Block of *Shaker*B K^+ channels by Pi1, a novel class of scorpion toxin

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Received 26 September 1996; revised version received 15 November 1996

Abstract Here we describe the basic features of the interaction of K^+ channels with Pi1, a recently described 35 amino acid scorpion toxin, which has four disulfide bridges instead of the three commonly found in all the other known scorpion toxins. We found that: (a) Pi1 blocks *Shaker*B from the outside with a 1:1 stoichiometry, and a K_d of 32 nM in zero external [K^+]; (b) extracellular K^+, Rb^+ and Cs^+ but not NH_4^+ ions strongly impede (destabilize) the block by this toxin; interestingly (c) the destabilizing binding of K^+, Rb^+, and Cs^+ is described by a Hill coefficient $n > 1$; (d) external K^+ is more effective than internal K^+ to reduce the block by Pi1.

Key words: *Shaker*B; *Pandinus*; Pi1; Potassium channel; Scorpion toxin

1. Introduction

Among the chemical agents that block K^+ selective channels a group of peptides contained in the venom of scorpions (henceforth referred to as K-toxins) have been particularly useful since they affect only K^+ channels with high affinity (for a review see Miller [1]).

Regardless of their primary sequence variability and corresponding differential affinity towards the several kinds of K^+ channels, all the K-toxins are known to be built around a common structural scheme: short, 32–39 amino acid residues, basic peptides cross-linked by three disulfide bridges formed between six highly conserved cysteines [1]. Recently, however, we purified a novel peptide, named Pi1, from the venom of the scorpion *Pandinus imperator*, which departs from this common scheme. Pi1 is a 35 amino acid basic peptide that presents an additional disulfide bridge (four instead of the three commonly found) [2].

In this communication we report that Pi1 reversibly blocks *Shaker*B from the outside, with a K_d of 32 nM in zero K^+. The block is not appreciably voltage dependent. Extracellular K^+, Rb^+ and Cs^+ strongly impede the block by Pi1 with a concentration dependence described by a Hill coefficient > 1. The extracellular pH has not much effect on the block.

2. Materials and methods

2.1. Toxin purification

The novel peptide Pi1 from the venom of the scorpion *Pandinus imperator* was purified and its amino acid sequence and disulfide bridges were determined as previously described [2].

2.2. Sf9 cell culture and ShakerB K^+ channel expression

The insect cell line Sf9 from *Spodoptera frugiperda* was kept in culture and *Shaker*B K^+ channels were expressed as previously reported [3,4].

2.3. Electrical recording

Macroscopic currents were recorded under whole-cell patch-clamp [5] with an Axopatch 1D (Axon Instruments, Inc.) as reported before [3]. The currents were sampled at 100 μs/point, and low pass filtered in line at 5 kHz.

The electrodes were pulled from borosilicate glass (KIMAX 51) to 1.2–2 MΩ resistance. About 80% of the series resistance was electronically compensated. The holding potential used throughout the work was −80 mV.

2.4. Solutions

The internal (K^+) solution used throughout the work was (in mM): 90 KF, 30 KCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES-K, pH 7.2, except where explicitly indicated. The external control (Na^+) solution was (in mM): 135 NaCl, 10 $CaCl_2$, 10 MES-Na, pH 6.4. All the other external solutions at pH 6.4 were prepared by replacing an isosmolar amount of NaCl by the chloride salt of the test cation. All the chemicals were of analytical grade.

3. Results and discussion

Fig. 1 illustrates the basic characteristics of the interaction of Pi1 with *Shaker*B. Fig. 1A shows macroscopic K^+ currents recorded in the control, zero K^+, Na^+ external solution (see Section 2), before (upper traces), during (middle traces), and after (bottom traces) the addition of 50 nM of Pi1 to the external solution. Pi1 caused a fully reversible 65% reduction in the amplitude of the outward K^+ currents through *Shaker*B. This is best seen in Fig. 1B, which shows the peak current versus voltage relationship (I-V) of the currents in Fig. 1A. The channels were activated by 30 ms pulses of the indicated amplitude delivered at a rate of one every 15 s from the holding potential of −80 mV, to allow a complete recovery from inactivation [6]. Notice that there is a 100% recovery of the current after the toxin is removed, by perfusing the cells with the control external solution. Pi1 merely scaled down the currents without altering its kinetics (not shown). This suggests that Pi1 does not alter the gating of the channels and interacts well with both closed and open channels. The reduction of the current is most probably due to a blockade of the K^+ conduction through the channels.

Pi1 block is not appreciably voltage dependent; this is shown in Fig. 1C where the fraction of the channels blocked by 50 nM Pi1 is plotted as a function of the voltage during the activation pulses. Also, the extent of the block does not depend on the holding potential between −80 and −120 mV (not shown).

