Inhibition of the Collapse of the *Shaker* K^+ Conductance by Specific Scorpion Toxins

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ABSTRACT The *Shaker* B K^+ conductance (G_K) collapses when the channels are closed (deactivated) in Na^+ solutions that lack K^+ ions. Also, it is known that external TEA (TEA_o) impedes the collapse of G_K (Gómez-Lagunas, F. 1997. *J. Physiol.* 499:3–15; Gómez-Lagunas, F. 2001. *J. Gen. Physiol.* 118:639–648), and that channel block by TEA_o and scorpion toxins are two mutually exclusive events (Goldstein, S.A.N., and C. Miller. 1993. *Biophys. J.* 65:1613–1619). Therefore, we tested the ability of scorpion toxins to inhibit the collapse of G_K in 0 K^+. We have found that these toxins are not uniform regarding the capacity to protect G_K. Those toxins, whose binding to the channels is destabilized by external K^+, are also effective inhibitors of the collapse of G_K. In addition to K^+, other externally added cations also destabilize toxin block, with an effectiveness that does not match the selectivity sequence of K^+ channels. The inhibition of the drop of G_K follows a saturation relationship with [toxin], which is fitted well by the Michaelis-Menten equation, with an apparent Kd bigger than that of block of the K^+ current. However, another plausible model is also presented and compared with the Michaelis-Menten model. The observations suggest that those toxins that protect G_K in 0 K^+ do so by interacting either with the most external K^+ binding site of the selectivity filter (suggesting that the K^+ occupancy of only that site of the pore may be enough to preserve G_K) or with sites capable of binding K^+ located in the outer vestibule of the pore, above the selectivity filter.

KEY WORDS: ion channel • conductance • *Shaker* • toxin • zero-K^+

INTRODUCTION

Potassium ions modulate the gating and stabilize the normal structure of voltage-dependent K^+ channels (Kv channels). The stabilizing role of K^+ ions, still not well understood, becomes evident when Kv channels are exposed to solutions that lack K^+ ions. In the case of *Shaker* B, the channels support well prolonged exposures to O K^+ (not added) solutions on both sides of the membrane. No noticeable changes are observed, as long as they are not gated while they are bathed in O K^+. However, when the channels are gated the K^+ conductance (G_K) collapses: the channels sink into a stable, noninactivated, nonconducting conformation (Gómez-Lagunas, 1997).

The drop of G_K is greatly accelerated by the presence of Na^+ ions in the 0 K^+ solutions (Gómez-Lagunas, 2001). In contrast, the collapse of G_K is prevented by some permeant or blocking ions, like for example external TEA (Gómez-Lagunas, 1997, 1999).

The extent of drop of G_K depends on the number of activating pulses delivered in 0 K^+ (hereafter referred to as pulsing), but it does not depend on the frequency of pulsing. On the other hand, pulsing from depolarized holding potentials does not affect G_K. Prolonged depolarizations reset nonconducting channels back into their normal conformation, capable of conducting K^+ ions (Gómez-Lagunas, 1997).

Altogether, the above observations were interpreted as indicating that G_k drops when the channels deactivate in 0 K^+, at the end of each activating pulse (Gómez-Lagunas, 1997). Posterior experiments, done with Shaker channels lacking the N-type inactivation, have given support to this hypothesis (Melishchuk et al., 1998; Loboda et al., 2001). The hypothesis is in accordance with the classical observations of Swenson and Armstrong (1981), showing that K^+ ions play an important role in the closing of K^+ channels.

The drop of G_K suggests that in 0 K^+ conditions the pore of the channels undergoes a structural modification. Moreover, recent observations suggest that this modification is not a discontinuous phenomenon, suddenly observed in 0 K^+, but that, on the contrary, it

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Abbreviation used in this paper: SF, selectivity filter.

likely develops continuously as the molar fraction of K^+ decreases (Gómez-Lagunas, 2001). These conclusions are supported by high-resolution crystallographic images of the pore of KcsA channels, recently obtained by MacKinnon and coworkers, which show that the selectivity filter (SF) presents structural differences in low versus high concentrations of K^+ ions (Zhou et al., 2001).

Scorpion toxins that block K channels (hereafter toxins) are basic peptides composed of 23–43 amino acid residues stabilized by 3 or 4 disulfide bridges, showing a conserved three-dimensional folding made by a stretch of α-helix and two or three strands of antiparallel β-sheet structure (Miller, 1995; Giangiacomo et al., 1999; Possani et al., 1999; Corona et al., 2002; Rodriguez de la Vega et al., 2003).

The extensive studies on the interaction between toxins and K channels performed in the last years have shown that these small peptides block different types of Kv channels, basically following the same mechanism: The binding to the channels is reversible, the stoichiometry is one toxin/one channel molecule, and the binding surface is the extracellular vestibule of the pore (Anderson et al., 1988; MacKinnon and Miller, 1988, 1989; Miller, 1995; MacKinnon et al., 1998).

It is important to note that although the general characteristics of the mechanism of block seem to be conserved some, physiologically significant, features of the interaction between toxins and channels are variable, as expected from their variable primary sequences. For example, and relevant for the present work, it has been reported that block of *Shaker* channels by native charybdotoxin (ChTx) is not altered by external K^+ ions (K_o^+), whereas block by a mutant R25Q (a glutamine substituting arginine in position 25) of ChTx is destabilized by K_o^+ ions (Goldstein and Miller, 1993), likely because the change R25Q allows the mutant ChTx to interact with a site(s) capable of binding K^+ in the outer vestibule of the pore (Goldstein and Miller, 1993).

Block of *Shaker* channels by toxins and external TEA (TEA_o) are two mutually exclusive processes (Goldstein and Miller, 1993). Therefore, considering that TEA_o impedes the drop of G_K in 0 K^+, with basically the same affinity (Kd) with which it blocks the K^+ current (I_K) through the channels (Gómez-Lagunas, 1997), the simple hypothesis immediately arises that scorpion toxins should also be effective inhibitors of the collapse of G_K in 0 K^+. This work was performed to test this simple idea.

We have found that, in contrast to our hypothesis, a high-affinity toxin against *Shaker* channels was unable to effectively inhibit the drop of G_K in 0 K^+. Searching an explanation for this fact, we found that scorpion toxins are indeed able to effectively inhibit the drop of G_K when their blocking capacity is destabilized by external K^+ ions. Moreover, we have found that externally added cations destabilize block with an effectiveness that does not match the selectivity sequence of K^+ channels. In other words, the toxins should make a significant functional contact with site(s) capable of binding K^+, which could be located in the external vestibule of the pore. Additionally, we show that protection against the drop of Gk as a function of [toxin] follows a saturation relationship. Among several possible models to explain our results, the application of the Michaelis-Menten equation, in principle, satisfies the experimental data obtained, and it shows a significant increment in the apparent Kd for protection in 0 K^+ compared with that of block of I_K. However, in DISCUSSION, another plausible model is presented and compared with the widespread known Michaelis-Menten model.

MATERIALS AND METHODS

Cell Culture and Shaker B Channels Expression

Insect Sf9 cells were kept in culture in Grace's Media (GIBCO-BRL) at 27°C. The cells were infected, with a multiplicity of infection of 10, with a recombinant baculovirus (*Autographa californica* nuclear ployhedrosis virus) containing the cDNA of *Shaker* B K^+ channels (Klaiber et al., 1990). Experiments were done 48 h after the infection, as reported previously (Gómez-Lagunas, 2001).

Scorpion Toxin Purification

The toxins Pi1 (α-KTx 6.1, see Tytgat et al., 1999) and Pi2 (α-KTx 7.1) were purified from the venom of the scorpion *Pandinus imperator* as reported previously (Olamendi-Portugal et al., 1996; Gómez-Lagunas et al., 1996). Tc30 (α-KTx 4.4) was purified from the venom of the scorpion *Tityus cambridgei* as reported (Batista et al., 2002).

Electrophysiological Recordings

Macroscopic currents were recorded under whole cell patch-clamp with an Axopatch 1D (Axon Instruments, Inc.). The currents were filtered at 5 KHz with the filter of the amplifier, and sampled every 100 ms with a TL1 interface (Axon Instruments, Inc). Electrodes were pulled from Borosilicate glass (KIMAX 51) to a 1.5–2 MΩ resistance; ~80% of the series resistance was electronically compensated.

Solutions

Solutions will be named according to their main cation and the side of its application, and represented as external/internal (e.g., Na_O/K_i). The external solutions contained (in mM), Na_o: 145 NaCl, 10 CaCl2, 10 HEPES-Na buffer, pH 7.1; K_o: 100 KCl, 45 NaCl, 10 $CaCl_2$, 10 HEPES-Na buffer, pH 7.1. The internal solutions contained (in mM), K_i: 90 KF, 30 KCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES-K buffer, pH 7.2; Na_i: 90 NaF, 30 NaCl, 2 $MgCl_2$, 10 EGTA, 10 HPES-Na buffer, pH 7.2. Other solutions were prepared by replacing NaCl with the corresponding equiosmolar amounts of XCl, where X stands for K^+, Cs^+, or NH_4^+ as indicated, keeping both the osmolarity and ionic strength constants. The purified, dried toxins were dissolved in the indicated external solution before being added to the recording chamber.

Data Analysis

Results are expressed as mean ± SEM of the indicated number of cells. Where necessary the *t* test was used to evaluate statistical significance ($\alpha = 0.05$).

