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

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Abstract: The Shaker B K⁺ conductance (Gk) 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 Gk (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, 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 Gk in 0 K⁺. We have found that these toxins are not uniform regarding the capacity to protect Gk. Those toxins, whose binding to the channels is destabilized by external K⁺, are also effective inhibitors of the collapse of Gk. 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 Gk 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 Gk 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 Gk) 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 (Gk) collapses: the channels sink into a stable, noninactivated, nonconducting conformation (Gómez-Lagunas, 1997).

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

The extent of drop of Gk 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 Gk. 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 Gk 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 (Melischuk 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 Gk 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
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; Rodríguez 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 (Kᵢ,⁺), whereas block by a mutant R25Q (a glutamine substituting arginine in position 25) of ChTx is destabilized by Kᵢ,⁺ ions (Goldstein and Miller, 1993), likely because the change R25Q allows the mutant ChTx to interact with a site(s) capable of binding Kᵢ,⁺ in the outer vestibule of the pore (Goldstein and Miller, 1993).

Block of Shaker channels by toxins and external TEA (TEAᵢ) are two mutually exclusive processes (Goldstein and Miller, 1993). Therefore, considering that TEAᵢ impedes the drop of Gᵢ in 0 K⁺, with basically the same affinity (Kd) with which it blocks the K⁺ current (Iᵢ) through the channels (Gómez-Lagunas, 1997), the simple hypothesis immediately arises that scorpion toxins should also be effective inhibitors of the collapse of Gᵢ 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 Gᵢ in 0 K⁺. Searching an explanation for this fact, we found that scorpion toxins are indeed able to effectively inhibit the drop of Gᵢ 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 Gᵢ 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 Kᵢ for protection in 0 K⁺ compared with that of block of Iᵢ. 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 polyhedrosis 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., Naᵢ/Oᵢ). The external solutions contained (in mM), Naᵢ: 145 NaCl, 10 CaCl₂, 10 HEPES-Na buffer, pH 7.1; Kᵢ: 100 KCl, 45 NaCl, 10 CaCl₂, 10 HEPES-Na buffer, pH 7.1. The internal solutions contained (in mM), Kᵢ: 90 KF, 30 KCl, 2 MgCl₂, 10 EGTA, 10 HEPES-K buffer, pH 7.2; Naᵢ: 90 NaF, 30 NaCl, 2 MgCl₂, 10 EGTA, 10 HEPES-Na buffer, pH 7.2. Other solutions were prepared by replacing NaCl with the corresponding equimolar amounts of XCl, where X stands for K⁺, Cs⁺, or NH₄⁺ 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 (α = 0.05).

RESULTS

GK Protection by Specific Scorpion Toxins

To test the hypothesis that scorpion toxins should be able to inhibit the drop of GK 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 GK in 0 K+ solutions.

Fig. 2 A presents inward K+ currents (I1) through Shaker B, evoked by +20-mV/30-ms activating pulses, in Ko/Nao solutions (see MATERIALS AND METHODS). Three traces are superimposed showing the stability of I1. Once the control I1 were recorded, the cell was superfused with the Nao solution containing 360 nM Tc30 (~5 times its Kd for blockage, see below), and 20 activating pulses were repeated to repeatedly gate the channels in the 0 K+ test solution (Na0 + Tc30/Na0). 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 Ko solution, and then the state of the channels was tested with the delivery of activating pulses. The traces in Fig. 2 C (I2), show that most of the channels were still able to conduct K+ ions.

The ~91% preservation of I1 in Fig. 2 C indicates that Tc30 is an effective inhibitor of the drop of GK in 0 K+. To better demonstrate the latter statement, and to show the collapse-recovery cycle of GK, once the currents in Fig. 2 C were recorded the cell was superfused with the Na0 solution (but this time without Tc30) and a round of 20 pulses was applied, with the cell bathed in Na0/Na0 solutions (Fig. 2 D), as in Fig. 2 B. Subsequently, the cell was superfused back with the Ko 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 GK. The fall of GK 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 I1 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 Ko (labeled I1) has a slower activation, and thus reaches a slightly smaller peak amplitude than the current recorded in the next pulses (collectively labeled as I2). The latter is best seen in Fig. 2 G, which compares the average time to peak of I1 versus I2 of four cells after pulsing in the presence of 360 nM Tc30. A similar behavior (I1 ≠ I2) was observed previously with Ba2+ ions as the protective agent in 0 K+, except that with Ba2+ the effect was much more evident (Gómez-Lagunas, 1999; see DISCUSSION).

