Energetics of *Shaker* K Channels Block by Inactivation Peptides

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ABSTRACT A synthetic peptide of the NH_2-terminal inactivation domain of the ShB channel blocks *Shaker* channels which have an NH_2-terminal deletion and mimics many of the characteristics of the intramolecular inactivation reaction. To investigate the role of electrostatic interactions in both peptide block and the inactivation process we measured the kinetics of block of macroscopic currents recorded from the intact ShB channel, and from ShBΔ6-46 channels in the presence of peptides, at different ionic strengths. The rate of inactivation and the association rate constants (k_{on}) for the ShB peptides decreased with increasing ionic strength. k_{on} for a more positively charged peptide was more steeply dependent on ionic strength consistent with a simple electrostatic mechanism of enhanced diffusion. This suggests that a rate limiting step in the inactivation process is the diffusion of the NH_2-terminal domain towards the pore. The dissociation rates (k_{off}) were insensitive to ionic strength. The temperature dependence of k_{on} for the ShB peptide was very high, ($Q_{10} = 5.0 \pm 0.58$), whereas k_{off} was relatively temperature insensitive ($Q_{10} \approx 1.1$). The results suggest that at higher temperatures the proportion of time either the peptide or channel spends in the correct conformation for binding is increased. There were two components to the time course of recovery from block by the ShB peptide, indicating two distinct blocked states, one of which has similar kinetics and dependence on external K^+ concentration as the inactivated state of ShB. The other is voltage-dependent and at −120 mV is very unstable. Increasing the net charge on the peptide did not increase sensitivity to knockoff by external K^+. We propose that the free peptide, having fewer constraints than the tethered NH_2-terminal domain binds to a similar site on the channel in at least two different conformations.

INTRODUCTION

Peptides of NH_2-terminal sequences of different K channels have been shown to be effective at blocking K channel ion conduction (Foster, Chung, Zagotta, Aldrich, and Levitan, 1992; Murrell-Lagnado and Aldrich, 1993; Ruppersberg, Stocker, Pongs,

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Heinemann, Frank, and Koenen, 1991; Toro, Stefani and Latorre, 1992; Zagotta, Hoshi, and Aldrich, 1990). For the *Shaker* K channel, peptide block appears to be similar to the intramolecular inactivation reaction (Hoshi, Zagotta, and Aldrich, 1990; Murrell-Lagnado and Aldrich, 1993; Zagotta et al., 1990). The blocking potency of the peptides is correlated with the rate of inactivation in the channel which has the corresponding sequence at it NH_2-terminus (Murrell-Lagnado and Aldrich, 1993). Choi, Aldrich, and Yellen (1991) showed that this form of inactivation was competed by internal tetraethylammonium (TEA) suggesting that the NH_2-terminal domain binds within the region of the channel pore. Similarly, we showed in the previous paper that TEA competes with blockade of ShBΔ6-46 channels by the ShB peptide suggesting that it also binds at the mouth of the open channel pore (Murrell-Lagnado and Aldrich, 1993). Amino acid substitutions within the peptide and within the NH_2-terminal domain produced qualitatively similar effects on the kinetics of current block, suggesting that structural features within the peptide that are important in determining binding and unbinding rates are also important within the NH_2-terminal domain in determining its interaction with a site at the pore entrance (Hoshi et al., 1990; Murrell-Lagnado and Aldrich, 1993; Zagotta et al., 1990).

Hoshi et al. (1990) examined the functional importance of the positively charged residues within the NH_2-terminal domain of ShB by making deletions and substitutions in this region. Deleting these charged residues (ShBΔ16-30) slowed the rate of inactivation as did substituting glutamines for arginines at positions 14 and 17. We showed in the previous paper that there is a clear relationship between the net charge on the peptide and the association rate constant (Murrell-Lagnado and Aldrich, 1993). Altering the net charge within the NH_2-terminal domain of ShB generally produced a smaller change in the rate of block compared with making the same change in the peptide, but the effects were qualitatively similar. These results suggest that long-range electrostatic interactions are involved in determining both the rate of peptide block and the rate of inactivation.

