

# Na<sup>+</sup> Interaction with the Pore of *Shaker* B K<sup>+</sup> Channels: Zero and Low K<sup>+</sup> Conditions

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**ABSTRACT** The *Shaker* B K<sup>+</sup> conductance ( $G_K$ ) collapses (in a reversible manner) if the membrane is depolarized and then repolarized in, 0 K<sup>+</sup>, Na<sup>+</sup>-containing solutions (Gómez-Lagunas, F. 1997. *J. Physiol.* 499:3–15; Gómez-Lagunas, F. 1999. *Biophys. J.* 77:2988–2998). In this work, the role of Na<sup>+</sup> ions in the collapse of  $G_K$  in 0-K<sup>+</sup> solutions, and in the behavior of the channels in low K<sup>+</sup>, was studied. The main findings are as follows. First, in 0-K<sup>+</sup> solutions, the presence of Na<sup>+</sup> ions is an important factor that speeds the collapse of  $G_K$ . Second, external Na<sup>+</sup> fosters the drop of  $G_K$  by binding to a site with a  $K_d = 3.3$  mM. External K<sup>+</sup> competes, in a mutually exclusive manner, with Na<sub>o</sub><sup>+</sup> for binding to this site, with an estimated  $K_d = 80$   $\mu$ M. Third, NMG and choline are relatively inert regarding the stability of  $G_K$ ; fourth, with  $[K_o^+] = 0$ , the energy required to relieve Na<sub>i</sub><sup>+</sup> block of *Shaker* (French, R.J., and J.B. Wells. 1977. *J. Gen. Physiol.* 70:707–724; Starkus, J.G., L. Kuschel, M. Rayner, and S. Heinemann. 2000. *J. Gen. Physiol.* 110:539–550) decreases with the molar fraction of Na<sub>i</sub><sup>+</sup> ( $X_{Na,i}$ ), in an extent not accounted for by the change in  $\Delta\mu_{Na}$ . Finally, when  $X_{Na,i} = 1$ ,  $G_K$  collapses by the binding of Na<sub>i</sub><sup>+</sup> to two sites, with apparent  $K_d$ s of 2 and 14.3 mM.

**KEY WORDS:** K<sup>+</sup> affinity • Na<sup>+</sup> block • conductance • selectivity • zero K<sup>+</sup>

## INTRODUCTION

In addition to permeate through voltage-dependent K channels (Kv channels)\* and modulate their gating, K<sup>+</sup> ions are an essential factor needed to keep these proteins in their normal, functional, state. It is now becoming clear however, that the K<sup>+</sup> requirements of Kv channels are highly variable, as revealed by the diversity of effects that K<sup>+</sup> depletion exerts on different kinds of channels. For example, in the absence of K<sup>+</sup> on both sides of the membrane, the activity of the delayed rectifier (DR) squid K channel is irreversibly lost (Chandler and Meves, 1970; Almers and Armstrong, 1980; Khodakhah et al., 1997). On the other hand, both DR channels of bullfrog sympathetic neurons (Block and Jones, 1997) as well as mammalian DRs, of the Kv2.1 subfamily, remain active in 0 K<sup>+</sup>, allowing a substantial permeation of Na<sup>+</sup>, but becoming anomalously immune to the addition of TEA (Zhu, and Ikeda, 1993; Ikeda and Korn, 1995). Similarly, Kv1.5 channels also remain active in 0 K<sup>+</sup>, although they only allow a small, fast inactivating, and TEA-sensitive, flux of Na<sup>+</sup> through them (Zhuren et al., 2000). In contrast, other K<sup>+</sup> channels are appar-

ently insensitive to K<sup>+</sup> depletion. For example, Kv1.3 channels, do not allow a measurable permeation of Na<sup>+</sup>, and remain active after the exposure to 0 K<sup>+</sup> (Immke et al., 1998; see also Jäger et al., 1998).

Recently, it was shown that in the absence of K<sup>+</sup> on both sides of the membrane, the *Shaker* B K<sup>+</sup> conductance ( $G_K$ ) collapses when the membrane is depolarized and then repolarized. Briefly, Gómez-Lagunas (1997, 1999) reported the following. First,  $G_K$  collapses if the channels are gated, by the delivery of standard activating pulses, while they are immersed in Na<sup>+</sup>-containing, 0 K<sup>+</sup>, solutions. The extent of collapse depends on the number of pulses but not on their frequency. Second, in contrast, the halt in the conductance is completely prevented if the channels are kept closed (are not gated) while they are in 0 K<sup>+</sup>. Third, depolarized holding potentials (HP, above  $-50$  mV) impede the drop of  $G_K$ . Fourth, external K<sup>+</sup> protects  $G_K$  by binding to a site in the channels with rather low, millimolar, affinity (apparent  $K_d = 2.9$  mM). Fifth, among divalent cations only Ba<sup>2+</sup> is able to replace K<sup>+</sup>, protecting  $G_K$ ; and finally, very prolonged depolarizations (seconds to minutes) recover the, previously lost, K<sup>+</sup> conductance.

The above observations were interpreted as meaning that closing without K<sup>+</sup> sinks the channels into a stable nonconducting, noninactivated, closed state(s) (Gómez-Lagunas, 1997). It seems that the channels need to close with a K<sup>+</sup> ion(s) bound to them, otherwise they sink into a reluctant conformation where they remain unable to

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\*Abbreviations used in this paper: DR, delayed rectifier; HP, holding potential; Kv channel, voltage-dependent K channel; m.c, maximal collapse.

conduct  $K^+$ , no matter how long  $V_m$  is kept at the HP or hyperpolarized potentials (Gómez-Lagunas, 1997, 1999; Melishchuk et al., 1998). Looking at the selectivity of the site(s) involved in the drop of  $G_K$ , preliminary observations have indicated that the presence of  $Na^+$  ions might significantly foster the collapse of  $G_K$  in 0- $K^+$  solutions, probably by promoting the displacement of  $K^+$  from the pertinent site(s) in the channels (Gómez-Lagunas, 1997; Melishchuk et al., 1998). Thus, to further understand the mechanism underlying the fall of  $G_K$ , in this work the role of  $Na^+$  ions in both the collapse of the *Shaker*  $K^+$  conductance in 0- $K^+$  solutions, and in the behavior of the channels in low  $K^+$ , is investigated.

Here, it is shown that  $Na^+$  ions foster the collapse of  $G_K$  in the absence of  $K^+$ . External  $K^+$  and  $Na^+$  compete, in a mutually exclusive manner, for binding to an externally located site (probably external to the selectivity filter), where  $G_K$  is modulated (available versus reluctant). On the other hand, to see if the collapse of  $G_K$  occurs as a discontinuous change in the properties of the channels in the limit of zero  $K^+$ , experiments with internal solutions containing both  $K^+$  and  $Na^+$  ions were done. The results show that as the molar fraction of  $Na_i^+$  ( $X_{Na,i}$ ) increases, the energy required to relieve  $Na_i^+$  block changes in an extent not accounted for by the change in the driving force of  $Na^+$ . This suggests that, as  $X_{Na,i}$  increases, there is either an increased electrostatic repulsion between the blocking  $Na^+$  and a neighbor ion, or, more likely, a deformation (change of the energy profile) of the pore, that could be maximal in the limit  $X_{Na,i} = 1$ . Under the latter conditions,  $G_K$  collapses, and  $Na_i^+$  interacts with the channels with a kinetics described by the noncooperative binding to two, kinetically distinguishable, sites.

A preliminary account of this work has been presented in abstract form (Gómez-Lagunas, 2000).

## MATERIALS AND METHODS

### Cell Culture and Channel Expression

Insect Sf9 cells kept in culture in Grace's media (GIBCO BRL) at 27°C were infected, with a multiplicity of infection of 10, with a recombinant baculovirus, *Autographa californica* nuclear polyhedrosis virus, containing the cDNA of *Shaker* B, as previously reported (Klaiber et al., 1990; Gómez-Lagunas, 1997). Experiments were performed 48 h after infection.