RESULTS

G_K Protection by Specific Scorpion Toxins

To test the hypothesis that scorpion toxins should be able to inhibit the drop of G_K in 0 K^+ (not added) solutions (see INTRODUCTION), we decided to use Tc30 (α-KTx 6.1, see Fig. 1), a recently described toxin (Batista et al., 2002) obtained from the venom of the scorpion *Tityus cambridgei* that blocks *Shaker* channels with nanomolar affinity (Kd = 68 nM, see below). Fig. 2 reports the test with Tc30 and, as a reference, also shows the collapse-recovery cycle of G_K in 0 K^+ solutions.

Fig. 2 A presents inward K^+ currents (I_K) through *Shaker* B, evoked by +20-mV/30-ms activating pulses, in K_o/Na_i solutions (see MATERIALS AND METHODS). Three traces are superimposed showing the stability of I_K. Once the control I_K were recorded, the cell was superfused with the Na_o solution containing 360 nM Tc30 (~5 times its Kd for blockage, see below), and 20 activating pulses were applied to repeatedly gate the channels in the 0 K^+ test solution (Na_o + Tc30/Na_i). As expected, there was no time-dependent current in any of the pulses (Fig. 2 B). Afterwards, the cell was immediately superfused with the control K_o solution, and then the state of the channels was tested with the delivery of activating pulses. The traces in Fig. 2 C (I_2), show that most of the channels were still able to conduct K^+ ions.

The ~91% preservation of I_K in Fig. 2 C indicates that Tc30 is an effective inhibitor of the drop of G_K in 0 K^+. To better demonstrate the latter statement, and to show the collapse-recovery cycle of G_K, once the currents in Fig. 2 C were recorded the cell was superfused with the Na_o solution (but this time without Tc30) and a round of 20 pulses was applied, with the cell bathed in Na_o/Na_i solutions (Fig. 2 D), as in Fig. 2 B. Subsequently, the cell was superfused back with the K_o solution and the state of the channels was tested. The traces in Fig. 2 E show that, in contrast to Fig. 2 C, there was a complete drop of G_K. The fall of G_K occurred because, without Tc30, the channels sank into the stable nonconducting, noninactivated conformation during the previous pulsing episode in 0 K^+. Fig. 2 F shows the recovery of I_k brought about by a 3-min depolarization to 0 mV (see Gómez-Lagunas, 1997, 2001).

In Fig. 2 C the first current recorded with the cell back in K_o (labeled I_1) has a slower activation, and thus reaches a slightly smaller peak amplitude than the current recorded in the next pulses (collectively labeled as I_2). The latter is best seen in Fig. 2 G, which compares the average time to peak of I_1 versus I_2 of four cells after pulsing in the presence of 360 nM Tc30. A similar behavior ($I_1 \neq I_2$) was observed previously with Ba^{2+} ions as the protective agent in 0 K^+, except that with Ba^{2+} the effect was much more evident (Gómez-Lagunas, 1999; see DISCUSSION).

The observations in Fig. 2 could be interpreted as meaning that Tc30 protects G_K just because it may trap K^+ ions in the pore of the blocked channels, impeding its exit toward the 0 K^+ solutions. If that were the case then it would be expected that any toxin capable of blocking the channels with high affinity should also be an effective inhibitor of the collapse of G_K. Therefore, we decided to test the effect of Pi2 (α-KTx 7.1), a toxin from the venom of the scorpion *Pandinus imperator* (Fig. 1) that blocks *Shaker* with an even higher affinity (Kd = 8 nM) than Tc30 (Gómez-Lagunas et al., 1996).

Fig. 3 A presents three superimposed control I_K, recorded in K_o/Na_i. Thereafter, the cell was superfused with the Na_o solution containing 150 nM Pi2 (~19 times its Kd for block, an over excess compared with [Tc30] in Fig. 2) and 20 activating pulses were delivered (Fig. 3 B), as in Fig. 2 B. Thereafter, the cell was superfused back with the control K_o solution and the state of the channels was tested. The traces in Fig. 3 C show that, in contrast to the result of pulsing with Tc30 (Fig. 2 C), this time there was a dramatic drop of G_K. The drop of G_K in Fig. 3 C was caused by gating the channels, with 150 nM Pi2, in 0 K^+ (Fig. 3 B). The latter is demonstrated in Fig. 3 D that shows the recovery of I_K after a 3-min depolarization to 0 mV.

Fig. 3 E compares the extent of Pi2 protection (4 ± 3%, $n = 5$) of G_K in 0 K^+ (in Na_o+Pi2/Na_i) against the extent of Pi2 block of I_K (92 ± 0.02%, $n = 4$) with 0 K^+ (not added) in only the external side of the membrane, which is the side of toxin blockage (in Na_o/K_i). The extent of drop of G_K with 150 nM Pi2 present (96 ± 3%) is not significantly different to the extent of drop in only Na_o/Na_i (99 ± 1%, Fig. 1 of Gómez-Lagunas,

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      1                                  27        37

Pi2:  TIS---CTNPKQCYPHCKKETGYPNAKCMNRKCKCFGR

Tc30: VFINVKCRGSKECLPACKAAVGKAAGKCMNGKCKCYP

Pi1:  L---VKCRGTSDCGRPCQQQTGCPNSKCINRMCKCYGC

ChTx: ZFTNVSCTTSKECWSVCQRLHNTSRGKCMNKKCRCYS
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FIGURE 1. Amino acid sequence of the scorpion toxins used in this work. The sequences are aligned according to the conserved cysteines (bold), for a reference the sequence of Charybdotoxin (ChTx) is also included. All toxins have the critical lysine (bold) at position 27 of ChTx (Miller, 1995; Gómez-Lagunas et al., 1996; Olamendi-Portugal et al., 1996; Possani et al., 1999; Batista et al., 2002).

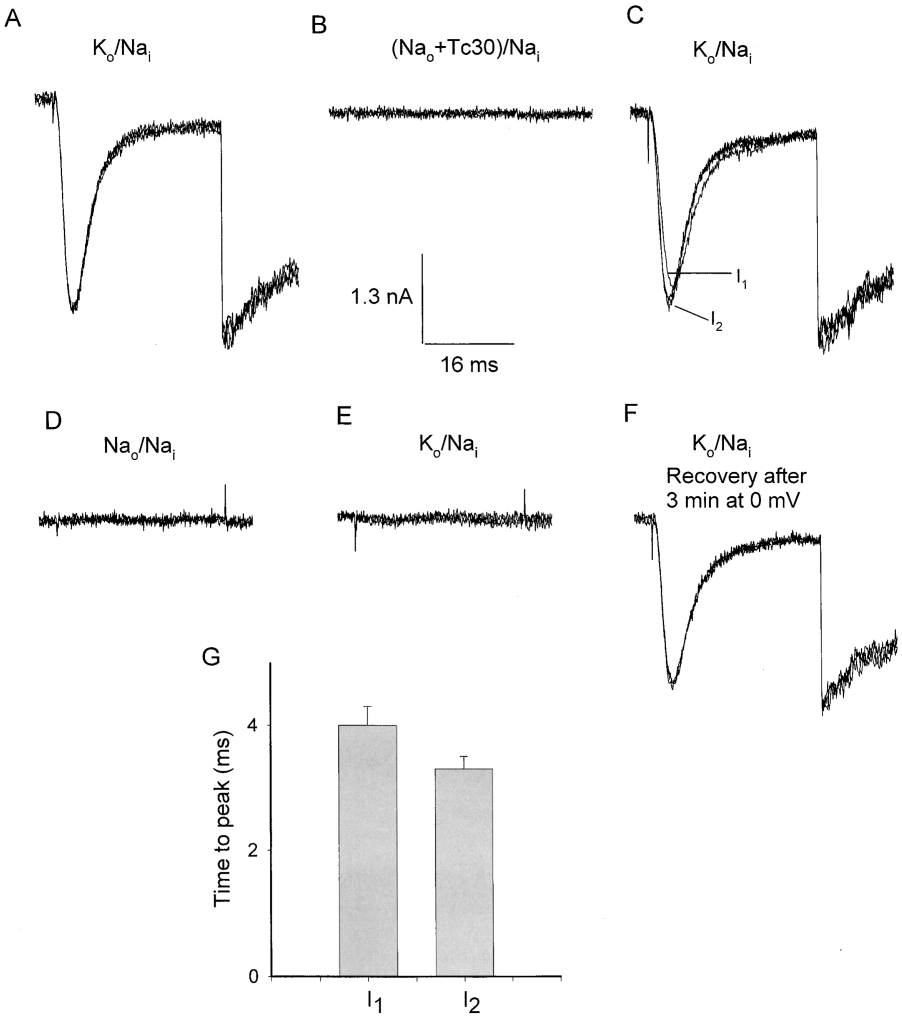

FIGURE 2. Tc30 inhibits the collapse of G_K in 0 K^+. (A) Control I_K evoked by three +20-mV/30-ms (activating) pulses in K_o/Na_i (see MATERIALS AND METHODS). The slow tails mark the end of the pulse. (B) Currents recorded by the delivery of 20 activating pulses applied at 1 Hz, in 0 K^+ (referred to as pulsing) with the cell bathed in (Na_o + Tc30)/Na_i, with [Tc30] = 360 nM. (C) I_K recorded with the cell back in K_o/Na_i immediately after pulsing in 0 K^+ in B. Tc30 inhibited the collapse of G_K. The current evoked by the first pulse applied back in K_o (I_1) differs from those evoked by the next pulses (together labeled as I_2) (see the text). (D) Currents recorded by pulsing in Na_o/Na_i (without Tc30), immediately after C. (E) Currents recorded with the cell back in K_o after the pulsing episode in D. Pulsing in 0 K^+ collapsed G_K. (F) I_K evoked by three activating pulses delivered 1 min after a 3-min depolarization period at 0 mV. Depolarization recovered G_K. (G) Comparison of the time to peak of I_1 versus I_2 after pulsing with 360 nM Tc30, as in C. The bars are the mean ± SEM of four cells. Scale bars are the same for all traces. HP, −80 mV.