The observations in Fig. 2 could be interpreted as meaning that Tc30 protects GK 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 GK. 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 I1 recorded in Ko/Na0. Thereafter, the cell was superfused with the Na0 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 Ko 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 GK. The drop of GK 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 I1 after a 3-min depolarization to 0 mV.

Fig. 3 E compares the extent of Pi2 protection (4 ± 3%, n = 5) of GK in 0 K+ (in Na0 + Pi2/Na0) against the extent of Pi2 block of I1 (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 Na0/K0). The extent of drop of GK with 150 nM Pi2 present (96 ± 3%) is not significantly different to the extent of drop in only Na0/K0 (99 ± 1%, Fig. 1 of Gómez-Lagunas, 2002).

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 Charybotoxin (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).
2001). Clearly, Pi2 is not an effective inhibitor of the collapse of GK.

The above observations strongly suggest that Tc30 protection of GK (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 GK. 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 GK. This possibility is tested below.

**Toxin-specific Interaction with K⁺ Binding Sites Correlates with their Effectiveness to Protect GK**

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

Fig. 4 reports the effect of the K⁺ distribution across the membrane on Pi2 block of IK. Fig. 4 A, left, presents a control IK at -50 mV, recorded in standard (Na⁺/K⁺) conditions. The subsequent addition of 150 nM Pi2 to the external Na⁺ solution blocked 90% of the channels (middle panel), as indicated. Pi2 block was abolished by perfusing the cell with the control Na⁺ 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 Ko/Ki or Ko/Nai solutions, respectively. Regardless of the K⁺ distribution across the membrane, Pi2 blocks IK 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⁺/K⁺; 92 ± 2%, n = 4) is not significantly different either to that with K⁺ in both sides of the membrane (K₀/K⁺; 88 ± 4%, n = 3) or to the extent of block with K⁺ in only the external solution (K₀/Na⁺; 86 ± 4%, n = 4).

**FIGURE 2.** Tc30 inhibits the collapse of GK in 0 K⁺. (A) Control IK evoked by three +20-mV/30-ms (activating) pulses in Ko/Nai (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⁺ + Tc30}/Na⁺ with [Tc30] = 360 nM. (C) IK recorded with the cell back in K₀/Nai immediately after pulsing in 0 K⁺ in B. Tc30 inhibited the collapse of GK. The current evoked by the first pulse applied back in K₀ (I₁) differs from those evoked by the next pulses (together labeled as I₂) (see the text). (D) Currents recorded by pulsing in Na⁺/Na⁺ (without Tc30), immediately after C. (E) Currents recorded with the cell back in K₀ after the pulsing episode in D. Pulsing in 0 K⁺ collapsed GK. (F) IK evoked by three activating pulses delivered 1 min after a 3-min depolarization period at 0 mV. Depolarization recovered GK. (G) Comparison of the time to peak of I₁ versus I₂ 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.
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-
external K⁺ ions exert a significantly destabilization of Tc30 block of Iₖ. 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 Iₖ 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 [Kᵢ⁺] 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 [Kᵢ⁺] 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,
Ki(K⁺) = 97 mM; and n(Cs⁺) = 2.3, Ki(Cs⁺) = 41 mM (see discussion). Csᵦ⁺, which is either poorly permeant or impermeant through K⁺ channels, destabilizes block more effectively than Kᵦ⁺. In contrast, NH₄⁺, which permeates K⁺ channels, is like the impermeant Na⁺ regarding the block of Iᵦ. The dotted line joining the extent of block in Naᵦ with that at the indicated [NH₄⁺] has no theoretical meaning. The traces in the bottom panel illustrate the block of Iᵦ in Naᵦ, with that at the indicated [NH₄⁺] has no theoretical meaning. The traces in the bottom panel illustrate the block of Iᵦ, with either 100 mM NH₄⁺ or 80 mM Cs⁺ ions in the external solution, as indicated. The above observations show that: (a) in addition to Kᵦ, 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 (Naᵦ/Kᵦ) with the third (Kᵦ/Naᵦ) 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 (Kᵦ), block is not significantly different with either Na⁺ (Naᵦ) or K⁺ (Kᵦ) internal solutions (second and third bars). That is: external K⁺ destabilizes the binding of Tc30 regardless of the internal K⁺ (Naᵦ or Kᵦ).