### Electrophysiology

Macroscopic currents were recorded under whole-cell patch clamp, with an Axopatch-1D (Axon Instruments). The currents were filtered at 5 KHz with the built in filter of the amplifier, and sampled at 100  $\mu$ s/point, with a TL1 interface (Axon Instruments). Electrodes were pulled from borosilicate glass (KIMAX 51) to a final resistance of 1–2 M $\Omega$ ;  $\sim$ 80% of the series resistance was electronically compensated. Activating pulses (unless otherwise indicated = +20 mV/30 ms) in 0- $K^+$  solutions, were delivered at 1 Hz. This procedure will be referred to as pulsing.

TABLE I  
Recording Solutions

Name	[KCl]	[KF]	[NaCl]	[NaF]	[CaCl <sub>2</sub> ]	[NMG-Cl]	[Choline-Cl]	[HF]
	mM	mM	mM	mM	mM	mM	mM	mM
$K_o$	100	0	40	0	10	0	0	0
$Na_o$	0	0	140	0	10	0	0	0
Choline <sub>o</sub>	0	0	0	0	10	0	140	0
NMG <sub>o</sub>	0	0	0	0	10	140	0	0
$K_i$	30	90	0	0	0	0	0	0
$Na_i$	0	0	30	90	0	0	0	0
NMG <sub>i</sub>	0	0	0	0	0	120	0	90

All the solutions also contained 10 mM HEPES-x pH 7.2 where x is either NaOH ( $K_o$ ,  $Na_o$ ,  $Na_i$ ), KOH ( $K_i$ ), HCl (NMG<sub>o</sub>), or TMA (Choline<sub>o</sub>, NMG<sub>i</sub>). All internal solutions also contained 2 mM MgCl<sub>2</sub> and 10 mM EGTA.

### Solutions

Solutions will be named by their main cation and represented as external/internal (e.g.,  $K_o/Na_i$ ). Their composition is listed in Table I. All other solutions in which the concentration of the test cation exceeded 5 mM, were made by the appropriate mixing of the listed solutions, keeping the osmolarity constant. Total exchange of the external solution was achieved in at most 15 s. Pulsing in 0  $K^+$  was performed under continuous perfusion, beginning 1 min after the start of the perfusion.

### Data Analysis

Where necessary, the *t* test was used to evaluate statistical significance. The results are expressed as mean  $\pm$  SEM of at least four cells. Curve fitting was performed with Sigmaplot 3.0 (Jandel).

## RESULTS

### $Na^+$ Ions Speed the Collapse of $G_K$ in 0 $K^+$ Conditions

With  $Na^+$ -containing, 0- $K^+$  solutions on both sides of the membrane ( $Na_o/Na_i$ ; MATERIALS AND METHODS), the delivery of depolarizing pulses that activate the channels followed by repolarization to a negative potential (usually the HP = -80 mV) collapse the *Shaker* B  $K^+$  conductance ( $G_K$ ; INTRODUCTION). Fig. 1 shows that  $Na^+$  ions foster the collapse of  $G_K$ . Fig. 1 A illustrates the collapse-recovery cycle of  $G_K$  in  $Na_o/Na_i$  solutions. The traces show inward  $K^+$  currents ( $I_K$ ), evoked by +20 mV/30-ms activating pulses, in  $K_o/Na_i$ . The left trace (Fig. 1 A, before) is a control  $I_K$ , recorded at the beginning of the experiment. The middle trace (Fig. 1 A, After) is the current left after the delivery of 20 activating pulses (a procedure hereafter referred to as pulsing) while the cell was bathed in the test  $Na_o/Na_i$  solutions (not shown). After pulsing in 0  $K^+$ ,  $Na^+$ -containing solutions, the channels become reluctant to conduct  $K^+$ . The right trace (Fig. 1 A, Recovery) shows the recovery of  $I_K$  brought about by a 3-min depolarization to 0 mV (Gómez-Lagunas, 1997).

In contrast to the dramatic effect of pulsing in  $Na_o/Na_i$ , the delivery of the same number of pulses in NMG-

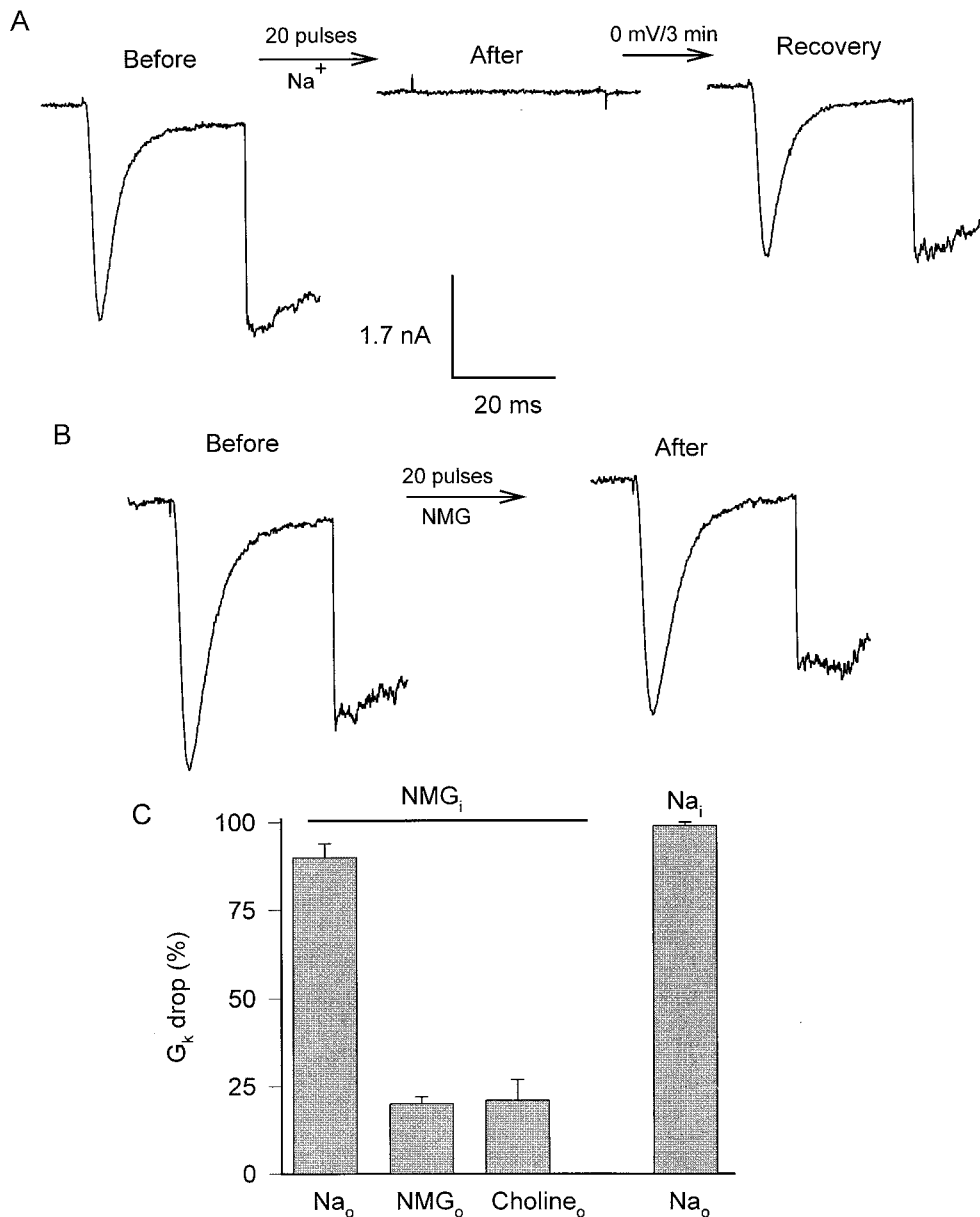


FIGURE 1.  $G_K$  collapse in 0- $K^+$  solutions of varying composition. (A)  $K^+$  currents evoked, by a +20 mV/30-ms activating pulse from the HP of  $-80$  mV, in  $K_o/Na_i$  solutions. Left panel (before), control  $I_K$ ; middle panel (after), current left after the delivery of 20 activating pulses (+20 mV/30 ms, referred to as pulsing) in  $Na_o/Na_i$  solutions (not shown); right panel (recovery),  $I_K$  recorded 1 min after the application of a 3-min depolarization to 0 mV. (B).  $K^+$  currents evoked by an activating pulse in  $K_o/NMG_i$  solutions. (left trace, Before, after),  $I_K$  left after pulsing in  $NMG_o/NMG_i$  solutions (not shown). Bars are the same for both A and B. (C). Percentage of  $G_K$  collapse,  $[1 - (I/I_0)] \times 100$ , after pulsing in the indicated solutions, as in A,  $I_0$  is the, control, peak  $K^+$  current before pulsing,  $I$  is the peak  $K^+$  current left after pulsing.