In the ShB channel there is good evidence that only one of the four NH_2-terminal domains binds to the mouth of the pore to produce inactivation. MacKinnon, Aldrich, and Lee (1993) showed that heteromultimeric *Shaker* channels composed of three subunits lacking the NH_2-terminal domain and only one with an intact NH_2-terminal domain inactivate at a rate about fourfold slower than the wild type channel, but recover from inactivation at a similar rate as the wild type channel. There is an additional inactivation process that remains after deleting the NH_2-terminal region of ShB, termed C-type inactivation (Hoshi, Zagotta, and Aldrich, 1991), which gives rise to a much longer lived closed state of the channel. In physiological solutions inactivation in ShB is voltage-independent between -30 and $+50$ mV, suggesting that the NH_2-terminal domain binds outside of the membrane electric field (Zagotta, Hoshi, and Aldrich, 1989). Demo and Yellen (1991) showed that the rate of recovery from inactivation increased in a voltage-dependent manner when the external K^+ concentration was raised and they proposed that a K ion entering the pore from the external side bound to a site within the pore and destabilized the NH_2-terminal domain by electrostatic repulsion. This suggests that in the inactivated state the NH_2-terminal domain physically occludes the pore, and

that charged residues within this domain face into the pore and interact with permeant ions.

The ShB peptide will have more degrees of freedom than the NH_2-terminal domain of the channel, in terms of both its conformation and orientation. Unlike the channel blocking peptide toxins (Bontems, Roumestand, Boyot, Gilquin, Doljansky, Menez, and Toma, 1991*a*; Bontems, Roumestand, Gilquin, Menez, and Toma, 1991*b*), the ShB peptide does not adopt a stable folded structure in solution and probably transiently visits many different conformations which are energetically equivalent (Lee et al., 1992). The peptide might therefore interact differently with the channel so forming a complex that blocks conduction but has a different conformation to that of the inactivated state of ShB. The ShB peptide applied to other K channels has been shown to produce at least two kinetically distinct blocked states (Foster et al., 1992; Toro et al., 1992). In this study, our aim was to address the following questions: (*a*) are long range electrostatic interactions involved in determining the rate of inactivation in ShB and the rate of block by the ShB peptide, and if so, is this a simple electrostatic mechanism of enhanced diffusion, (*b*) what other factors determine the rate of block by the ShB peptide and thus may be important in determining the rate of inactivation in the intact ShB channel; (*c*). Do the additional degrees of freedom of the peptide compared to the tethered NH_2-terminal domain affect the way that the inactivation particle interacts with the channel to block conduction. To address this final question we have compared how membrane voltage and the external permeant ion concentration affect the stability of the peptide-channel complex, with how they have been shown to influence the stability of the inactivated state of ShB (Zagotta et al., 1989, Demo and Yellen, 1991).

MATERIAL AND METHODS

These are as described in the preceding paper (Murrell-Lagnado and Aldrich, 1993).

RESULTS

The rate of block of ShBΔ6-46 channels by the ShB peptide is proportional to concentration within the range that we have used (1–100 μM), suggesting that we can account for the reaction on the basis of a bimolecular collision (Murrell-Lagnado and Aldrich, 1993; Zagotta et al., 1990*b*). The blocking rate is increased with increasing net positive charge within the COOH-terminal half of the peptide (Murrell-Lagnado and Aldrich, 1993) suggesting that the effective concentration of the peptide is dependent on its valence. Either the actual concentration of peptide molecules in the vicinity of the binding site is increased by a simple electrostatic mechanism of enhanced diffusion, or the proportion of time a peptide molecule spends in the correct conformation and orientation to bind is increased. If it is a simple electrostatic mechanism involving long range interactions between charges in the vicinity of the binding site and charges on the peptide, then changes in ionic strength should affect the association rate of peptides of different valence in a predictable way. Increasing ionic strength reduces long range electrostatic interactions because of the screening of surface charges by the ions in solution. We have investigated the effects

of ionic strength on both the blocking and unblocking rates of the peptide and the intact inactivation domain of ShB.