2001). Clearly, Pi2 is not an effective inhibitor of the collapse of G_K.

The above observations strongly suggest that Tc30 protection of G_K (Fig. 2) is not due to the presence of K^+ ions that may have remained trapped in the pore of the channels that were blocked by Tc30 in the 0 K^+ solutions. That does not seem to be the case. Toxins are not uniform regarding the capacity to protect G_K. Thus, our data suggest that differences in specific contact points between particular toxins and the outer vestibule of *Shaker* may be of importance regarding the ability of the toxins to prevent the collapse of G_K. This possibility is tested below.

Toxin-specific Interaction with K^+ Binding Sites Correlates with their Effectiveness to Protect G_K

Previous observations have suggested the involvement of externally located K^+ binding site(s) in the collapse of G_K in 0 K^+ (Gómez-Lagunas, 1999, 2001). Thus, in order to understand the differences in the effectiveness of Tc30 and Pi2 to protect G_K, we decided to test the effect of the K^+ distribution across the membrane on the I_K block by both toxins.

Fig. 4 reports the effect of the K^+ distribution across the membrane on Pi2 block of I_K. Fig. 4 A, left, presents a control I_K at +50 mV, recorded in standard (Na_o/K_i) conditions. The subsequent addition of 150 nM Pi2 to the external Na_o solution blocked ~90% of the channels (middle panel), as indicated. Pi2 block was abolished by perfusing the cell with the control Na_o solution, as shown in the right panel (see also Gómez-Lagunas et al., 1996). Fig. 4, B and C, report the results of the same manipulation, but this time performed in cells placed in either K_o/K_i or K_o/Na_i solutions, respectively. Regardless of the K^+ distribution across the membrane, Pi2 blocks I_K with comparable efficiency. The latter is best seen in Fig. 4 D, which shows that the average extent of Pi2 block in the absence of K^+ in the external solution (Na_o/K_i; 92 ± 2%, n = 4) is not significantly different either to that with K^+ in both sides of the membrane (K_o/K_i; 88 ± 4%, n = 3) or to the extent of block with K^+ in only the external solution (K_o/Na_i; 86 ± 4%, n = 4).

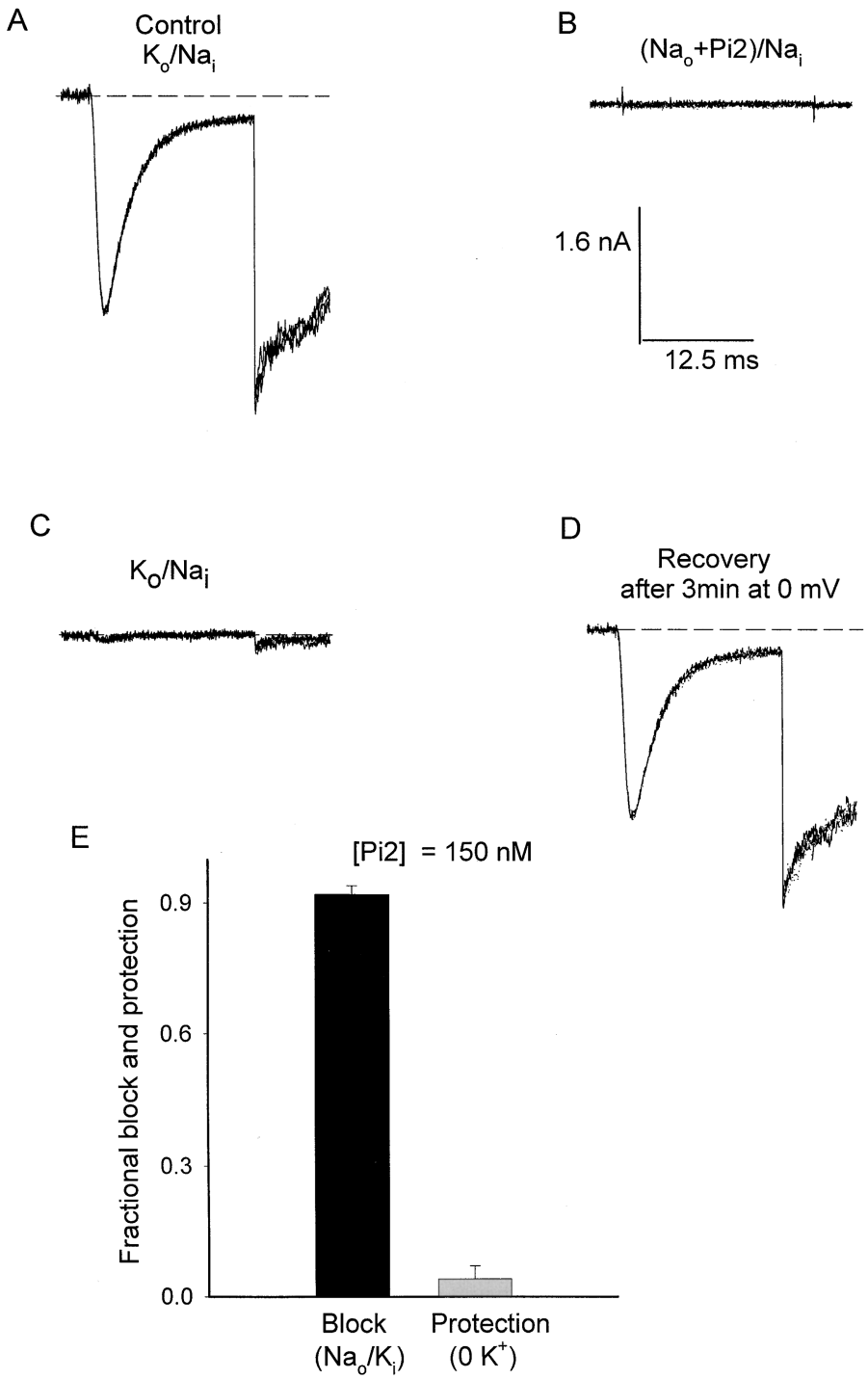

FIGURE 3. Pi2 is unable to inhibit the collapse of G_K in 0 K^+. (A) Control I_K evoked by three activating pulses in K_o/Na_i. (B) Currents evoked by pulsing in 0 K^+, with 150 nM Pi2 in the Na_o solution (Na_o + Pi2)/Na_i. (C) Four superimposed I_K, recorded every 20 s, with the cell back in K_o after pulsing in B. Pi2 was unable to impede the drop of G_K. (D) I_K recovery after a 3-min depolarization (like in Fig. 2 F), the panel shows four superimposed I_K. (E) Comparison of the average extent of Pi2 block of I_K in Na_o/K_i (92 ± 2%, n = 4) versus the extent of Pi2 protection of G_K in 0 K^+ (4 ± 3%, n = 5). Scale bars are the same for all traces. HP, −80 mV.

The above observations can be explained by the following nonexclusive possibilities: (a) Pi2 does not interact, in a functionally significant manner with K^+ binding sites of the channels; or (b) these K^+ sites are equally occupied by K^+ ions in all of the three conditions of Fig. 4 (see below).

We next turned our attention to Tc30 in order to determine if its blocking capacity was sensitive to the distribution of K^+ ions across the membrane. Fig. 5 A shows Tc30 block of I_K in standard recording conditions, as in Fig. 4 A. In Na_o/K_i solutions, 360 nM Tc30 block ~90% of the channels (see below). In contrast, with K^+ ions in both the internal and the external solutions (K_o/K_i) the extent of block is substantially reduced (~41%, Fig. 5 B). The decrease in the strength of Tc30 block is also observed when K^+ ions are present in only the extracellular solution (~49% block, K_o/Na_i, Fig. 5 C). It is clear that, although with low affinity, ex-