containing, 0  $K^+$ , and 0  $Na^+$  (not added) solutions scarcely affects  $G_K$ . This is shown in Fig. 1 B, which presents two  $K^+$  currents recorded in  $K_o/NMG_i$ , before (left trace) and after (right trace) pulsing in  $NMG_o/NMG_i$  (not shown). There was only a 13% reduction of  $I_K$ .

Fig. 1 C compares the reduction of  $G_K$  after pulsing in a variety of solutions. Notice that, whereas in  $Na_o/Na_i$  (last bar)  $G_K$  is basically erased ( $99 \pm 1\%$  reduction,  $n = 4$ ), in  $NMG_o/NMG_i$  (second bar)  $G_K$  drops only  $20 \pm 2\%$  ( $n = 16$ ). Thus, pulsing in 0  $Na^+$ , NMG-containing, solutions collapses  $G_K$ , but far less than in the presence of  $Na^+$  ions (in fact, in  $Na_o/Na_i$ , only 10–15 pulses are needed to completely eliminate  $G_K$ , see Gómez-Lagunas, 1997, 1999). Moreover, after pulsing in NMG most of the channels ( $\approx 80\%$ ) remain in a state from which they

readily collapse upon the addition of  $Na^+$  (not shown). The third bar in Fig. 1 C, shows that external choline, another impermeant and nonblocking cation, is as inert as NMG ( $G_K$  drop =  $21 \pm 6\%$ ,  $n = 5$ ; see DISCUSSION).

In summary, the combined condition, presence of  $Na^+$  and lack of  $K^+$ , makes  $G_K$  more liable to collapse. Finally, notice that pulsing with  $Na^+$  ions present (added) in only the external solution (in  $Na_o/NMG_i$ , first bar) drops  $G_K$  in about the same extent ( $90 \pm 4\%$ ,  $n = 9$ ) as it does with  $Na^+$  on both sides of the membrane ( $99 \pm 1\%$ , last bar). So, even when under physiological conditions, external  $Na^+$  neither permeates nor blocks Kv channels (but see Block and Jones, 1996), it effectively fosters the collapse of  $G_K$  in 0- $K^+$  solutions.

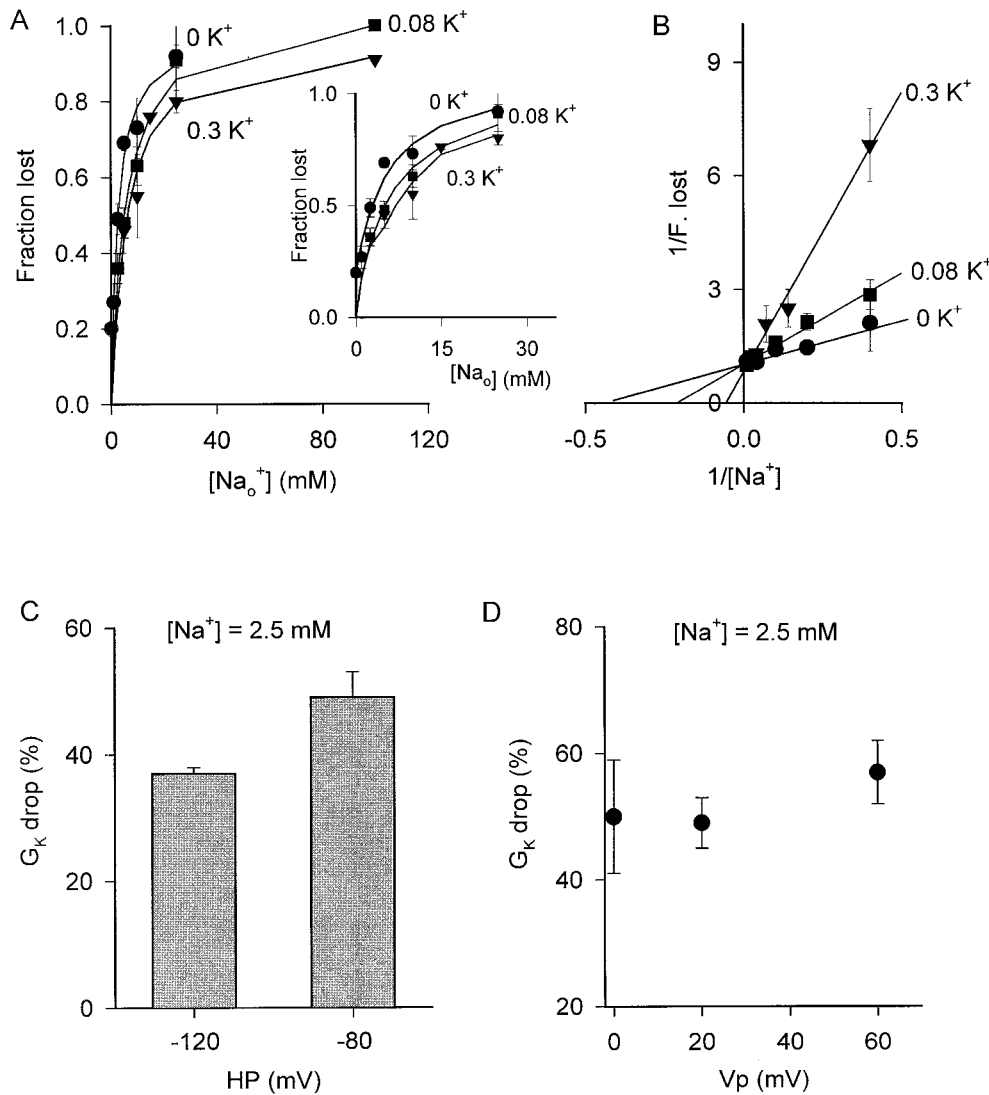


FIGURE 2.  $G_K$  drop as a function of the external  $[Na^+]$ . (A). Extent of  $G_K$  reduction  $[1 - (I/I_0)]$ , like in Fig. 1 C, after pulsing in either  $(NMG_o - [Na_o^+])/NMG_i$ , 0- $K^+$  solutions, with the indicated  $[Na_o^+]$  (●), or in the same solutions but with 0.08 (■) or 0.3 mM  $K_o^+$  (▼). The lines are the fit of the points with a Hill equation with parameters (●):  $n = 0.97$ ,  $K_d = 3.3$  mM, and maximal collapse (m.c) = 1.05; and (■):  $n = 0.91$ ,  $K_d = 4.7$  mM, m.c = 1.02; (▼):  $n = 1.10$ ,  $K_d = 5.3$  mM, m.c = 0.97. The inset shows the plot in an expanded  $[Na_o^+]$  scale (B). Double reciprocal plot of the points in A. 0  $K^+$ ,  $r = 0.996$ ; 0.08  $K^+$ ,  $r = 0.988$ ; 0.3  $K^+$ ,  $r = 0.997$  (see text for details). (C). Percentage of  $G_K$  collapse as a function of the HP during pulsing in  $(NMG_o - [Na_o^+])/NMG_i$ , with  $[Na_o^+] = 2.5$  mM. (D).  $G_K$  drop as a function of the pulse potential ( $V_p$ ), during pulsing from the HP of  $-80$  mV,  $[Na_o^+] = 2.5$  mM as in C. The points are the mean  $\pm$  SEM of at least four measurements at each  $[Na^+]$ .