Ionic Strength Dependence of Association and Dissociation Rates

The ionic strength dependence of peptide blocking and unblocking rates was examined by either keeping the internal concentration of K^+ constant at 50 mM and increasing the concentration of *N*-methyl glucamine (NMG), or by varying the concentration of K^+ from 50 to 600 mM. The association and dissociation rate constants, k_{on} and k_{off}, were calculated from the decay time course of the macroscopic currents at +50 mV as described in the previous paper (Murrell-Lagnado and Aldrich, 1993). Similar values were obtained with the concentration of either ion being varied although with very high concentrations of NMG^+ (300–600 mM) there appeared to be significant block of outward current. We compared two peptides; the parent ShB peptide and a peptide which has lysines substituted for the two acidic residues at positions 12 and 13 (ShB:E12KD13K). The net charge on the ShB peptide is +2, not including the positive charge at the NH_2-terminus and discounting any charge on the histidine at position 16, which we think is predominantly uncharged under the conditions of our experiments (Murrell-Lagnado and Aldrich, 1993). The net charge on the ShB:E12KD13K peptide is +6. Fig. 1 shows k_{on} and k_{off} for the ShB and ShB:E12KD13K peptides plotted on a log scale versus the ionic strength of the internal solution. The association rate constants decreased with increasing ionic strength whereas there was no clear relationship between k_{off} and ionic strength, suggesting that long-range electrostatic interactions are involved in determining k_{on} but not k_{off}. The association rate constant of the more positively charged peptide, ShB:E12KD13K, was more steeply dependent upon ionic strength which is what would be predicted for a simple electrostatic mechanism of enhanced diffusion. The lack of any clear dependence of k_{off} on ionic strength is consistent with our previous findings, that there was no clear relationship between the net charge on the peptide and k_{off} (Murrell-Lagnado and Aldrich, 1993). It suggests that between the bound state and the transition state there is little change in either short- or long-range electrostatic interactions.

The rate of inactivation in ShB channels with an intact NH_2-terminal domain was also affected by ionic strength. Values for k_{on} and k_{off} are included in the plot in Fig. 1. The rate constants were estimated from the decay time course of macroscopic currents recorded from the wild type ShB channel in a similar way as for the peptides (Murrell-Lagnado and Aldrich, 1993), although in this case the values for k_{on} are in units of s^{-1}. The association rate decreased with increasing ionic strength suggesting that, similar to peptide block, long-range electrostatic interactions are involved in determining the rate of inactivation. The steepness of the dependence of k_{on} on ionic strength was less for the tethered NH_2-terminal domain than for the free ShB peptide. In view of the results with peptides, we propose that long-range electrostatic interactions increase the frequency of encounters of the NH_2-terminal region of the channel protein with the region at the internal mouth of the pore. This suggests that a rate determining step in inactivation is the diffusion of the NH_2-terminal domain to the binding site. Similar to our findings with the peptides, there was no apparent dependence of the dissociation of the tethered domain on ionic strength.

The dashed lines through the data points for k_{on} versus ionic strength for the two peptides represent fits to Eq. 1 based on the Gouy-Chapman model. The Gouy-Chapman model describes how the local potential for a surface with a uniformly distributed charge density varies with the concentration of ions in solution (Grahame, 1947). Using this model to approximate the local potential at the binding site on the channel one can calculate how the association rate of a point charged species should vary with ionic strength (MacKinnon, Latorre, and Miller, 1989) from the relation:

$$k_{on} = k_{on}(0)\left\{\frac{\sigma}{\sqrt{c}} + \left[\left(\frac{\sigma}{\sqrt{c}}\right)^2 + 1\right]^{1/2}\right\}^{2z} \quad (1)$$