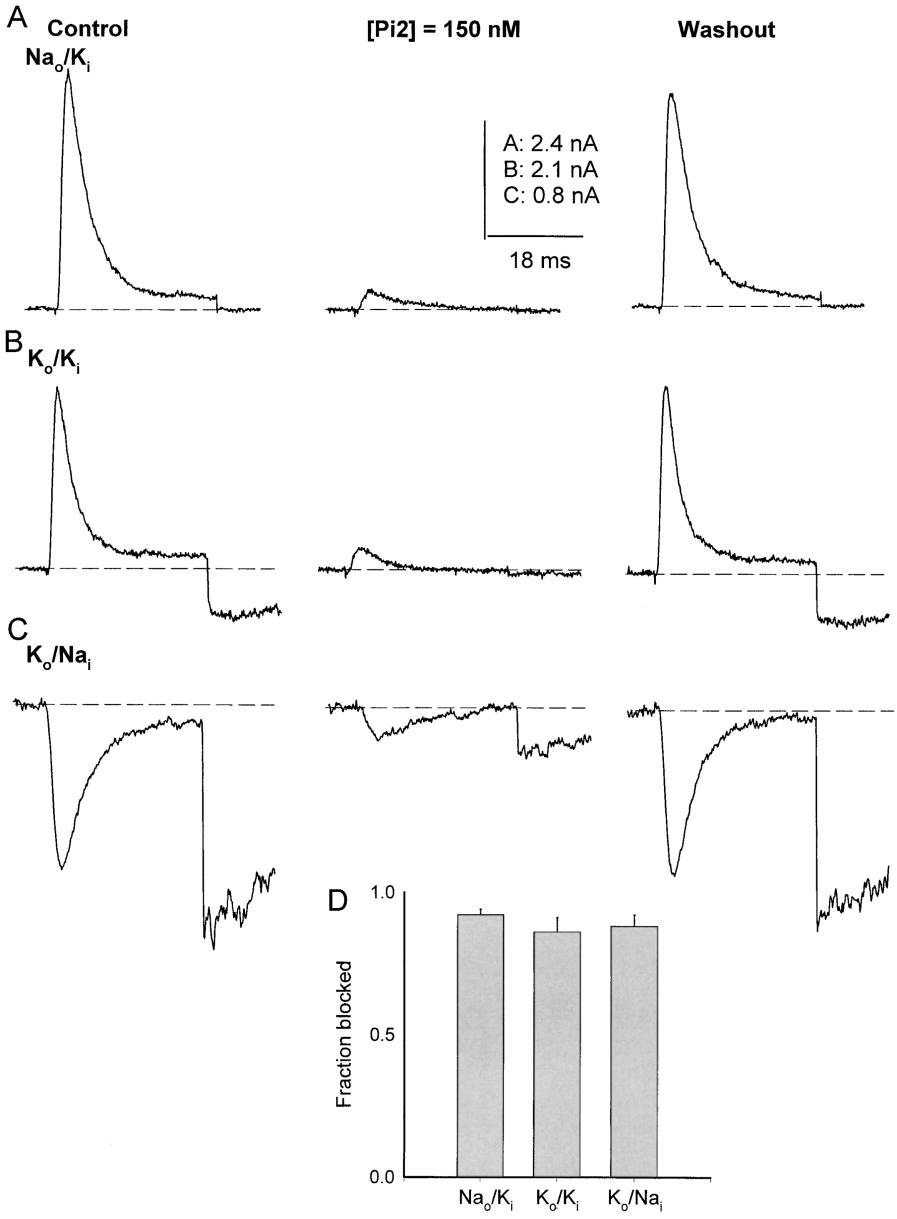

FIGURE 4. Pi2 blocks I_K with comparable efficiency regardless of the K^+ distribution across the membrane. (A) Pi2 block of I_K in Na_o/K_i: the left panel shows a control I_K activated by a +50-mV/30-ms pulse, the middle panel is the I_K left upon the addition of 150 nM Pi2 to the external solution; the right panel shows the recovery of I_K after washing the cell with the control external solution, as indicated. (B) Pi2 block in K_o/K_i as in A. (C) Pi2 block in K_o/Na_i, as in A except that the currents were activated by a +20-mV pulse. (D) Comparison of the average extent of Pi2 block from experiments like those in A–C. See text for details. The dashed lines signal de 0 current level. With K_O slow tails are seen at pulse end. HP, −90 mV.

ternal K^+ ions exert a significantly destabilization of Tc30 block of I_K. The observations also suggest that the insensitivity of Pi2 block of *Shaker* to the K^+ distribution across the membrane is not due to an equal occupancy of the pertinent K^+ sites in the three recording conditions of Fig. 4, but rather to a lack of a functional interaction between Pi2 and externally located K^+ binding sites.

Fig. 6 shows peak-current versus voltage relationships (left panel) of experiments like those in Fig. 5. The right panel presents the average extent of block, assessed from plots like those in the left panel, at a [Tc30] that blocks about half the channels. The relatively large standard error at the more negative pulse on each panel is due to the small size I_K at those voltages. Regardless of the K^+ distribution across the membrane, Tc30 block of wild-type Shaker channels is not appreciably affected by the membrane potential (see DISCUSSION).

The $[K_o^+]$ dependence of Tc30 block is further studied in Fig. 7. Additionally, in order to get some insight into the possible location of the site(s) involved in the destabilization of block, we also tested the effect of other externally added cations.

With physiological $[K^+]$ in the internal solution (like in Fig. 5 B), when either external $[K^+]$ or $[Cs^+]$ increases the extent of block by Tc30 decreases (Fig. 7 A, top). The experimental points follow a modified Hill equation (solid line through the points, see figure legend), with Hill number n for K^+ $n(K^+) = 1.44$,

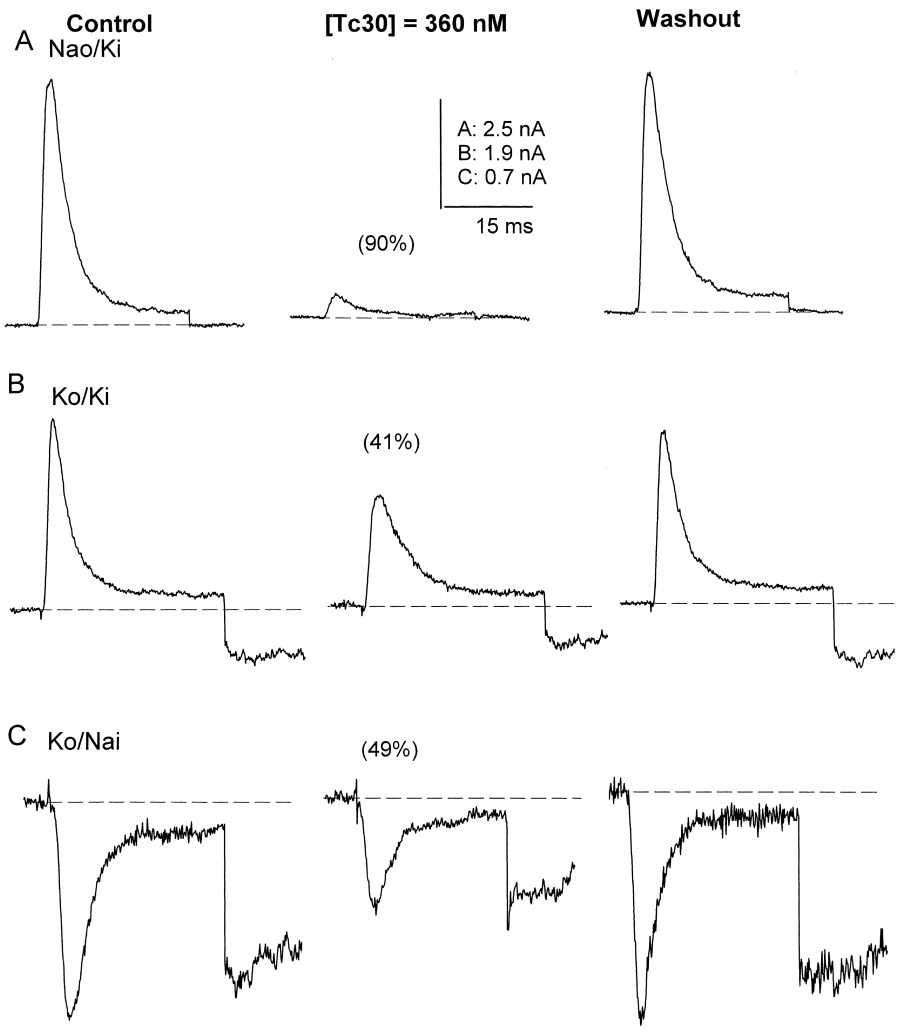

FIGURE 5. Tc30 block of I_K is destabilized by external K^+ ions. (A) Tc30 block of I_K in Na_o/K_i, like in Fig. 4 A. (B) Tc30 block of I_K in K_o/K_i, like in Fig. 4 B. (C) Tc30 block in K_o/K_i, like in Fig. 4 C. K_o^+ destabilizes Tc30 block of Shaker. HP, −90 mV.

$Ki(K^+)$ = 97 mM; and $n(Cs^+)$ = 2.3, $Ki(Cs^+)$ = 41 mM (see DISCUSSION). Cs_o^+, which is either poorly permeant or impermeant through K^+ channels, destabilizes block more effectively than K_o^+. In contrast, NH_4^+, which permeates K^+ channels, is like the impermeant Na^+ regarding the block of I_K. The dotted line joining the extent of block in Na_o with that at the indicated $[NH_4^+]$ has no theoretical meaning. The traces in the bottom panel illustrate the block of I_k, with either 100 mM NH_4^+ or 80 mM Cs^+ ions in the external solution, as indicated. The above observations show that: (a) in addition to K_o^+, other externally added cations (like Cs^+) destabilize Tc30 block, and (b) the effectiveness with which the tested cations destabilize block does not match the selectivity sequence of K^+ channels. The latter suggests that the ions may be acting in sites located outside the conduction pathway of the channels (see DISCUSSION).

Fig. 7 B compares the average extent of Tc30 block as a function of the K^+ distribution across the membrane, from experiments like those in Fig. 5. It is seen that: (a) a comparison of the first (Na_o/K_i) with the third (K_o/Na_i) bar suggests that external K^+ destabilizes Tc30 binding more effectively than internal K^+ and, consistent with this observation, (b) with 100 mM K^+ in the external solution (K_o), block is not significantly different with either Na^+ (Na_i) or K^+ (K_i) internal solutions (second and third bars). That is: external K^+ destabilizes the binding of Tc30 regardless of the internal K^+ (Na_i or K_i).