#### External $Na^+$ Interaction with Shaker Channels

The interaction of external  $Na^+$  with *Shaker* was further studied by looking at the extent of  $G_K$  collapse produced by pulsing in solutions of variable  $[Na_o^+]$ , with  $NMG_i$  as the internal solution (in  $(NMG_o + [Na_o^+])/NMG_i$ , see MATERIALS AND METHODS). Fig. 2 A shows that as  $[Na_o^+]$  increases,  $G_K$  drops following a Hill saturation curve (line through the points, labeled 0  $K^+$ ), with a  $K_d = 3.3$  mM, and maximal extent of collapse = 1.05 (Hill number  $n = 0.97$ ). When the same measurements are done in the presence of either 0.08 or 0.3 mM  $K_o^+$ , it is seen that the apparent  $K_d$  for  $Na^+$  increases to either 4.7 or 5.3 mM, respectively, without a significant change in the maximal extent of collapse (0.98 or 0.91 with 0.08 or 0.3 mM  $K^+$ , respectively). This indicates that  $K^+$  protection (reduction of  $G_K$  drop) is more efficient at the lower  $[Na_o^+]$ , and thus shows that  $K^+$  inhibits in a competitive manner the

binding of  $Na^+$ , to the site where  $G_K$  is modulated. The inset shows the plot in an expanded  $[Na_o^+]$  scale.

The competitive, mutually exclusive, binding of  $Na^+$  and  $K^+$  is best seen in Fig. 2 B, that presents the double reciprocal plot of the points in A. The presence of either 0.08 or 0.3 mM  $K_o^+$  increases the slope of the least-square lines (i.e., the apparent  $K_d$  for  $Na_o^+$ ), without significantly changing the  $(1/F.lost) -$  axis intercept (0.95 in 0  $K^+$  vs. 1.02 or 1.1 with 0.08 or 0.3 mM  $K^+$ , respectively). The above results, allow the actual affinity for  $K_o^+$  (i.e.,  $K_d(K_o^+)$ , in the absence of the competing  $Na^+$  ions) to be estimated, with the use of the known equation for the apparent  $K_d(K_{app})$  of a ligand ( $K^+$ ) in the presence of a competitive inhibitor I ( $Na^+$ ) of known  $K_i = K_d(Na_o^+)$ , by taken  $K_{app}(K_o^+) = 2.9$  mM, the previously reported  $K_o^+$  affinity, that had been obtained in the presence of saturating  $[Na^+] = 140$  mM (Gómez-Lagunas, 1997), as (Segel, 1993):  $K_d(K_o^+) \approx K_{app}(K_o^+) / (1 + ([I]/K_i)) = 80 \mu M$ .

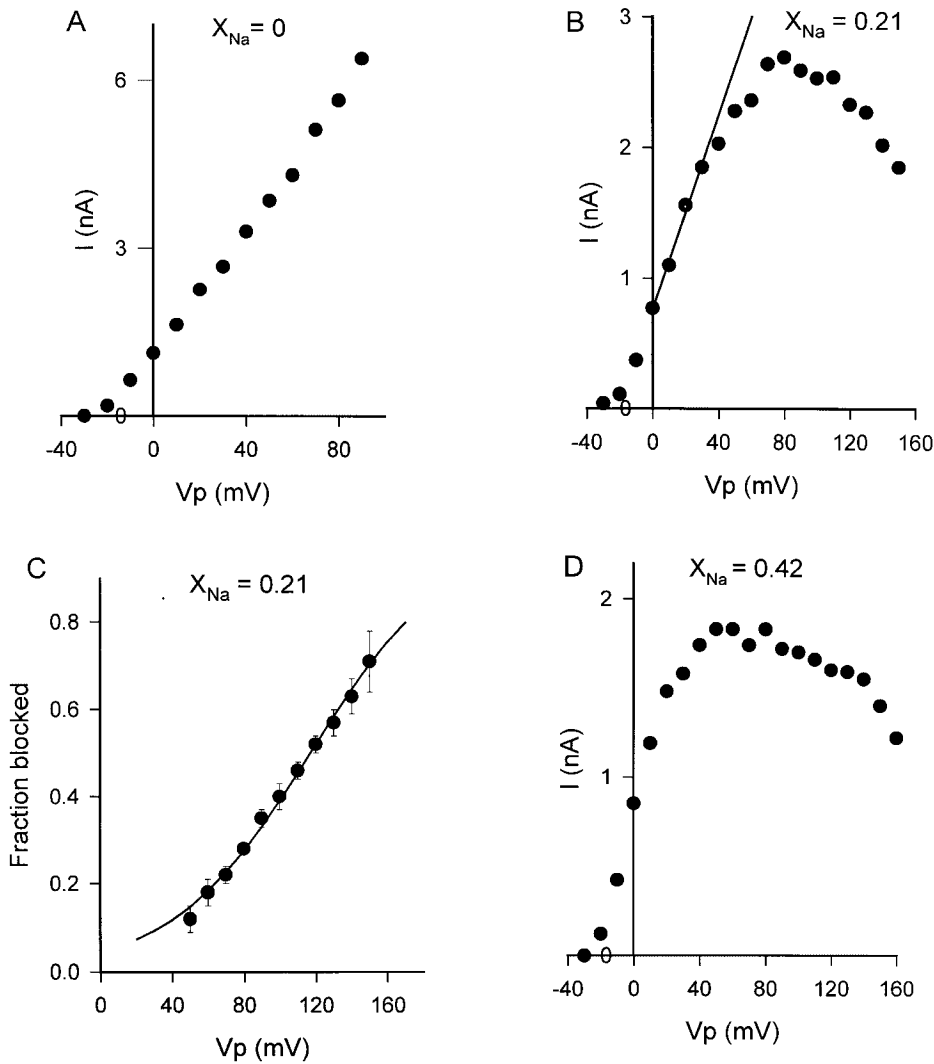


FIGURE 3. Internal Na<sup>+</sup> block at small  $X_{Na} \leq 0.42$ . (A). Current-voltage (I-V) relationship in standard recording conditions ( $Na_o/K_i$ ). (B). I-V relationship obtained with the  $Na_o$  solution, and with 25 mM Na<sup>+</sup> plus 95 mM K<sup>+</sup> ( $X_{Na,i} = 0.21$ ; MATERIALS AND METHODS) in the internal solution. The straight line is the least squares fit of the points between 0 and +30 mV ( $r = 0.996$ ). The difference between the line (expected current if  $X_{Na,i} = 0$ ,  $I_e$ ) and the points (observed  $I_K$ ) was used to estimate the fractional block (fb) of the channels as a function of voltage as:  $fb = 1 - (I/I_e)$ . (C). Fraction blocked against pulse potential, estimated as in B. The points are the average  $\pm$  SEM of four cells. The line is the fit of the points with a Woodhull equation:  $fb = 25/(25 + K_o \times \exp(-\delta FV/RT))$ , with  $\delta = 0.7$  and  $K_o = K_i(0 \text{ mV}) = 538.3 \text{ mM}$ ;  $25 = [Na_i^+]$ . (D). I-V relationship obtained with the  $Na_o$  solution and  $X_{Na,i} = 0.42$ . Pulses were applied every 20 s to allow full recovery from inactivation. HP = -90 mV.

However, it is pertinent to mention that considering the possibility of ion permeation through the channels, deviations from the equilibrium conditions, on which the saturation Michaelis-Menten or Hill equations are based (Segel, 1993), might be expected, particularly at the higher  $[K^+]$ , so both the  $K^+$  and  $Na^+$   $K_i$ s are likely to be overestimations of their true equilibrium values.