The absence of an appreciable voltage dependence in the block by Pi1 indicates that this peptide binds to a most external facing region of the channels, which do not sense the voltage drop across the membrane. By analogy with the block by the other known K-toxins, this region should most prob-

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PII S0014-5793(96)01387-7

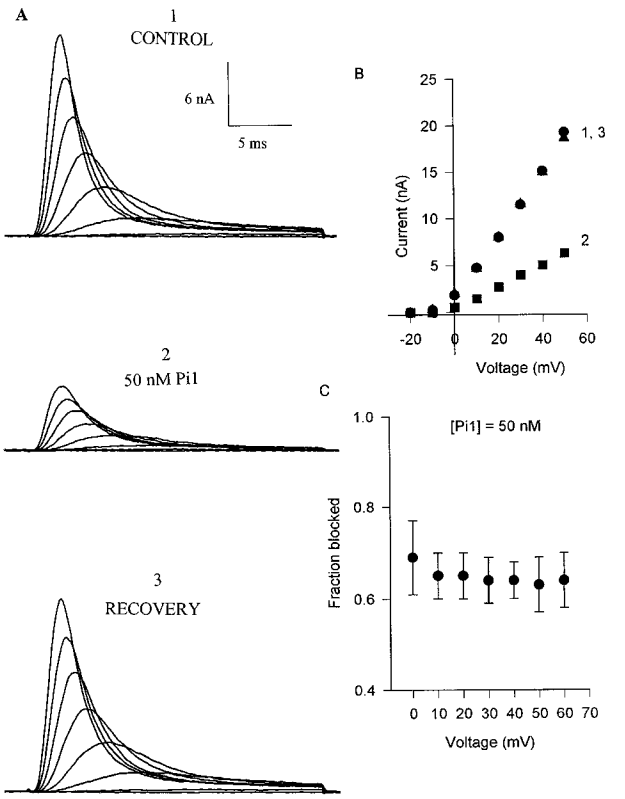

Fig. 1. Pi1 reversibly blocks *Shaker*B K^+ channels. A: Currents elicited by 30 ms pulses of −20 to +50 mV, delivered in increments of 10 mV, at a rate of 1 pulse every 15 s from the HP of −80 mV. The upper panel shows the currents in the control, zero K^+, Na^+ external solution (see Section 2). The middle panel shows the currents with 50 nM Pi1 added to the external solution. The bottom panel shows the currents after removing the toxin by perfusing the external Na^+ control solution. B: Peak current versus voltage relationship of the currents in A. C: Fraction of the channels blocked by 50 nM Pi1 as a function of the voltage during the activation pulses. The points are the mean ± S.E.M. of five experiments.

ably be the outer 'mouth' of the pore. Regarding this point it is pertinent to mention that the block of *Shaker* channels by charybdotoxin (CTX) (by far the most extensively studied K-toxin) exhibits a slight voltage dependence [7], due to an enhanced dissociation of the toxin by membrane depolarization. Part of this slight voltage dependence comes from a destabilizing interaction between internal K^+ ions and a lysine residue at position 27 on CTX [7]. In the case of Pi1 the external K^+ exerts a bigger destabilization of the toxin block than the internal K^+ (see below).

Fig. 2A illustrates the concentration dependence of the interaction of Pi1 with *Shaker*B. The block follows an adsorption hyperbolic curve with a K_d of 32 nM in zero external K^+. The inset shows the linear relationship of the double reciprocal plot which suggests a binding reaction with a 1:1 stoichiometry.

The above results indicate that the block by Pi1 (a peptide with four disulfide bridges) conforms to the common scheme of interaction between scorpion toxins (peptides with three disulfide bridges) and K^+ channels [1], i.e. Pi1 reversibly blocks from the outside following a simple bimolecular reaction, as all the other known K-toxins do.

The results in Figs. 1 and 2A were obtained with an extracellular solution buffered at pH 6.4. This pH is commonly used with Sf9 cells. Fig. 2B shows that the external pH does not significantly affect the potency of the block by Pi1 in the range of 5.5–8.1.

Since the classic study about the TEA block of K^+ channels by Armstrong [8], it has been recognized that K^+ ions can destabilize the binding of pore blocking compounds, therefore we examined the effect of the ionic composition of the external solution in the block by Pi1. Fig. 3A shows the fraction of the channels blocked by 100 nM Pi1 added to the control Na^+ external solution as a function of the indicated concentrations of K^+, Rb^+, NH_4^+ and Cs^+ ions (see Section 2).