where $k_{on}(0)$ is the association rate constant with zero local potential, c is the ionic strength of the internal solution, z is the effective valence of the peptide and σ is a constant related to the surface charge density. The charges on the surface of proteins are clustered rather than being uniformly distributed and neither the structure or charge distribution of the channel is known so we can not predict what the potential profile will be in the vicinity of the binding site. For this reason the values for σ obtained from the fit do not have any easily interpretable physical meaning. The ShB peptide is not a point charge although, we showed in the preceding paper (Murrell-Lagnado and Aldrich, 1993) that charged residues within the COOH-terminal half of the peptide act fairly equivalently. Alterations within this region of the ShB peptide that conserved the net charge produced only small changes in k_{on} and k_{off}. This suggests that modeling the peptide as a point charge may not be completely unreasonable. When fitting Eq. 1 to the data we allowed z to vary to find the best fit by eye, rather than constraining z to be the net charge on the peptide. The lines shown are for $z = 1.8$ for the ShB peptide and 3.3 for the ShB:E12KD13K peptide. The extrapolated values that we obtained from these fits for k_{on} at infinitely high ionic strength [$k_{on}(0)$] were 1.3×10^5 M^{-1} s^{-1} for the ShB peptide and 1.0×10^5 M^{-1} s^{-1} for the ShB:E12KD13K peptide. Presumably at infinitely high ionic strength the distribution of charges on either the peptide or the surface of the channel should be unimportant because they will be completely shielded. There is good agreement between these values for $k_{on}(0)$ and the value for k_{on} for the neutral peptide, ShB:R17QK18QK19Q, in physiological solutions (Murrell-Lagnado and Aldrich, 1993), consistent with a simple electrostatic mechanism. In the absence of long range-electrostatic interactions the association rate constant for the ShB peptides is between three to four orders of magnitude lower than a diffusion limited rate. Thus, upon encountering the channel the peptide molecule has about a 1 in 10,000 chance of binding.

For a simple bimolecular reaction in order for two molecules to react upon encountering each other, they have to be in the correct orientation and have sufficient energy. According to the Arrhenius Law, the rate of a reaction depends exponentially upon the necessary energy, or activation energy (E_a) of the reaction:

$$k = Ae^{(-E_a/RT)} \quad (2)$$

where R is the gas constant, T is the absolute temperature and A is the preexponential factor characteristic for the reaction. The preexponential factor is proportional to