If the site where  $Na_o^+$  (and  $K_o^+$ ) binds was located within the electric field of the membrane, it would be expected that pulsing from a hyperpolarized HP should increase the drop of  $G_K$ , by favoring the  $Na_o^+$  occupancy of the site.

In contrast to the above prediction, Fig. 2 C shows that, with a nonsaturating  $[Na_o^+] = 2.5 \text{ mM}$ , the drop of  $G_K$  is slightly, although significantly, reduced ( $P < 0.05$ ), instead of increased, by pulsing from a more negative HP (from  $49 \pm 4\%$  [ $n = 7$ ] at HP -80 mV; to  $37 \pm 1\%$  [ $n = 6$ ] at HP = -120 mV). On the other hand, Fig. 2 D shows that within the range of 0 to +60 mV, the pulse potential (Vp) does not play a significant

role on the extent of collapse. The dependence on holding potential of  $Na_o^+$  action is qualitatively equivalent to that of  $Ba_o^{2+}$  protection (Gómez-Lagunas, 1999). The latter decreases by pulsing from hyperpolarized HPs, and, like  $Na_o^+$  action, it is not dependent on Vp. The following nonexcluding possibilities had been proposed as an explanation of this voltage dependence (Gómez-Lagunas, 1999): (1) hyperpolarized HPs might promote ion ( $Na_o^+$  or  $Ba^{2+}$ ) permeation through the channels, thus reducing the strength of their corresponding effects; or (2) there could be more than one site where the state of  $G_K$  is modulated, a negative HP might favor the occupancy of innermost and less effective site, in terms of the corresponding effect of the ions (see Fig. 6). Whatever the case, the effect of voltage on  $Na_o^+$  and  $Ba_o^{2+}$  actions, along with the previous finding that the potency with which ions other than  $K^+$  protect  $G_K$ , is not related to the permeability sequence of the channels (Gómez-Lagunas, 1997, 1999) suggests that the external site, whose state of occupancy deter-

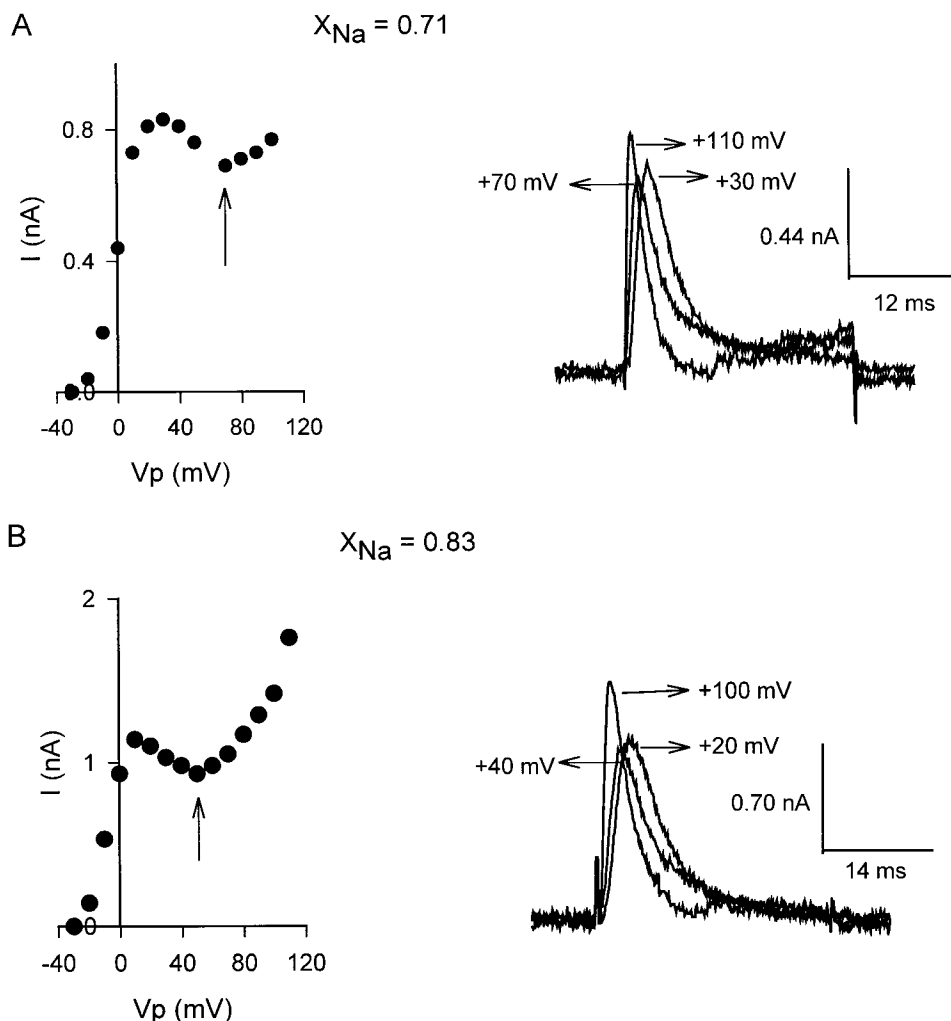


FIGURE 4. Internal  $\text{Na}^+$  block at high  $X_{\text{Na},i} > 0.42$ . (A) I-V relationship obtained with  $\text{Na}_o$  and an internal  $X_{\text{Na},i} = 0.71$  solution. The traces in the right illustrate the N shape of the I-V (B) as in A but with  $X_{\text{Na},i} = 0.83$ . The pulses were applied every 20 s, as in Fig. 4. HP,  $-90$  mV.

mines the state of  $G_K$  (available or reluctant), may not be part of the selectivity filter, but instead that it might be externally located to it (see DISCUSSION).

#### Internal $\text{Na}^+$ Interaction with Shaker Channels

It is known that internal  $\text{Na}^+$  interacts with the pore of K channels blocking  $I_K$  (Bezanilla and Armstrong, 1972; French and Wells, 1977; Marty, 1983; Yellen, 1984; Neyton and Pelleschi, 1991; Starkus et al., 2000). To see if the collapse of  $G_K$  occurs as a discontinuous change only observed in the limit of  $0 \text{ K}^+$  (molar fraction of  $\text{Na}^+$ ,  $X_{\text{Na}} = 1$ ), or if there is a gradual change in the properties of the channels as  $X_{\text{Na}}$  increases, the behavior of the channels as the molar fraction of  $\text{Na}_i^+$  ( $X_{\text{Na},i}$ ) approached 1 was studied, in internal solutions where  $X_{\text{Na},i} + X_{\text{K},i} = 1$ .

For a reference, Fig. 3 A shows a typical I-V relationship obtained under standard conditions ( $\text{Na}_o/\text{K}_i$ ). Above 0 mV, where the probability of opening is 1,  $I_K$  increases linearly with the voltage. With the  $X_{\text{Na},i} = 0$ , this is always observed. An I-V obtained with a low

$X_{\text{Na},i} = 0.21$  is shown in Fig. 3 B. First  $I_K$  increases with the voltage, but at about +40 mV, the current starts to deviate from the linearity and after that a region of negative conductance develops, as  $\text{Na}_i^+$  blocks the channels. The departure from linearity, at positive voltages ( $\geq +40$  mV), is more clearly seen by comparing the experimental points with the straight line in the plot. The latter is the least-square fit of the points between 0 and +30 mV, and therefore gives an estimate of the expected  $I_k$  if  $X_{\text{Na},i}$  was zero. Thus, the difference between the line and the points in the graph is an estimate of the extent of block at each voltage, the latter is plotted in Fig. 3 C, which presents the mean  $\pm$  SEM of four cells. The line is the fit of the points with a Woodhull equation (Fig. 3 legend), with electrical distance  $\delta = 0.7$ , and  $K_i(0 \text{ mV}) = 538.3 \text{ mM}$  (Woodhull, 1973). A representative I-V ( $n = 6$  cells) obtained with a higher  $X_{\text{Na},i} = 0.42$  is shown in Fig. 3 D. See that, qualitatively, it looks like that with  $X_{\text{Na},i} = 0.21$ . With the  $\text{Na}_o$  solution, and  $X_{\text{Na},i}$  up to 0.42,  $\text{Na}_i^+$  block becomes continuously stronger as the voltage is made more positive, for voltages up to +160 mV.