The data in Fig. 3A show that: (a) the potency of block by Pi1 was not changed when 100 mM of the impermeant Na^+ ions was replaced by an equiosmolar amount of the slightly permeant NH_4^+ ions; however, (b) addition of the permeant K^+ ions to the external solution clearly reduced the block by

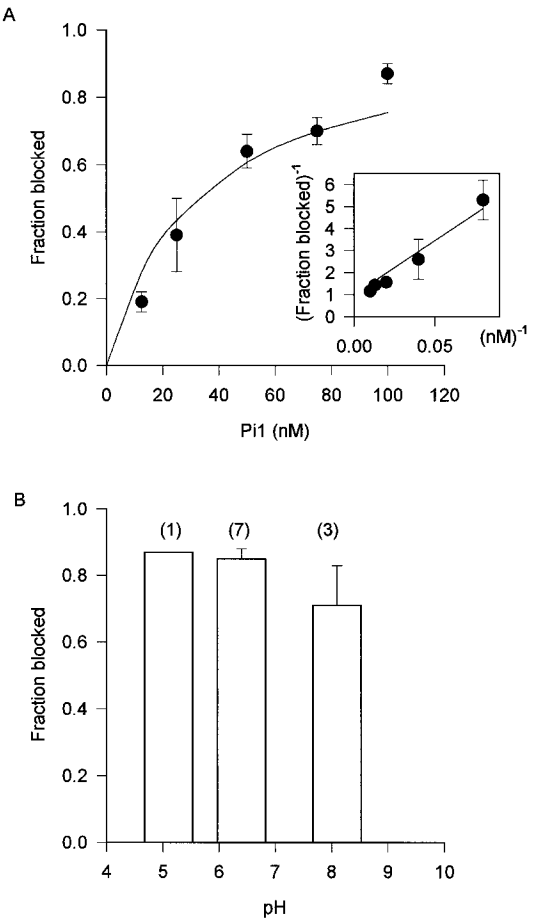

Fig. 2. Concentration and pH dependence of the block by Pi1. A: The main figure shows the fraction of the channels blocked by the indicated concentrations of Pi1 added to the external Na^+ control solution. The line through the points is the fitted Langmuir adsorption isotherm with a K_d of 32 nM. The inset shows the double reciprocal plot of the points. The points are fitted by a least squares line yielding the K_d of 32 nM in zero K^+ ($r = 0.989$). B: Fraction of the channels blocked by 100 nM Pi1 as a function of the indicated pH of the external solution. The external, zero K^+, solution at pH 6.4 was the same as in A. The solution at pH 8.1 was the same as that at pH 6.4 except that the buffer was 10 mM Trizma Base pH 8.1. The solution at pH 5.5 was like that at pH 6.4 except that the buffer was 10 mM citrate-NaOH pH 5.5. The bars represent the S.E.M. of the indicated number of experiments (numbers in the parentheses).