Fig. 8 A reports the extent of I_K block in standard recording conditions (Na_o/K_i, i.e., with 0 K^+ in the side of toxin action), as well as the extent of G_K protection in O K^+ (Na_o/Na_i), as a function of the indicated [Tc30]. It is seen that both the Ik block and Gk protection points follow a saturation relationship with [Tc30]. Therefore, as a first approach we fitted both of them with the Michaelis-Menten equation (lines through the points), which is commonly used to describe the interaction between toxins and channels. Notice that: (a) the Michaelis-Menten equation describes well both I_K block (as expected) and G_k protection by Tc30. Nonetheless, at this

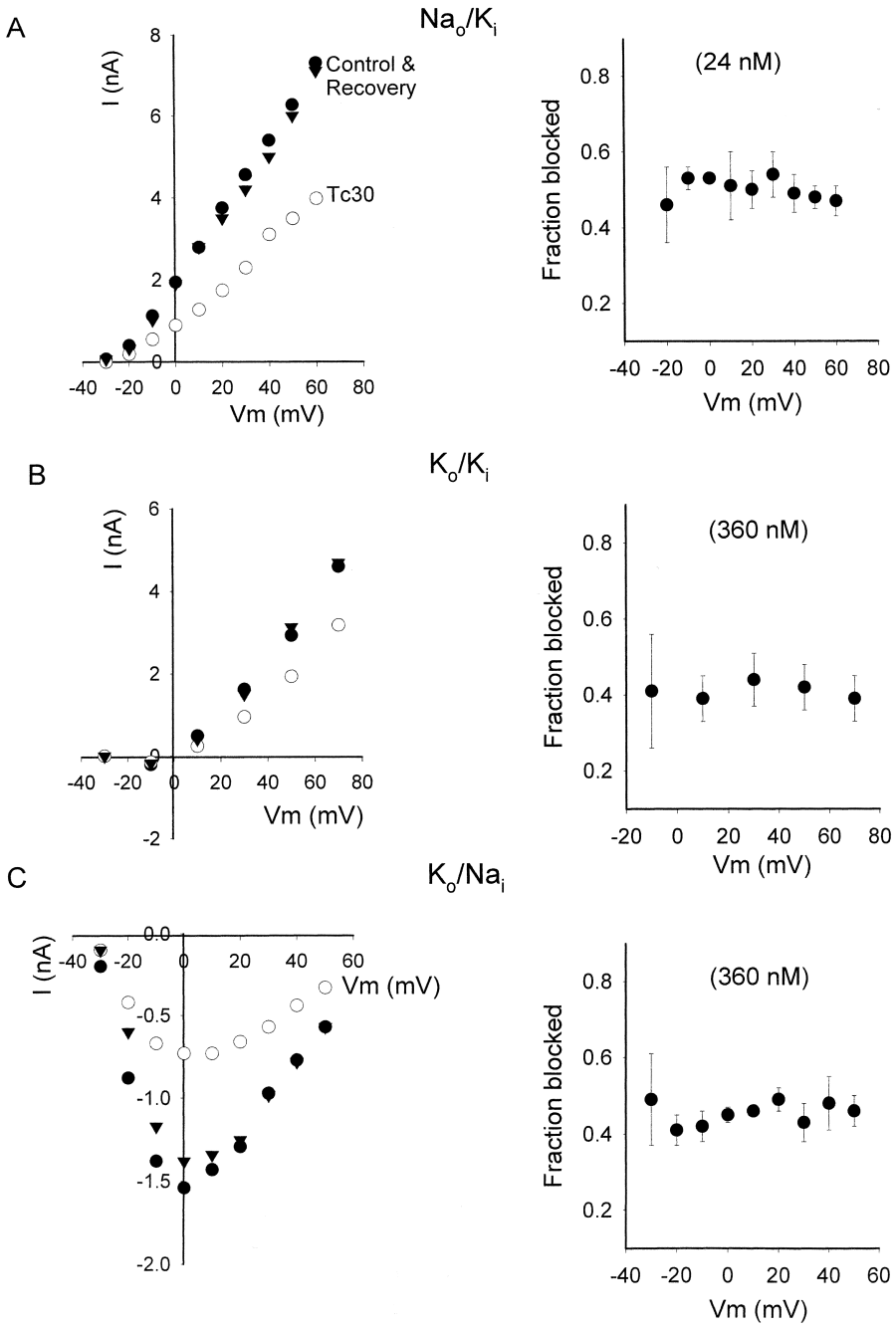

FIGURE 6. Tc30 block as a function of the membrane potential. (A, Left panel) I-V relationship, in Na_O/K_i, of an experiment like in Fig. 5 A. Filled circles, control I_K, open circles: I_K left after adding [Tc30] = 24 nM. Filled triangles, recovery of I_K after toxin removal. (Right) Fraction of the channels blocked as a function of the pulse potential. Fraction blocked = 1 − (I/Io), where Io is the control peak current and I is the current left in the presence of Tc30. (B) As in A, but with the cell in K_o/K_i, and [Tc30] = 360 nM. (C) As in B, but with the cell in K_o/Na_i. The points in the right panels are the mean ± SEM of at least three cells on each condition.

point it should be mentioned that Tc30 protection in 0 K^+ can also be described by alternative models that may lead to conclusions different from those of the Michaelis-Menten equation (see DISCUSSION). (2) Although Tc30 is an effective inhibitor of the collapse of G_K, according to the Michaelis-Menten model the apparent Kd for G_K protection (188 nM) is ~3 times that of I_K block (68 nM). This is best seen in Fig. 8 B, which shows the double-reciprocal plot of the points in A.

The latter observation could be interpreted in at least two ways: it could be argued that at nonsaturating [Tc30], where a fraction of the channels collapse during pulsing in 0 K^+, there is a partition of the population of toxin molecules between noncollapsed and collapsed channels, this would be formally equivalent to a competitive inhibition assay of enzyme kinetics, and would bring about an apparent increase in the Kd of toxin protection of G_k. On the other hand, the increase in the apparent Kd for G_K protection suggests that, with 0 K^+ on both sides of the membrane, there could be a conformational change in the outer vestibule of the pore that decreases the binding affinity of Tc30 (see DISCUSSION).

The reduced apparent affinity of Tc30 in 0 K^+ (protection of G_K in Fig. 8 B) suggests that the ineffective-