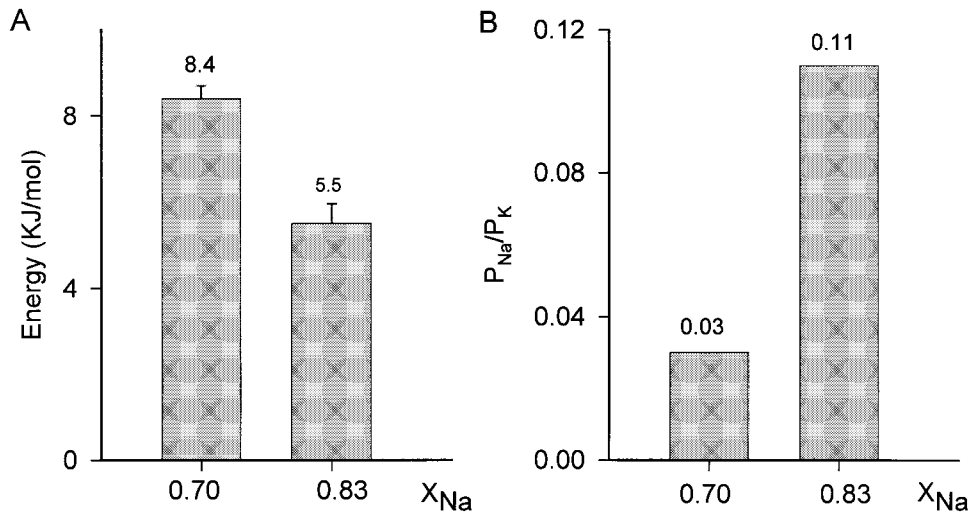


FIGURE 5. Energy needed to relieve  $Na_i^+$  block, and the estimated change in  $P_{Na}/P_K$ , as a function of  $X_{Na,i}$ . (A) Minimal energy needed to relieve  $Na_i$  block as a function of the indicated  $X_{Na,i}$ . (B) Permeability ratio  $P_{Na}/P_K$  as a function of  $X_{Na}$ . See text for details.

In contrast to the behavior with  $X_{Na,i} \leq 0.42$ , Fig. 4 A shows that with  $X_{Na,i} = 0.71$ , the I-V relationship acquires an N shape (French and Wells, 1977, Starkus et al., 2000) within the voltage range  $V_p \leq +160$  mV.  $I_K$  first increases, but starts to deviate from the linearity as  $Na_i^+$  blocks the channels, and thereafter a region of negative conductance develops, until a “critical” voltage ( $V_c$ ) is reached (in this case  $V_c = +70$  mV) where  $Na_i^+$  block is relieved and the current increases again with the voltage (pointed out by the arrow), giving the I-V its N shape. The traces in the right panel of Fig. 4 A illustrate the N shape of the I-V relationship. See that the peak currents are in the following order:  $I(+110$  mV) >  $I(+30$  mV) >  $I(+70$  mV). Finally, Fig. 4 B shows an I-V obtained with  $X_{Na,i} = 0.83$ . Notice that although its overall shape is like that observed with  $X_{Na,i} = 0.71$ ,  $V_c$  is smaller ( $V_c = +50$  mV). The traces in the right panel illustrate the N shape of the I-V relationship.

In the N-shaped I-Vs, there is a voltage ( $V_c$ ) where  $Na_i^+$  block is overcome (French and Wells, 1977). From the value of  $V_c$ , the minimal energy required to unblock the channels ( $B_{Na}$ ) can be estimated as follows (Latorre and Miller, 1983):  $B_{Na} = |-FV_c|$ , where  $F$  is the Faraday constant.

Fig. 5 A compares  $B_{Na}$  at the two  $X_{Na,i}$  where a corresponding  $V_c$  could be reached within the range  $V_p \leq +160$  mV. In going from a  $X_{Na,i}$  of 0.71 to 0.83,  $B_{Na}$  changes from  $8.4 \pm 0.3$  ( $n = 5$ ) to  $5.5 \pm 0.5$  kJ/mol ( $n = 5$ ). On the other hand, the electrochemical gradient of  $Na^+$  ( $\Delta\mu_{Na}$ ), evaluated at  $V_c$ ,  $\Delta\mu_{Na} = F(V_c - V_{Na})$ , where  $V_{Na}$  is the Nernst potential of  $Na^+$ , changes from 5.5 ( $X_{Na,i} = 0.71$ ) to 4.0 kJ/mol ( $X_{Na,i} = 0.83$ ). So, whereas  $B_{Na}$  decreases 2.9 kJ/mol,  $\Delta\mu_{Na}$  decreases only 1.5 kJ/mol, this indicates that the change in  $V_c$ , with  $X_{Na,i}$ , cannot be entirely accounted for by the change in the driving force of  $Na^+$ .

The lack of coincidence between the change of  $B_{Na}$  and  $\Delta\mu_{Na}$  is also seen when Figs. 3 and 4 are compared.

For example, with  $X_{Na,i} = 0.42$  the I-V is not N-shaped, so  $V_c \geq +160$  mV, then when  $X_{Na,i}$  increases to for example 0.83,  $B_{Na}$  changes from at least 15.5 ( $X_{Na,i} = 0.42$ , underestimating  $V_c$  as +160 mV) to  $5.5 \pm 0.5$  kJ/mol ( $X_{Na,i} = 0.83$ ) that is 10 kJ/mol, whereas  $\Delta\mu_{Na}$  changes from at least 12.9 to 4.0 kJ/mol, that is 8.9 kJ/mol.

By looking at  $Na_i^+$  block of the squid K channel, N-shaped I-Vs were first observed by French and Wells (French and Wells, 1977), whom after observing that block was overcome by very positive potentials, proposed that the relative permeability of  $Na^+$  to that of  $K^+$  ( $P_{Na}/P_K$ ) increased at those voltages (more than +160 mV). This proposal has received support from a recent study of the change of the  $P_{Na}/P_K$  ratio of *Shaker* channels, during the onset of slow inactivation (Starkus et al., 2000). In the simple case where a single, rate limiting, barrier  $B$  limits ion permeation through a channel, the relative permeability of a test ion ( $Na^+$ ) to that of a reference ion ( $K^+$ ) can be estimated with the relation (Reuter and Stevens, 1980; Latorre and Miller, 1983):  $P_{Na}/P_K = \exp[(B_{Na} - B_K)/RT]$ , where  $R$ ,  $T$  have their usual meanings.

Following this simplifying approach, and taking  $B_K = 0$ , and  $B_{Na}$  as in Fig. 5 A, it is seen that when  $X_{Na,i}$  increases from 0.71 to 0.83 (1.2 times),  $P_{Na}/P_K$  increases about four times (Fig. 5 B). The results in Figs. 3–5 show that as  $X_{Na,i}$  increases there is an actual reduction (i.e., a reduction not entirely accounted for by the change in  $\Delta\mu_{Na}$ ) of the energy required to relieve  $Na_i^+$  block, yielding a substantial change of the  $P_{Na}/P_K$  ratio.