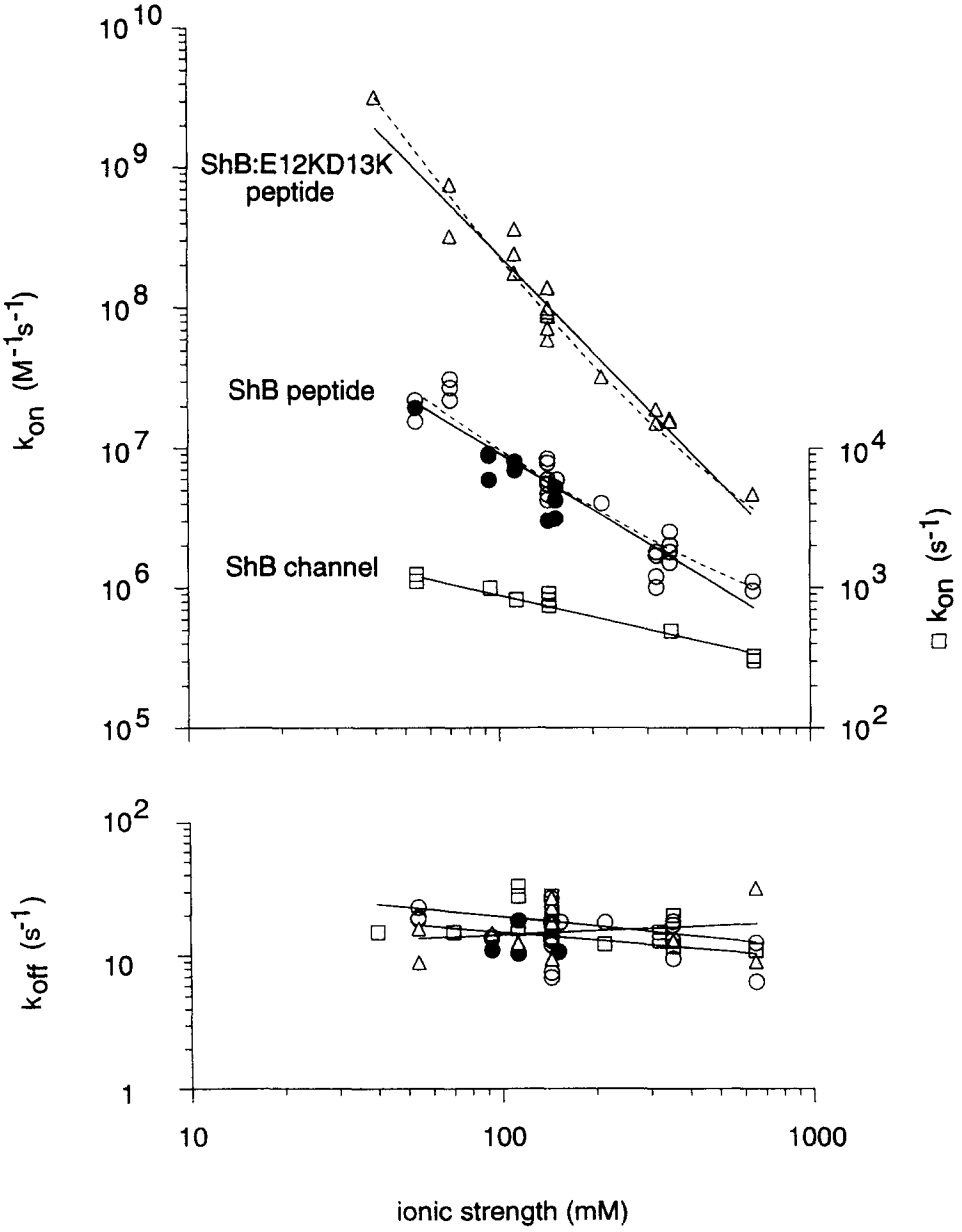

FIGURE 1. The effects of ionic strength on the association and dissociation rate constants of the ShB and ShB:E12KD13K peptides, and on the rate of inactivation in the ShB channel. Ionic strength of the internal solution was varied either by keeping the internal concentration of KCl at 50 mM and varying the concentration of *N*-methyl glucamine (*filled symbols*), or by varying the concentration of KCl, (*open symbols*). Macroscopic currents from either ShB or ShBΔ6-46 channels, in inside-out patches, were elicited by stepping the voltage to +50 mV from a holding voltage of −100 mV. Peptides were applied to the bath solutions. The rate constants k_{on} and k_{off} were calculated from the time course of the decay phase of the currents

the encounter frequency, which depends upon diffusion and thermal energy, and the fraction of time the reactants are in the correct orientation to bind. The rate of binding of the peptide to the channel will also depend upon what fraction of the time these molecules are in the correct conformations to react. The peptide does not appear to adopt a single stable structure in solution and probably transiently visits many different conformations (Lee, Aldrich, and Gierasch, 1992). Single channel records from ShBΔ6-46 in the absence of peptide, show that at depolarized voltages there are at least two kinetically distinct short lived closed states that the channel enters after the initial opening transition, so that the open probability is ~0.8 (Hoshi, Zagotta, and Aldrich, 1993).

A relatively low k_{on} suggests that either the preexponential factor is low, or the activation energy is high. The activation energy and preexponential factor can be estimated from the temperature dependence of the rate constant, if Arrhenius law (Eq. 2) is obeyed and the overall temperature dependence of the reaction is dominated by the exponential term. A plot of ln k against $1/T$ will have a slope of E_a/R:

$$\ln k = \ln A - \frac{E_a}{RT}. \tag{3}$$

We compared the temperature dependence of block by the ShB peptide and the ShB:E12KD13K peptide. If the extra charges on the altered peptide simply enhance diffusion this should be reflected in the value of the preexponential factor only. Fig. 2 shows macroscopic currents recorded from ShBΔ6-46 channels in the presence of ShB peptide at three different temperatures. The currents were from the same patch and were elicited by stepping the voltage from −120 to +50 mV. Lowering the temperature clearly slowed the rate of decay of the macroscopic currents and also reduced the peak current amplitude and the rate of activation.