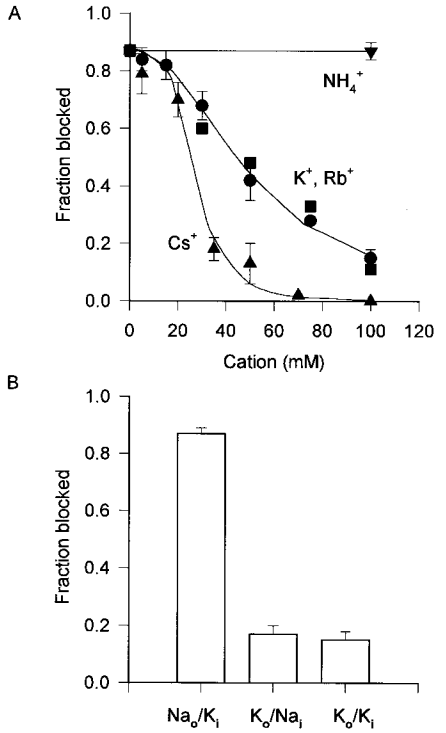

Fig. 3. External cations specifically affect the block by Pi1. A: Fraction of the channels blocked by 100 nM Pi1 added to the control Na^+ external solution, with the indicated concentrations of K^+, Rb^+, Cs^+ and NH_4^+ added by isosmolar replacement for Na^+ (see Section 2). The block was progressively reduced as the external $[K^+]$ (circles), $[Rb^+]$ (squares) and $[Cs^+]$ increased. The lines are the fit to the points with the equation $fb = f_{max}\ (k'/(k'+[I]^n))$, where fb is the fraction blocked, f_{max} is the extent of block in the reference Na^+ solution (0.87) and k′ and n are the fitted parameters: K^+, Rb^+: $n = 2.2$, $k' = 6639$, which gives a $[I]_{0.5}$ of 46.1 mM, where $[I]_{0.5}$ is the concentration of the ions at which 50% of the channels are still blocked. Cs^+: $n = 4.1$, $k' = 1\,141\,200$, which gives a $[Cs]_{0.5} = 30.4$ mM. Replacement of 100 mM Na^+ by 100 mM NH_4^+ had no effect on the block by Pi1, the horizontal line was manually adjusted. The points are the mean ± S.E.M. of at least four measurements. B: Comparison of the fraction of the channels blocked by 100 nM Pi1 added to the extracellular solution in the reference Na^+ external and K^+ internal solutions (Na_o/K_i), in the Na^+ external solution with 100 mM K^+ (K_o), and the reference K^+ internal solution (as in A, labeled K_o/K_i), and in the Na^+ external solution with 100 mM K^+, and a Na^+ internal solution (K_o/Na_i). The composition of the Na^+ internal solution was (in mM): 90 NaF, 30 NaCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES-Na pH 7.2.

Pi (note that fewer than 20% of the channels were still blocked by Pi1 with 100 mM K^+ outside, out of the average 87% blocked in the zero K^+, Na^+ control solution); (c) the permeant Rb^+ ions were, as expected, as effective as K^+ in impeding the block by Pi1; and (d) the impermeant Cs^+ ions also impede the block of the channels by Pi1. Surprisingly, Cs^+ was clearly more effective than K^+. With 100 mM Cs^+ none of the channels is blocked by the toxin. Finally and obviously, (e) the effect of the ions cannot be ascribed to an unspecific ionic strength effect.

The potency with which the tested cations destabilize (impede) the binding of Pi1 ($Cs^+ > K^+ = Rb^+ \gg NH_4^+ = Na^+$) is not related to the permeability sequence of the channels [9,10]. This suggest that the mechanism by which the ions interfere with the block of the toxin might not be a simple competition for entry into the most external face of the conduction pore. If this were the case we would have expected NH_4^+ ions to behave differently than Na^+ ions. In contrast, it seems that the ions impede or destabilize the block by Pi1, by binding to sites located in each of the four subunits that are known to compose the channel [11]. The line through the points in Fig. 3A was fitted with an equation that simply considered that the fraction of the channels blocked by Pi1 (fb) decreased as the fraction of the channels with bounded K^+, Rb^+ or Cs^+ ions (f_{ion}) increased, namely: $fb = fb_{max}(1-f_{ion})$. Where fb_{max} is the fraction of the channels blocked in the Na^+ control solution. The binding of the ions (f_{ion}) turned out to follow a Hill equation with $n > 1$ (see the legend of Fig. 3A): Cs^+: $n = 4.1$, $[I]_{0.5} = 30.4$ mM; K^+ and Rb^+: $n = 2.2$, $[I]_{0.5} = 46.1$ mM, where $[I]_{0.5}$ is the concentration of the ions at which only half of the channels are still blocked by Pi1. The difference in the fitted values of n suggests that Cs^+ probably binds to sites different to those to which K^+ and Rb^+ bind. The difference in the value of n seems to account for the clearly stronger effect of Cs^+ over K^+ and Rb^+, given that the $[I]_{0.5}$ values are not very different.

Regarding the above point, it is pertinent to mention that it is known that cations of the external solution can bind to sites located in the external 'vestibule' of the pore with a potency that differs from that of the permeability of the channels. This has been seen by measuring the effects on the various cations on the gating of the channels [12].

Given the notorious effect of the cations of the external solution on the block by Pi1, it was interesting to compare the effect of the external versus the internal K^+ on the block of *Shaker*B by Pi1. Fig. 3B shows that compared with an average 87% block exerted by 100 nM Pi1 with zero external K^+ (Na^+ control solution), and 120 mM K^+ in the normal K^+ internal solution (see Section 2), only 17% of the channels were blocked with 100 mM K^+ in the external solution and zero K^+ in the internal solution (see the legend to Fig. 3B). Interestingly, this latter amount of block is not significantly different from that observed with 100 mM K^+ outside and 120 mM K^+ inside (15%). This indicates that the external K^+ is more efficient than the internal K^+ in impeding or destabilizing the block of *Shaker*B by Pi1.

In contrast to the notorious effect of the monovalent cations shown in Fig. 3, we did not see any significant effect of external Ca^{2+} ions in a range of 3–10 mM (not shown).

It will be of interest to pursue the molecular basis of the interference of the ions on the block by Pi1, with the use of mutated versions of both Pi1 and the channels.

Acknowledgements: Supported in part by grants from: European Commission (CI1*-CT94-0045), National Autonomous University of Mexico (DGAPA-IN-206994), and Howard Hughes Medical Institute (N:75191-527104).

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