The above effect could be explained as the result of an increase in the electrostatic repulsion between the blocking  $Na^+$  and a neighbor ion, as  $X_{Na}$  increases. However, it is not easy to see why this repulsion should be bigger as the molar fraction of the permeant  $K^+$  ion decreases. Alternatively, it could be that as  $X_{Na,i}$  increases, there is a deformation of the pore, measured as a reduction of the energy required to allow  $Na^+$  permeation. If that were

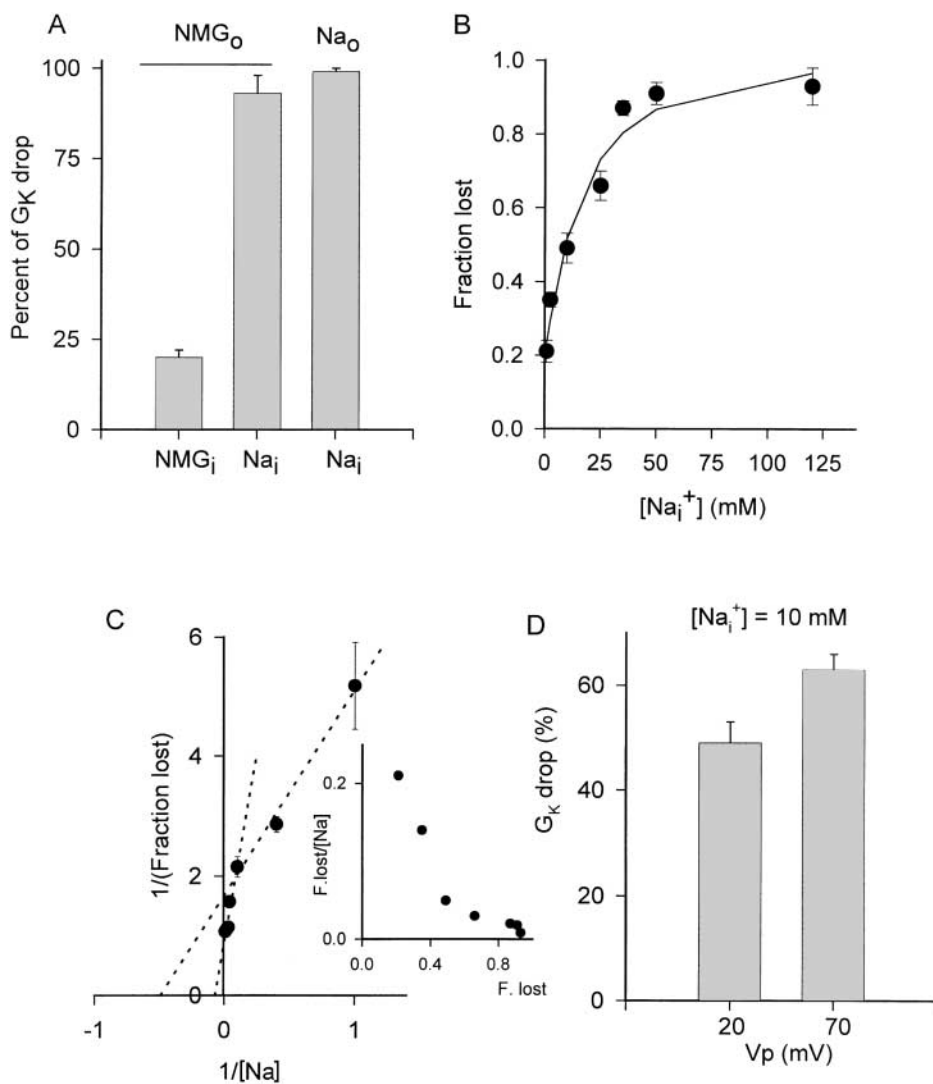


FIGURE 6.  $G_K$  drop in, 0  $K^+$ ,  $Na^+$ -containing internal solutions. (A).  $G_K$  reduction after pulsing with the cell bathed in either  $NMG_o/NMG_i$ ,  $NMG_o/Na_i$ , or  $Na_o/Na_i$  solutions, as indicated. (B).  $G_K$  reduction,  $1 - (I/I_o)$ , as a function of  $[Na_i^+]$ .  $I$  is the control  $I_K$  at +20 mV, and  $I_o$  is the current left after pulsing with the cell in  $NMG_o/(NMG_i - [Na_i^+])$  with the indicated  $[Na_i^+]$ , like in Fig. 2 (MATERIALS AND METHODS). The line is the fit of the points with a Hill equation with  $n = 1.16$  and an offset = 0.2 that accounts for the drop in, 0- $Na^+$ ,  $NMG$  solutions. The points are the mean  $\pm$  SEM of at least four cells at each  $[Na_i^+]$ . (C) Double reciprocal plot of the points in B. The two dotted lines are the least-squares fit of the points, on them, with the following parameters:  $r = 0.994$ ,  $K_{d1} = 2$  mM, maximal collapse<sub>1</sub> (m.c)<sub>1</sub> = 0.6; and:  $r = 0.970$ ,  $K_{d2} = 14.3$  mM, (m.c)<sub>2</sub> = 0.36. The inset shows the Eadie-Scatchard plot of the points in B. (D)  $G_K$  drop as a function of the pulse potential, during pulsing in  $NMG_o/(NMG_i - [Na_i^+])$ , with  $[Na_i^+] = 10$  mM.

the case, it could be that this deformation were maximal in the limit where  $X_{Na,i} = 1$ . Pulsing under the latter conditions ( $Na_o/Na_i$ ) collapses  $G_K$  (Gómez-Lagunas, 1997). Finally, as aforementioned (Fig. 2), the voltage dependence of external  $Na^+$  and  $Ba^{2+}$  actions, as well as that of internal  $Ba^{2+}$  (Gómez-Lagunas, 1999), might be explained by the involvement of two sites in the drop of  $G_K$ ; thus, to explore this point, the role of internal  $Na^+$  on the fall of  $G_K$ , when  $X_K = 0$ , was studied.

Fig. 6 A shows that pulsing with  $Na^+$  present (added) in only the internal solution ( $NMG_o/Na_i$ ), drops  $G_K$  in about the same extent ( $93 \pm 5\%$ ,  $n = 6$ ) as it does with  $Na^+$  present on both sides of the membrane (last bar,  $99 \pm 1\%$ ,  $n = 4$ ). For comparison, the figure also shows the collapse in  $NMG_o/NMG_i$ , as in Fig. 1. The above observation suggests that  $Na^+$  could possibly act on the same site regardless of the side of the membrane from where it comes. To further explore this point the collapse of  $G_K$  as a function of  $[Na_i^+]$  (in  $NMG_o/(NMG_i - [Na_i^+])$ ) was determined. Fig. 6 B shows that as  $[Na_i^+]$  increases  $G_K$  drops following a Hill curve (line through the points)

with  $n = 1.16$  (which indicates that there is, at least, one site where  $Na_i^+$  binds) plus an offset, that accounts for the collapse in  $NMG_o/NMG_i$  (0.20, Fig. 6 A). However, Fig. 6 C shows that the double-reciprocal plot of the points in B is best fitted by two straight lines, which indicates that  $Na_i^+$  binds to two sites: one with a maximal collapse (m.c) of 60% and  $K_d = 2$  mM, and a second, lower affinity site, with m.c = 36% and  $K_d = 14.3$  mM. The presence of two sites is also clearly distinguished in the inset, which presents the Eadie-Scatchard plot of the points in B (Segel, 1993) (see DISCUSSION). The voltage dependence of  $Na_i^+$  action is presented in Fig. 6 D, which shows that with a nonsaturating  $[Na_i^+] = 10$  mM, increasing  $V_p$  from +20 to +70 mV significantly increases ( $P < 0.05$ ) the drop of  $G_K$ : as if promoting the  $Na^+$  occupancy of the most externally located site, fostered the fall of  $G_K$ . On the other hand, the HP during pulsing (-80 vs. -120 mV), does not exert a significant effect on the extent of collapse (not shown).

It is pertinent to mention that by contrast to the qualitative similarity of the voltage dependence of the  $Na_o^+$



and  $\text{Ba}_o^{2+}$  effects, the voltage dependence of  $\text{Na}_i^+$  and  $\text{Ba}_i^{2+}$  actions are different. The latter decreases markedly with hyperpolarized HPs, and it is not dependent on  $V_p$  (Gómez-Lagunas, 1999). Further work is needed to understand this difference.