Fig. 3 *A* shows ln k_{on} plotted against $1{,}000/T$ for two separate experiments, one in the presence of ShB peptide and the other in the presence of ShB:E12KD13K peptide. From the least squares linear fit to the data we calculated that for the association of the ShB peptide, E_a was 26.8 kcal mol^{-1} and A was 3.1 × 10^{26} M^{-1} s^{-1}, and for the ShB:E12KD13K peptide, E_a was 21.6 kcal mol^{-1} and A was 1.9 × 10^{24} M^{-1} s^{-1}. The mean value for E_a for the association of the ShB peptide was 27 ± 3.1 kcal mol^{-1} (SEM, n = 3) and for the ShB:E12KD13K peptide it was 21 ± 1.4 kcal mol^{-1} (SEM, n = 2). This gives values for the Q_{10} over this temperature range of

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and from the steady state level of current remaining (Murrell-Lagnado and Aldrich, 1993). The data for the two peptides and the intact ShB channel were from between 3–6 experiments in each case. The log functions were fitted by the method of least squares. For k_{on} versus ionic strength, the slopes of the lines were: for the ShBE12KD13K peptide, −2.27; for the ShB peptide, −1.35; for inactivation in ShB, −0.52. For k_{off} versus ionic strength, the slopes were between −0.2 and 0.1. The dashed lines represent fits to equation 1 in the text and the values for the parameters are also given in the text. The external solution contained 140 mM NaCl, 6 mM $MgCl_2$, 2 mM KCl and 5 mM HEPES (pH = 7.1) and the internal solution contained 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES and either 50 mM KCl and NMG (0–600 mM) or KCl (50–600 mM), (pH 7.2). The temperature was 20°C.

5.0 ± 0.58 (SEM, n = 3) for the ShB peptide and 3.6 ± 0.2 (SEM, n = 2) for the ShB:E12KD13K peptide. By contrast the dissociation rate constants were very insensitive to temperature (Fig. 3 *B*). The data shown in Fig. 3 *B* are from the same experiments as in Fig. 3 *A*. For the ShB peptide the values for E_a and A for dissociation were 0.51 kcal mol^{-1} and 45 s^{-1} respectively, and for the ShB: E12KD13K peptide they were 1.4 kcal mol^{-1} and 370 s^{-1} respectively, giving values for Q_{10} of < 1.1 in both cases. In three separate experiments the values for E_a for dissociation for the ShB peptide ranged from −0.37 kcal mol^{-1} to +4.3 kcal mol^{-1}.