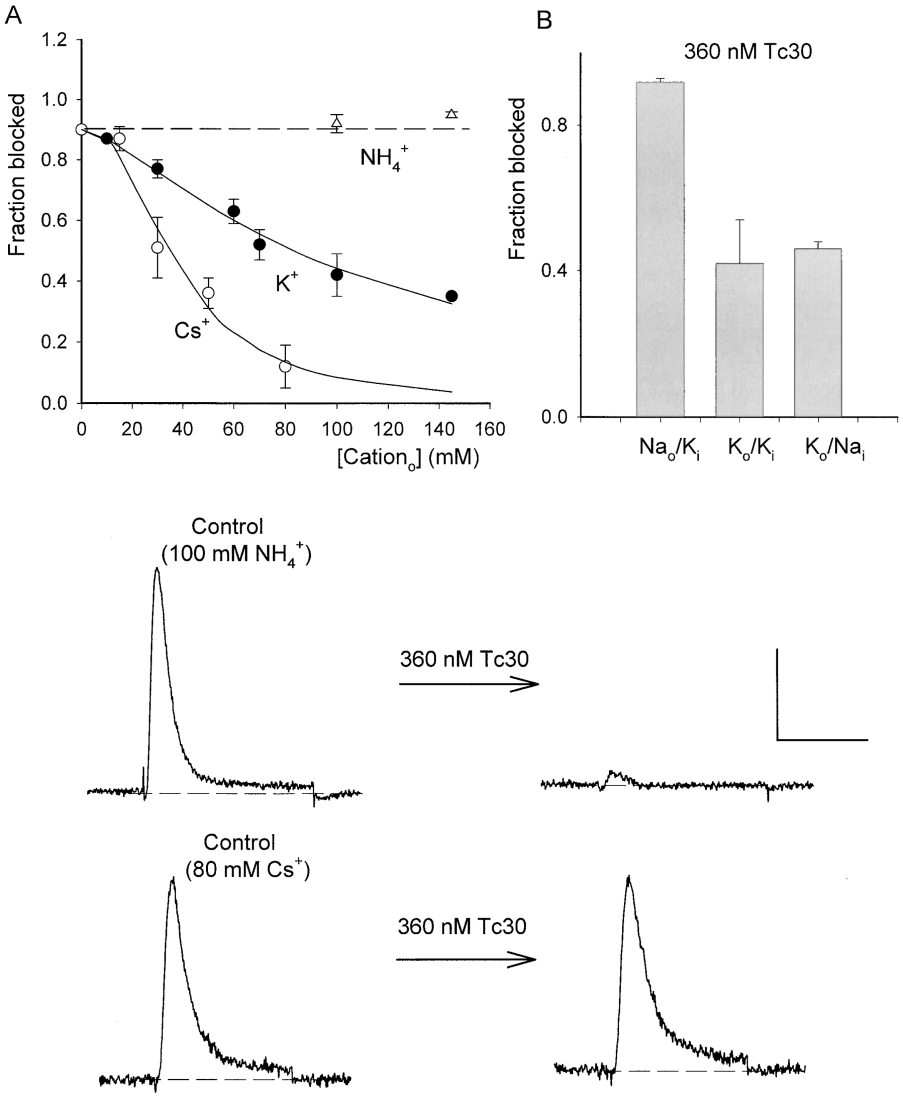

FIGURE 7. Destabilization of Tc30 block by externally added cations. (A, top) fraction of the channels blocked (fb) by 360 nM Tc30 as a function of either $[K_o^+]$, $[Cs_o^+]$, or $[NH_{4o}^+]$ as indicated. The solid lines are the fit of the points with a modified Hill equation: $fb = f_{max}(1 - f_X)$, where f_{max} is the maximal block (with 0 K^+, in the Na_o solution), and f_X is the fraction of channels occupied with competing K_o^+ or Cs_o^+ ions, as indicated; from the Hill equation: $f_X = [X^+]^n/(K_i' + [X_o^+]^n)$, where X stands for either K_o^+ or Cs_o^+, $(K_i')^{1/n} = K_i$ the inhibition constant, and n is the Hill number; $n(K^+) = 1.4$, $K_i(K^+) = 97$ mM; $n(C_s^+) = 2.3$, $Ki(Cs^+) = 41$ mM. The dotted line that joins the fb with 0 K^+ (Na_o^+) with the fb at the two $[NH_4^+]$ tested has no theoretical meaning. The left traces in the bottom panel are control currents at +40 mV with either 100 mM NH_4^+ or 80 mM Cs^+ in the external solution, as indicated. The right traces show the current left after the addition of 360 nM TC30. The horizontal scale bar indicates 16 ms, and the vertical bar indicates 2.5 nA for the top traces (NH_4^+) and 1.5 nA for the bottom traces (Cs^+). (B) Block of I_K by [Tc30] = 360 nM as a function of the K^+ distribution across the membrane, as in Fig. 5. Regardless of the internal solution (Na_i or K_i), external K^+ destabilizes Tc30 block of Shaker. The points are the mean ± SEM of at least three cells on each condition.

ness of Pi2 to inhibit the drop of G_K (Fig. 3) could be due to a major decrement in its apparent affinity toward the channels in 0 K^+, such that at 150 nM concentration (~19 times its Kd for block) at most ~4% of the channels would have a toxin bound to them (Fig. 3). The latter would mean that a ~150-fold increase in the apparent Kd of Pi2 (to ~3,600 nM) took place in 0 K^+. To test this possibility we look at the extent of G_K drop after pulsing in 0 K^+ with both Pi2 and Tc30 present in the Na_o solution (as in Fig. 2 B).

The results of the test (Fig. 8 C) show that 150 nM Pi2 significantly reduces the extent of G_K protection exerted by a saturating (1,200 nM) amount of Tc30 (60 ± 10% vs. 97 ± 2%, respectively, $n = 3$). This clearly indicates that the Kd of Pi2 in 0 K^+ could not have possibly increased 150-fold; that is: the ineffectiveness of Pi2 to protect G_K (Fig. 3) was not the result of the absence of binding of Pi2 to the channels. Instead, the results obtained thus far suggest that, as opposed to Tc30, the lack of a significant functional contact between Pi2 and K^+ binding sites on the channels (Fig. 4) might be the reason of the lack of protection of G_K.

A prediction of the last statement is that a toxin whose block of I_K is significantly destabilized by external K^+ ions should be able to inhibit the collapse of G_K in 0 K^+, like Tc30 does.

We have previously found that block of Shaker channels by Pi1 (α-KTx 6.1, see Fig. 1), another toxin present in the venom of the scorpion Pandinus imperator (Olamendi-Portugal et al., 1996), is destabilized by external K^+. Furthermore, we also found that externally added cations destabilized Pi1 block with an effectiveness ($Cs^+ > K^+ \cong Rb^+ >> NH_4^+ \cong Na^+$) similar to that found for Tc30 (Gómez-Lagunas et al., 1997) (see DISCUSSION). Therefore, we decided to test the effect of Pi1 in 0 K^+.

Fig. 9 A, left, shows a control I_K. The right panel is the current left after pulsing in 0 K^+ with 100 nM Pi1 in the Na_o solution (a concentration only ~3 times its reported Kd for blockage). Pi1 inhibited well the drop of G_K. The

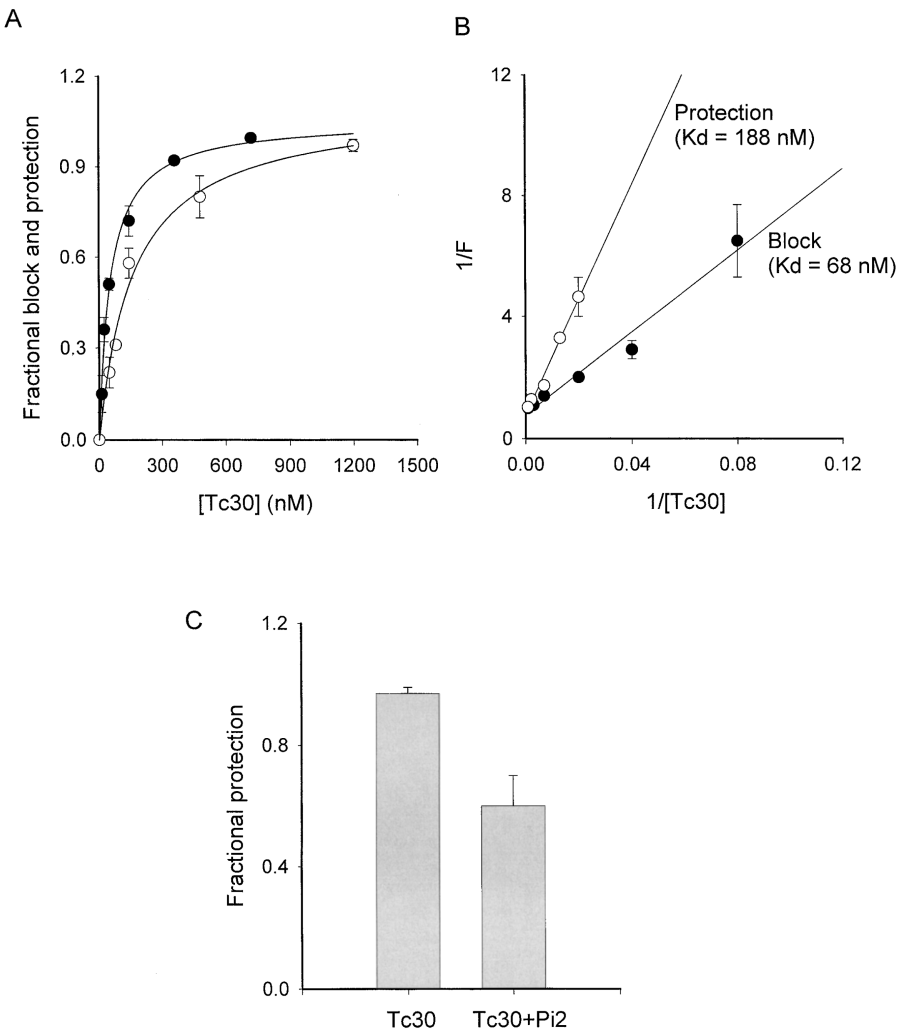

FIGURE 8. Block of I_K, and G_K protection in 0 K^+ as a function of [Tc30]. (A) Extent of G_K protection in 0 K^+ (Na_o/Na_i), as in Fig. 2 (open circles), and block of I_K in standard recording conditions (Na_o/K_i), as in Fig. 5 A (closed circles), as a function of [Tc30]. (B) Double reciprocal plot of the points in A. The lines are least square fit of the points. r(protection) = 0.991, r(block) = 0.988 (C) Extent of G_K protection in 0 K^+ by 1,200 nM Tc30 (left bar) alone (97 ± 2%, n = 3) and by 1,200 nM Tc30 plus 150 nM Pi2 (right bar) (60 ± 10%, n = 3) added to the Na_o solution during pulsing, like in Fig. 2. Pi2 reduces the extent of protection afforded by saturating Tc30. In the protection experiments pulsing in 0 K^+ started 3 min after the addition of Tc30 to the recording chamber.

latter can be better appreciated in Fig. 9 A where the extent protection of G_K in 0 K^+ (70 ± 7%, n = 3) is compared with the extent of I_K block (90 ± 2%, n = 3) with 0 K^+ in only the external solution (in Na_o/K_i).

The results in Fig. 9 also suggest that there was a decrement in the apparent Kd of Pi1 toward the channels in 0 K^+. However, in this case from the single concentration for protection in Fig. 9 B, and from the reported Kd (32 nM) for I_K block (Gómez-Lagunas et al., 1997), the apparent Kd of Pi1 seems to have had an increment of only ~1.5 times in 0 K^+ (see DISCUSSION).

Finally, observe that in Fig. 9 B, like in Fig. 2 C, the current evoked by the first pulse applied with the cell back in K_o (I_1) has smaller final amplitude than that of the following currents (I_2).

DISCUSSION

Scorpion toxins block K channels with a conserved bimolecular mechanism. Therefore, it could have been expected that all toxins that block *Shaker* channels would be uniform regarding the capacity to inhibit the collapse of G_K in 0 K^+. Instead, here we have shown that Pi2 was unable to impede the drop of G_K, whereas Tc30 and Pi1, which block *Shaker* channels with lower affinity than Pi2, were effective inhibitors of the drop of G_K.

