 12 ms pulse to 0 mV from a V_h of −80 mV in K_o^+–Na_i^+. At the end of the pulse the membrane was repolarized to −60 mV for 20 ms, to compensate for the large inward tail currents. The currents were recorded 14 min after breaking into the cell with a Na_i^+ solution containing the proteolytic enzyme papain at 0·1 mg ml^{-1}. Note that the currents did not show fast inactivation. At the end of the pulse, the deactivation tails are so large that they go off the scale. *B*, currents elicited by ten 30 ms pulses to +20 mV delivered from a V_h of −80 mV at 1 Hz in Na_o^+–Na_i^+. *C*, currents evoked by 12 ms pulses to 0 mV in K_o^+–Na_i^+, after the ten pulses in zero K^+ shown in *B*. The conductance was dramatically reduced. Note the very small deactivation tails at −60 mV. *D*, current evoked by a 30 ms pulse to 0 mV from −80 mV, recorded after having changed the V_h to −20 mV for ~1 min (as in Fig. 8*A*). Note the clear recovery of the conductance (~60%); again the deactivation tail is off the scale.

several minutes for completion. Even at +70 mV, more than 3 s are required to see some recovery (not shown).

Finally, Fig. 10 shows that the same basic results are also qualitatively observed in *Shaker* B channels with N-type inactivation removed by the addition of papain (0·1 mg ml^{-1}) to the pipette Na_i^+ solution (see Gómez-Lagunas & Armstrong, 1995).

Figure 10*A* illustrates two control currents evoked by 12 ms pulses to 0 mV in K_o^+–Na_i^+. The currents were recorded 14 min after the establishment of the whole-cell configuration. Note that the channels no longer presented N-inactivation. Due to the large driving force of K^+ and the lack of fast inactivation, the amplitude of the deactivation tails, at the end of the pulse, goes off the scale (even when at the end of the 0 mV pulse the membrane was first stepped to −60 mV for 20 ms). Next, the cell was changed to Na_o^+–Na_i^+ and ten 30 ms pulses to +20 mV were delivered at 1 Hz from −80 mV (Fig. 10*B*). Afterwards, the cell was brought back to the K_o^+–Na_i^+ solutions, and 45 s later two 15 ms pulses to 0 mV were delivered (1 pulse every 3 min) to test the state of the channels. Figure 10*C* shows that the current was dramatically reduced. Note the very small deactivation tails at −60 mV. Finally, V_h was changed to −20 mV for ~1 min, and after another minute at −80 mV, a 30 ms pulse to 0 mV was applied. The resulting current in Fig. 10*D* shows that there was a marked recovery (~60%). Note that again the deactivation tail goes off the scale.

Thus the fall in conductance is not due to either N-inactivation or any interaction between the N- and C-inactivation gates. Also, these results indicate that the state where the channels remain unconductive, after pulsing in zero K^+, is equally visited with or without N-inactivation. The same results were observed in all of three cells treated with papain.

Finally, it is pertinent to mention that during the sustained depolarizations that allowed the recovery of the conductance (Figs 8–10), no noticeable K^+ current was observed (not shown). This was probably due to the strikingly slow rate of exit from the non-conducting sate.

DISCUSSION

In zero K^+ external solutions, stable K^+ currents through *Shaker* B channels are recorded. At −80 mV or negative holding potentials, and low frequencies of pulsing (< 1 pulse every 9 s), the outward currents are of a size that is insensitive to small, millimolar changes in the concentration of extracellular K^+. Similarly, inward K^+ currents can be recorded in the absence of K^+ ions in the internal solution.

However, when the channels are exposed at both sides of the membrane to zero K^+ solutions, a new phenomenon appears: the conductance is reduced if the channels go through a gating cycle, but it is unchanged if the channels are kept closed. The fall in K^+ conductance has the notable characteristic of being strikingly stable. At −80 mV or hyperpolarized holding potentials, the conductance never recovers. This indicates that there must be a conformational change, which impedes the K^+ ions of the external solution to destabilize the non-conducting state.

The results of the experiments with monovalent cations and TEA indicate that the binding of an appropriate ion (K^+ under physiological conditions) to a binding site probably located towards the external side of the pore, is required to avoid the collapse of conductance.

The mechanism of the fall in *Shaker* conductance is different from that reported for other K^+ channels, upon exposure to a partial (one side of the membrane), or a total absence of K^+: (a) it differs from the run-down of the delayed rectifier channel of the squid giant axon in the absence of K^+ ions at both sides of the membrane, where the collapse of conductance is irreversible (Almers & Armstrong, 1980); (b) it is also different (for all the reasons already indicated) from the fall in conductance that some K^+ channels experience in the absence of external K^+, as a result of an increased rate of slow inactivation (Labarca & MacKinnon, 1992; Pardo *et al.* 1992; López-Barneo *et al.* 1993; Baukrowitz & Yellen, 1995).

Is the non-conducting state the result of a block of the pore?

It seems unlikely that the fall in conductance could be due to a simple block of the pore by an ion, because: (a) recovery requires long-lasting depolarizations, although a change in the voltage should be sensed immediately by a blocking charge located in the transmembrane electric field; and (b) once back in the K_o^+ solution, it would be expected that the 100 mM K^+ on the outside should destabilize the binding of a positive charge blocking the conduction pathway (e.g. Armstrong, 1971). In contrast, the fraction of non-conducting channels remains constant in K_o^+, as long as the membrane is not subject to long-lasting depolarizations.

Hypothesis

The halt in conductance could happen during the closing of the channels at −80 mV. In the absence of K^+, the channels could fall into a strikingly stable, non-inactivated, closed state. Prolonged depolarizations would be needed to open the channels from the abnormally stable closed state.

This hypothesis serves to explain the characteristics of the halt in conductance. Firstly, the extent of reduction of the conductance depends on the number of pulses, because the more times the channels pass through a gating cycle, the proportion of them remaining closed increases.

Secondly, the frequency of pulsing does not have much effect, because recovery from both types of inactivation ends when the channels are finally closed. Then of the fraction of channels still capable of conducting after a pulse, both those that recovered from inactivation, during the interpulse at −80 mV (and that will be activated and inactivated by the next pulse), and those that did not, will have to finally close.

Thirdly, pulsing from a depolarized V_h does not reduce the conductance, because a sustained depolarization destabilizes the closed state. Also, closing could preclude external K^+ from having access to the site where it destabilizes the non-conducting state.

This idea agrees with the occupancy hypothesis of Armstrong and co-workers (Swenson & Armstrong, 1981; Armstrong & Matteson, 1986; Matteson & Swenson, 1986) according to which, external K^+ slows (inhibits) the rate of closing of K^+ channels by binding to a site in the pore. Thus it seems that in zero K^+ (at both sides of the membrane), the channels fall into an unusually stable closed state.

The results of this work could be equally explained if the channels are thought to be either frozen in a particular (unknown) closed state, or if they could freely gate along closed states but without being able to finally open.

Whatever the case, the results indicate that a gating, non-inactivated state (possibly a closed state) is modulated by the binding of K^+ to a site located towards the extracellular side of the pore.

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