Fig. 8 A reports the extent of Iᵦ block in standard recording conditions (Naᵦ/Kᵦ, i.e., with 0 K⁺ in the side of toxin action), as well as the extent of Gᵦ protection in O K⁺ (Naᵦ/Naᵦ), as a function of the indicated [Tc30]. It is seen that both the Iᵦ block and Gᵦ 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 Iᵦ block (as expected) and Gᵦ protection by Tc30. Nonetheless, at this
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 Gk, according to the Michaelis-Menten model the apparent Kd for Gk protection (188 nM) is ~3 times that of Iₖ 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 Gk. On the other hand, the increase in the apparent Kd for Gk 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 Gk in Fig. 8 B) suggests that the ineffective-
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^+ > > Rb^+ > > NH_4^+ > > Na^+) similar to that found for Tc30 (Gomez-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
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latter can be better appreciated in Fig. 9 A where the extent protection of \( G_K \) in 0 K\(^+\) (70 \( \pm \) 7\%, \( n = 3 \)) is compared with the extent of \( I_K \) block (90 \( \pm \) 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 \( \sim 1.5 \) 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 biomolecular 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
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; Teplau et al., 1999).

High millimolar [Ko$^+$] 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$^+$ competes with Na$^+$ 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 $\mu$M (Gómez-Lagunas, 2001).

The markedly different Ki values of Ko$^+$ 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 (0 K$^+$) experiments, which could influence the binding of Ko$^+$ 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 Ko than in Na$^+$ solutions, regardless of the internal solution (Na or K$^+$), 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 Ko$^+$ 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_+^+. It has also been reported that binding of ChTx to Shaker channels is not destabilized by K_+^+ either, but that in contrast block by the ChTx mutant R25Q is decreased by K_+^+ (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_+^+ 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_+^+ destabilizes block more effectively than K_+^+ (K(Cs+)/K(K+) = 0.42, 0.66 for Tc30 and Pi1, respectively), whereas, on the other hand, block seems equally strong with either Na_+^+ or NH_4+ 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_+^+ destabilizes block with Hill numbers n of 1.4 (Tc30) or of 2.0 (Pi1), whereas Cs_+^+ 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_+^+/Na_+^+) than in K+ -containing standard conditions (Na_+^+/K_+^+). This suggests that in 0 K+ solutions (Na_+^+/Na_+^+) 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 K_d 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) = 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)/F_u, where F_u = [unblocked fraction of channels], and from the Michaelis-Menten model: F_u = 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))F_u I_0, that is: I_2 = (1 - C(2)F_u)(1 - C(1)F_u) I_0, 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

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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:

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

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 $K_{app} = 70$ 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 $F_u = 1$). 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 $0 \text{ K}^+$ 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 $0 \text{ K}^+$. 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, I1) of Ba\textsuperscript{2+} ions, that could had been trapped in the channels during pulsing in 0 K\textsuperscript{+}; or, (b) to a conformational rearrangement of the channels back in the K\textsubscript{o} solution (Gómez-Lagunas, 1999). In the present case, the relation I\textsubscript{1} \neq I\textsubscript{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\textsubscript{1} \neq I\textsubscript{2} here reports a slight (as judged from the small difference of the currents) rearrangement of the channels once they are back in K\textsubscript{o} after pulsing in 0 K\textsuperscript{+}.

This work was supported in part by CONACyT (National Conseil of Science and Technology, Mexican Government) grant numbers Z005 and 40690-Q and DGAPA-UNAM (Direccion General de Asuntos del Personal Academico – National Autonomous University of Mexico) grant numbers: IN-216900 and IN216503-2 to F. Gómez-Lagunas and L.D. Possani.

Olaf S. Andersen served as editor.

Submitted: 19 May 2003
Accepted: 23 January 2004

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