These observations suggest that toxin (Tc30 or Pi1) inhibition of the collapse of G_K in 0 K^+ is not due to a nonspecific steric inhibition of the conformational change that takes place when G_K collapses. It also seems not to be the result of the permanence of K^+ ions bound to the channels in the 0 K^+ solutions, as the result of toxin block. Because if any of these two mechanisms of inhibition would be the case, then any toxin capable of blocking the channels should inhibit the collapse of G_K.

More likely, our observations suggest that the preservation of G_K in 0 K^+ depend on specific interactions of the toxin with critical residues, or microdomains (like ion-binding sites) on the channels. The presence of toxin-specific interactions was demonstrated by the differential effect of K^+ ions on toxin block of I_K. Moreover, toxins that protect G_K in 0 K^+ were found to be

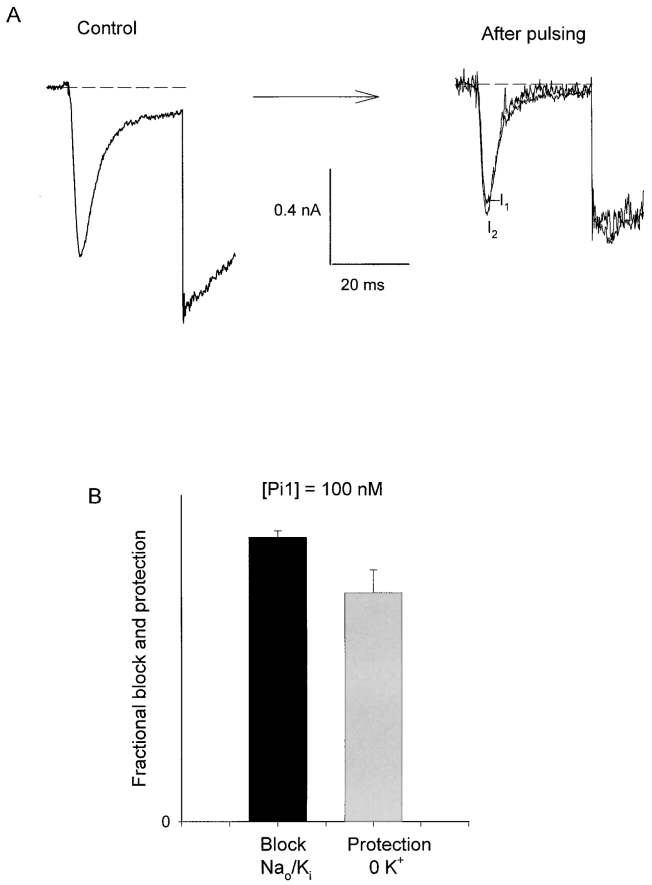

FIGURE 9. Pi1 protects G_K in 0 K^+. (A, left) Control I_K in K_o/Na_i. (Right) I_K left after pulsing in $(Na_o+Pi1)/Na_i$, with [Pi1] = 100 nM (not depicted). Most of the channels (~77%) were protected by Pi1. See that $I_1 < I_2$, as in Fig. 2. (B) Comparison of the extent of G_K protection in 0 K^+ versus the extent of Pi1 block of I_K with 0 K^+ in only the external solution (Na_o/K_i), as indicated. The bars are the mean ± SEM of three experiments.

those for which the blockage of I_K was significantly destabilized by extracellular K^+ ions.

Therefore, it seems that the toxin-specific protection of G_K in 0 K^+ somehow operates through the interaction of the toxins with sites capable of binding K^+, whose probable location is discussed below.

Toxin Block in the Presence of K^+ Ions

Considering that in multi-ion pores, the apparent voltage dependence of block by a charged blocker depends on the state of occupancy of the pore (Neyton and Miller, 1988a,b), we looked at the voltage dependence of I_K block by Tc30 as a function of the K^+ distribution across the membrane. Our results show that independently of the K^+ distribution, Tc30 block of the wild-type Shaker channels is not noticeably affected by the pulse potential. The latter, however, does not necessarily mean that upon binding to the channels Tc30 does not actually sense the voltage drop across the membrane, as the slow kinetics of toxin binding to K channels could have concealed the detection of voltage effects on block (e.g., see Goldstein and Miller, 1993; Terlau et al., 1999).

High millimolar $[K_o^+]$ is needed to destabilize toxin binding to the channels (Ki of 95 and 46 mM for Tc30 and Pi1, respectively) (this work; Goldstein and Miller, 1993; Goldstein et al., 1994; Ranganathan et al., 1996; Gómez-Lagunas et al., 1997). In contrast, K_o^+ competes with Na_o^+ for a site (likely located in the SF) that when occupied by Na^+ makes G_K prone to collapse upon channel deactivation, with a Ki of only 80 μM (Gómez-Lagunas, 2001).

The markedly different Ki values of K_o^+ for destabilization of toxin block and for protection of G_K could be interpreted as the result of K^+ binding to different sites on the channels. However, the difference could also be due to the different ionic conditions of the toxin block (high internal $[K^+]$) and of the G_K protection (O K^+) experiments, which could influence the binding of K_o^+ to its site of action.

Toxin Interaction with K^+ Binding Sites and Protection of G_K

A lysine at position 27 (K27, using the ChTx sequence) is a critical and highly conserved residue of scorpion toxins (Goldstein et al., 1994; Aiyar et al., 1995; Miller, 1995). In the case of ChTx, it has been shown that K27 participates in a destabilizing interaction that K^+ ions, coming from the internal solution, through the pore, exert over the binding of the toxins. Therefore, when a toxin binds to a channel, the lateral chain of K27 is thought to point at the central axis of the pore (Anderson et al., 1988; Park and Miller, 1992; Goldstein and Miller, 1993; Goldstein et al., 1994; Aiyar et al., 1995; Hidalgo and MacKinnon, 1995).

The three toxins tested in this work have a conserved K27, in equivalent positions. However, those toxins that inhibited the drop of G_K in 0 K^+ block I_K less in K_o than in Na_o solutions, regardless of the internal solution (Na_i or K_i), clearly indicating an interaction between those toxins and external K^+ sites.

It has been shown that K27 of Agitoxin2 (AgTx2), an isoform of ChTx, is energetically coupled (interacts with) to Y445 of *Shaker* (Ranganathan et al., 1996). The latter residue participates in the formation of the most external K^+ binding site of the SF of the pore (Heginbotham et al., 1994; Morais-Cabral et al., 2001; Zhou et al., 2001). Binding of AgTx2 to *Shaker* channels is destabilized by K_o^+ ions, and this effect depends on the presence of K27 on AgTx2 (Ranganathan et al., 1996). Thus, it seems that binding of a K^+ ion, coming from the external solution, to the most external K^+ binding site of the SF destabilizes the binding of AgTx2.

On the other hand, in this work, we have shown that Pi2 (a toxin that presents K27) block of *Shaker* channels is not affected by K_o^+. It has also been reported that binding of ChTx to *Shaker* channels is not destabilized by K_o^+ either, but that in contrast block by the ChTx mutant R25Q is decreased by K_o^+ (Goldstein and Miller, 1993). Therefore, not all K27-toxins are destabilized by external K^+ ions.

The aforementioned observations suggest that: (a) not all K27 residues of scorpion toxins interact in the same K^+-dependent manner with Shaker Y445, perhaps because of differences in the average distance between these partner residues (K27-Y445) and, (b) K^+ binding sites located outside the SF of the channels could bind K_o^+ ions and destabilize the binding of some toxins, like ChTx R25Q (Goldstein and Miller, 1993).

Therefore, regarding the mechanism of inhibition of the collapse of the K^+ conductance in 0 K^+, the following two nonexclusive possibilities exist. Most likely, (a) Tc30 and Pi1 inhibit the drop of G_K by interacting with the more external K^+ binding site of the SF. Interestingly, this possibility suggests that: only the K^+ occupancy of the more external site of the SF may be needed in order to preserve G_K. On the other hand, (b) it could be that toxins that protect G_K do so by interacting with K^+-binding sites located in the outer vestibule of the pore, outside the SF. In other words, that binding of K^+ ions to sites outside the SF could preserve G_K, possibly through an allosteric mechanism.

Supporting the latter possibility is the observation that external monovalent cations destabilize toxin binding to the channels (Tc30 and Pi1) with an effectiveness that does not match the selectivity sequence of K^+ channels: Cs_o^+ destabilizes block more effectively than K_o^+ ($Ki(Cs^+)/Ki(K^+) = 0.42$, 0.66 for Tc30 and Pi1, respectively), whereas, on the other hand, block seems equally strong with either Na_o^+ or NH_{4o}^+ ions (Fig. 7; Gómez-Lagunas et al., 1997). Therefore, it seems likely that sites capable of binding K^+ with low affinity may exist in the outer vestibule of the pore, outside the SF of the channels (e.g., see also Pardo et al., 1992; Goldstein and Miller, 1993; Lopez-Barneo et al., 1993; Gómez-Lagunas et al., 1997; Jäger et al., 1998; Thompson et al., 2000; Wang et al., 2000; Consiglio et al., 2003).

On the other hand, the kinetics of block destabilization by external cations is complex. K_o^+ destabilizes block with Hill numbers n of 1.4 (Tc30) or of 2.0 (Pi1), whereas Cs_o^+ does it with n of 2.3 (Tc30) or of 4 (Pi1) (Fig. 7; Gómez-Lagunas et al., 1997). Thus, it seems that the minimal numbers of ions which upon binding to the channels destabilize block, depends both on the ion and on the toxin, probably because of differences in physiologically relevant contact points of different toxins with the channels.