#### DISCUSSION

The *Shaker* B  $G_K$  collapses when the channels close in 0-K<sup>+</sup> solutions (Gómez-Lagunas, 1997, 1999; Melishchuk et al., 1998). Prolonged depolarizations are needed to recover  $G_K$  (Gómez-Lagunas, 1997). The drop of  $G_K$  seems to involve major changes in the conformation of the channels including not only the pore (Gómez-Lagunas, 1997), but also the voltage sensor (Melishchuk et al., 1998). So it could be that recovery is so slow due to the stability of the collapsed conformation, and to the number of rearrangements needed to reset the channels in their normal conducting state.

Here, it was shown that the combined condition 0 K<sup>+</sup> and presence of  $\text{Na}^+$  makes  $G_K$  more liable to collapse, as  $\text{Na}^+$  ions speed (in number of pulses) the drop of  $G_K$ .

Nonetheless,  $\text{Na}^+$  is not necessary for  $G_K$  to collapse, as it also falls in NMG or choline solutions. Considering that  $\text{Na}^+$  binds with millimolar affinity from both sides of the membrane, it seems unlikely that the drop of  $G_K$  in the 0- $\text{Na}^+$  solutions, could be produced by contaminant  $\text{Na}^+$  ions. More likely, the binding of  $\text{Na}^+$ , instead of  $\text{K}^+$ , or just the absence of  $\text{K}^+$  in the pertinent site(s) when the channels close, elicits the drop of  $G_K$ , although with significantly different speeds that might be accounted for by a smaller dwelling time of  $\text{K}^+$ , in the pertinent site(s), in the presence of  $\text{Na}^+$  ions than in their absence, in agreement with the observed mutually exclusive binding of  $\text{K}^+$  and  $\text{Na}^+$  ions to the externally located site.

#### *Extracellular $\text{Na}^+$ and the Drop of $G_K$*

Under physiological conditions,  $\text{Na}_o^+$  neither permeates nor blocks Kv channels (but see Block and Jones, 1996), however, it effectively fosters the drop of  $G_K$ , by binding to a site with an apparent  $K_d = 3.3$  mM. External  $\text{Na}^+$  and  $\text{K}^+$  compete, in a mutually exclusive manner, for binding to this site, and from this observation a high, micromolar, affinity for  $\text{K}_o^+$  was estimated,  $K_d = 80$   $\mu\text{M}$ . The presence of high affinity binding sites for  $\text{K}^+$  in the selectivity filter of the channels, from where, under physiological conditions,  $\text{Na}^+$  is excluded because of its lower affinity, has been shown to underlie the structural basis of the mechanism of selectivity of K channels (e.g., Korn and Ikeda, 1995; Doyle et al., 1998; Ogielska and Aldrich, 1998).

Could the site where  $\text{K}_o^+$  and  $\text{Na}_o^+$  bind modulating  $G_K$  be located within the selectivity filter? The protection exerted by TEA<sub>o</sub> suggests that the site is externally located (Gómez-Lagunas, 1997), as the selectivity filter

is (Doyle et al., 1998). Nonetheless, the voltage dependence of  $\text{Na}_o^+$ ,  $\text{Ba}_o^{2+}$ , and  $\text{K}_o^+$  actions (Gómez-Lagunas, 1999; Fig. 2), along with the observation that the potency with which monovalent cations protect is not related to the selectivity of the channels (Gómez-Lagunas, 1997), together suggest that this site could be external to the selectivity filter.

However, considering that in multi-ion pores, the voltage dependence of ligand binding depends on ion occupancy (Neyton and Miller, 1988a,b; Spassova and Lu, 1999; Thompson and Begenisich, 2001), and that selectivity of Kv channels depends on the  $[\text{K}^+]$  (Korn and Ikeda, 1995; Ogielska and Aldrich, 1998; Fig. 5), the selectivity filter cannot yet be ruled out as the externally located place where  $G_K$  is modulated (Melishchuk et al., 1998). Although, the drop of  $G_K$  with  $[\text{Na}_o^+]$  follows a Hill equation with  $n = 0.97$  and a linear double reciprocal plot, the reduced effectiveness of  $\text{Na}_o^+$  at hyperpolarized HPs, suggested the involvement of more than one site in the collapse of  $G_K$ . This possibility was reinforced by the kinetics of the drop of  $G_K$  with  $[\text{Na}_i^+]$  (see below).

#### *Internal $\text{Na}^+$ Interaction with *Shaker**

For voltages up to +160 mV, I-V relationships obtained with the  $\text{Na}_o$  solution, and with a variable  $X_{\text{Na},i}$ , have a shape that depends on  $X_{\text{Na},i}$ . With  $X_{\text{Na},i} \geq 0.71$ , the I-Vs are N-shaped. In contrast to the behavior of *Shaker* channels, French and Wells (1977) did not find a significant change of  $V_c$  with the internal  $\text{Na}^+$  in the squid K channel. It would be interesting to determine if the differential dependence of the I-V relationships on  $X_{\text{Na},i}$  of these channels, keeps any relation to their different behavior in 0-K<sup>+</sup> solutions (Almers and Armstrong, 1980; Khodakhah et al., 1997; Gómez-Lagunas, 1997, 1999).

Recently, by looking at the onset of the slow inactivation of *Shaker* channels lacking the NH<sub>2</sub> terminus domain, Starkus et al. (2000) afforded evidence showing that once  $V_c$  is reached and the conductance becomes again positive,  $\text{Na}^+$  actually flows throughout the channels, carrying current from the internal to the external solution. This supports the proposal of French and Wells (1977) that selectivity is voltage-dependent. The results presented here show that the *Shaker* selectivity also varies with  $X_{\text{Na},i}$ . It is pertinent to point out that an analogous statement has been made for the squid  $\text{Na}^+$  channel (Cahalan and Begenisich, 1976).

It has been shown that mammalian Kv2.1 channels as well as DR channels of bullfrog neurons although remain stable in 0 K<sup>+</sup>, conducting  $\text{Na}^+$ , undergo a conformational change of the pore region, which is observed as a reduction of the capability of TEA to block the channels (Korn and Ikeda, 1995; Block and Jones, 1997). Likewise, it seems that as  $X_{\text{Na},i}$  increases there is a deformation of the *Shaker* pore, which is observed as a reduction of the energy barrier that limits  $\text{Na}_i^+$  efflux.

That deformation could be maximal when  $X_K = 0$ , but further studies are needed to determine its relation with the collapse of  $G_K$  here studied.

When  $X_{K,i} = 0$ , pulsing with  $Na^+$  ions added to the internal solution alone drops  $G_K$  in about the same extent as it does with  $Na^+$  on both sides of the membrane, suggesting that  $Na_i^+$  might be able to reach the externally located site where  $G_K$  is modulated, in agreement with the observation that  $V_c$  decreases as  $X_{Na,i}$  approaches one, and with reports showing  $Na^+$  currents through Kv channels in 0  $K^+$  (Zhu and Ikeda, 1993; Korn and Ikeda, 1995; Block and Jones, 1997; Starkus et al., 1997, 2000; Melishchuk et al., 1998; Ogielska and Aldrich, 1998; Zhuren et al., 2000). On the other hand, the  $[Na_i^+]$  dependence of  $G_K$  drop indicates that there are actually two binding sites, of which the one with the higher affinity ( $K_d = 2$  mM) could be the externally located site ( $K_d = 3$  mM). It might be that presence of two sites could form the structural basis of the  $X_{Na,i}$  dependence of  $V_c$ . Whatever the case, it is pertinent to mention that the possible involvement of two sites in the collapse of  $G_K$  was previously suggested by the characteristics of the protection afforded by internal  $Ba^{2+}$  (Gómez-Lagunas, 1999).

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