Scorpion Toxin Binding to the Channels in 0 K^+

External K^+ destabilizes both Tc30 and Pi1 block of Shaker. Nonetheless, according to the Michaelis-Menten model, the apparent binding affinity of both toxins is smaller in 0 K^+ (Na_o/Na_i) than in K^+-containing standard conditions (Na_o/K_i). This suggests that in 0 K^+ solutions (Na_o/Na_i) there is a conformational change in the external vestibule of the pore, which decreases toxin affinity. Binding of the appropriate toxins (Tc30, Pi1) then seems to prevent a more extensive conformational change that otherwise would lead to the collapse of G_K in 0 K^+.

It is opportune to mention that a conformational change in the outer region of the pore has been well documented in Kv2.1 channels under 0 K^+ conditions, which in this case precludes the binding of TEA_o (Ikeda and Korn, 1995). On the other hand, in *Shaker* channels we did not find a significant change in the binding affinity of TEA_o in 0 K^+ (Gómez-Lagunas, 1997). Thus, it could be that the possible conformational change that affects toxin binding neither includes the blocking site of TEA_o nor affects the access of TEA_o to that site at the entrance of the SF (Luzhkov and Aqvist, 2001).

The inhibition of the drop of G_K as a function of [Tc30] is fitted well by the Michaelis-Menten equation, with an apparent Kd bigger than that of block. Nonetheless, taking into account the differences between the block and the collapse of G_K experiments, we considered that it would be of interest to discuss a second model that explicitly takes into account some of the characteristics of both the toxin binding and of the collapse experiments.

If I_1 is the current left after the delivery of one pulse in 0 K^+ then: $I_1 = (1 - C(1))\, I_0$, where I_0 is the control current, before pulsing in 0 K^+ and $C(1)$ is the fraction of the channels collapsed by one (the first) pulse; we know that: $C(1) \approx 0.34$ (see Fig. 4 of Gómez-Lagunas 1997). Now, in the presence of a given [Tc30], the fraction of channels collapsed by one (the first pulse) will be: C(1)Fu, where Fu = {unblocked fraction of channels}, and from the Michaelis-Menten model: Fu = Kapp/(Kapp+[Tc30]). That is, we are assuming that the probability of collapse by a pulse and the probability of toxin binding are independent of each other.

When a second pulse is applied in 0 K^+: $I_2 = (1 - C(2)Fu)I_1$, that is: $I_2 = (1 - C(2)Fu)(1 - C(1)Fu)I_o$, where C(2) = {fraction of channels collapsed by the second pulse} = {fraction collapsed by a pulse after one pulse was already applied}.

At the end of the first activating pulse a fraction of the channels will deactivate—another fraction will remain inactivated—and a fraction of these deactivating channels will collapse. At the moment of the delivery of the second pulse, the distribution of the population of

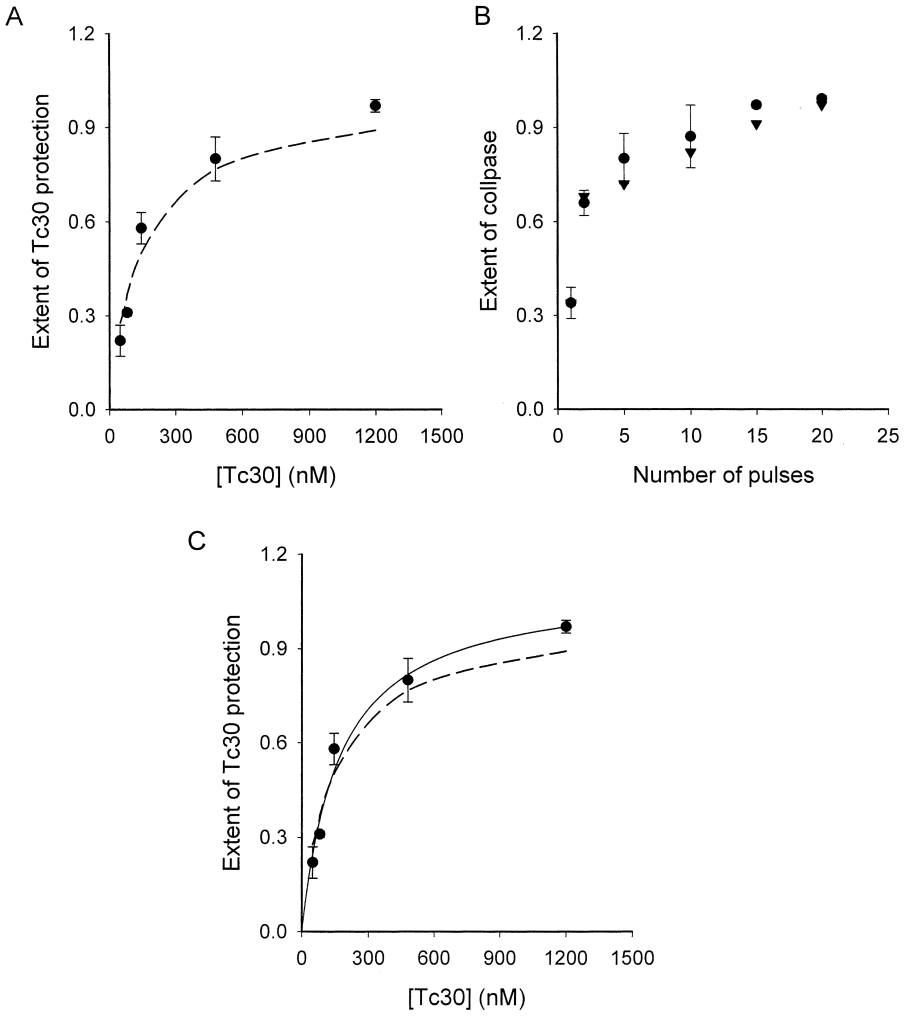

FIGURE 10. Tc30 protection and extent of G_K drop as a function of the number of pulses in 0 K^+. (A) G_K protection in 0 K^+ as a function of [Tc30] (circles, experimental points in Fig. 8 A). The dashed line is the fit of the points with the model in the text, m = 0.01 and Kapp = 70 nM. (B) G_K drop as a function of the number of +20-mV/30-ms pulses delivered from −80 mV (circles, Figs. 5 A and 1 of Gómez-Lagunas, 1997, 2001, respectively). The inverted triangles are the fit of the observations by the model in the text, with m = 0.01 (as in A) and Fu = 1. (C) Comparison of the fit of the points in A by either the Michaelis-Menten model (solid line) or the model in the text (dashed line).

channels will clearly be different to that before the first pulse, so it is expected that the fraction of channels that will deactivate, and therefore be amenable to collapse, at the end of the second pulse will be different to that at the end of the first pulse, therefore we expect that $C_2 \neq C_1$. Here, for simplicity, we will assume that once the first pulse has been applied C changes linearly with the number of pulses: C(n) = mn, for $n \geq 2$, thus after the delivery of $n = 20$ pulses the extent of Tc30 protection will be:

$$I_{20}/I_0 = (1 - C(1)Fu)\prod_{n=2}^{19}(1 - mnFu).$$

Fig. 10 A shows that the model fits reasonably well the protection afforded by varying [Tc30] (experimental points in Fig. 8 A), with m = 0.01 and Kapp = 70 nM (line through the points). To further validate the model we tested if it could fit (with the value of m obtained in Fig. 10 A) the extent of collapse produced by a variable number of pulses (Figs. 5 and 1 of Gómez-Lagunas, 1997, 2001, respectively), in the absence of toxin (i.e., with Fu = 1). Fig. 10 B shows that indeed the model (triangles) fits reasonably well the experimental observations (circles). Finally, Fig. 10 C compares the fit provided by the Michaelis-Menten model (solid line) to the protection exerted by varying [Tc30] (Fig. 8 A), with that of the above model (dashed line). Although both models fit well the experimental points, it is readily seen that the fit provided by the simplest, Michaelis-Menten model is more accurate. Nonetheless, it is interesting that the above model fits well the observations with an apparent Kd equal to that of block, suggesting that at least part of the apparent increase in the Kd for protection, seen with the Michaelis-Menten model, could be due to the binding of toxin to collapsed channels, which is a possibility embodied in the hypothesis of the model.

Finally, it is pertinent to point out that the difference between the first (I_1) and the following (I_2) K^+ currents recorded after pulsing in 0 K^+ in the presence of either Tc30 or Pi1, was previously observed (although the difference was much more noticeable) with Ba^{2+} ions as the protective agent in 0 K^+. In the case of Ba^{2+}, it was hypothesized that the difference in the currents could

be due to either of two nonexclusive possibilities: (a) to the slow exit (at the first pulse, I_1) of Ba^{2+} ions, that could had been trapped in the channels during pulsing in 0 K^+; or, (b) to a conformational rearrangement of the channels back in the K_o solution (Gómez-Lagunas, 1999). In the present case, the relation $I_1 \neq I_2$ is not likely to report the exit of toxin from the channels, because scorpion toxins bind to an external, superficial receptor surface from which they readily diffuse away. Therefore, it seems possible that the relation $I_1 \neq I_2$ here reports a slight (as judged from the small difference of the currents) rearrangement of the channels once they are back in K_o after pulsing in 0 K^+.

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