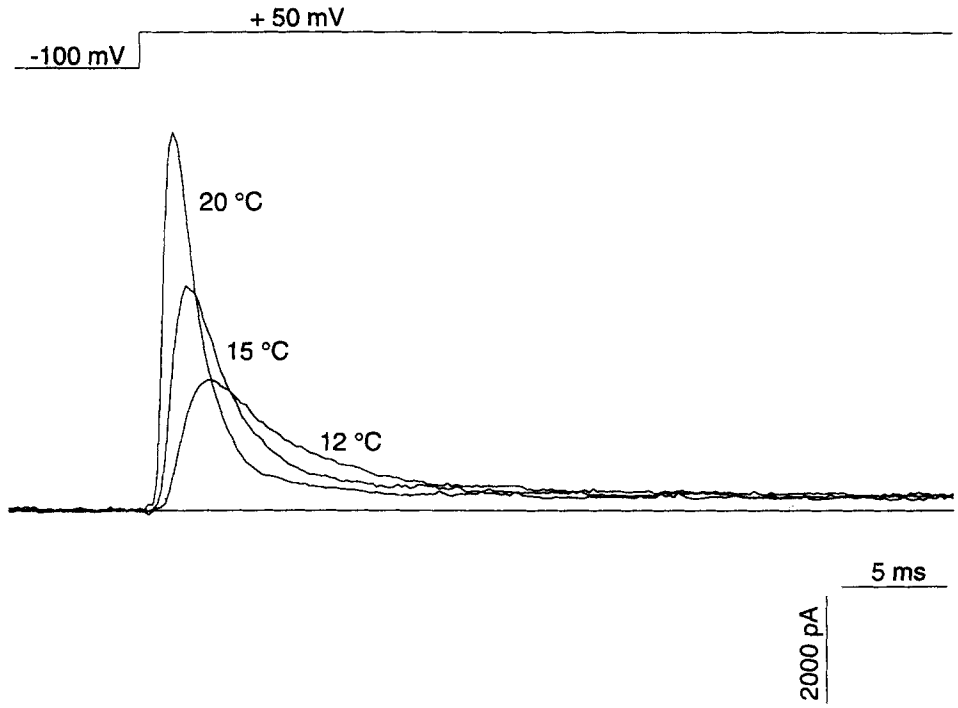

FIGURE 2. The effects of temperature on the rate of block of ShBΔ6-46 channels by the ShB peptide. Macroscopic currents were recorded from an inside-out membrane patch with 200 μM ShB peptide present in the bath, at the temperatures indicated. The decay phase of each of the traces was fitted with the sum of two exponentials. The time constants of the fast components were: 1.4 ms at 20°C; 2.2 ms at 15°C; and 3.9 ms at 12°C. The currents were low pass filtered at 1.2 kHz and digitized at 100 μs per point.

The high temperature dependence of k_{on} and the low temperature dependence of k_{off} is consistent with the findings of Nobile, Olcese, Chen, Toro, and Stefani (1993) that the rate of inactivation in ShB is very temperature dependent but recovery from inactivation is relatively temperature insensitive.

If the peptide and channel were always in the correct conformation and orientation for binding, then even allowing for the possibility of favorable electrostatic interactions, the value of A for the association of the peptides would not be expected to exceed 10^{11} M^{-1} s^{-1} (Fersht, 1985). The anomalously high temperature dependence

of the association rate constant indicates that the binding of the peptide does not obey the Arrhenius law. One possibility is that the effective concentration of the peptide is very temperature-dependent. If we assume that conformational changes that the peptide and the channel undergo in the unbound state are on a much faster time scale than the association-dissociation steps, then the binding of the peptide is better accounted for on the basis of a consecutive reaction with preequilibria, between the correct and incorrect conformations of the peptide and channel, rather than in terms of a simple bimolecular reaction. According to this scheme the measured association rate constant will be proportional to what fraction of the molecules are in the correct conformation for binding at any given time. The high temperature dependence of k_{on} suggests that there is a temperature dependent shift in one or more of the preequilibria, so that at higher temperatures the fraction of time that either the peptide or the channel spend in the correct conformation for binding is increased.

Fig. 3 *C* shows the van't Hoff plots of ln K_d versus the reciprocal of temperature, where K_d is equal to k_{off}/k_{on}. The slope of the lines provide an estimate of the enthalpic contribution to the free energy of binding (ΔH) according to the expression:

$$\ln K_d = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \tag{4}$$

where ΔS is the change in entropy upon binding. For the ShB peptide the value for ΔH was +27 kcal mol^{-1} and the value for ΔS, calculated from the intercept with the *y*-axis, was +0.12 kcal mol^{-1}K^{-1}, which at 20°C gives a value for $T\Delta S$ of +34 kcal mol^{-1}. For the ShB:E12KD13K peptide the values for ΔH and $T\Delta S$ were +23 kcal mol^{-1} and +32 kcal mol^{-1}, respectively. Hydrophobic interactions are entropically driven and therefore the positive value for TΔS for peptide binding is consistent with the results from the previous paper suggesting that hydrophobic interactions stabilize the binding of the ShB peptide to ShBΔ6-46 (Murrell-Lagnado and Aldrich, 1993). However, given the anomalous temperature dependence of the rate constants it is difficult to interpret the observed thermodynamic parameters in terms of the driving forces for peptide binding. One would predict that the binding of the unstructured peptide would involve a considerable loss of conformational entropy.

The Blocking Potency of the Peptides Increases with Increasing Depolarization

The NH_2-terminal domain of the intact ShB channel, by virtue of being tethered to the channel protein will be more constrained than the free peptide both in terms of its conformation and orientation. Therefore the conformation that it adopts upon binding to the mouth of the pore may differ from that adopted by the ShB peptide. Single channel recordings from ShB show no apparent voltage-dependence to inactivation between −30 mV and +50 mV (Zagotta et al., 1989) suggesting that either the tethered domain binds completely out of the membrane electric field or that only the nonpolar side groups of the hydrophobic residues enter into the pore of the channel. In agreement with these results we found that there was no voltage-dependence to the rate of inactivation of macroscopic currents recorded from ShB between +20 and +100 mV (not shown). To determine whether there was any

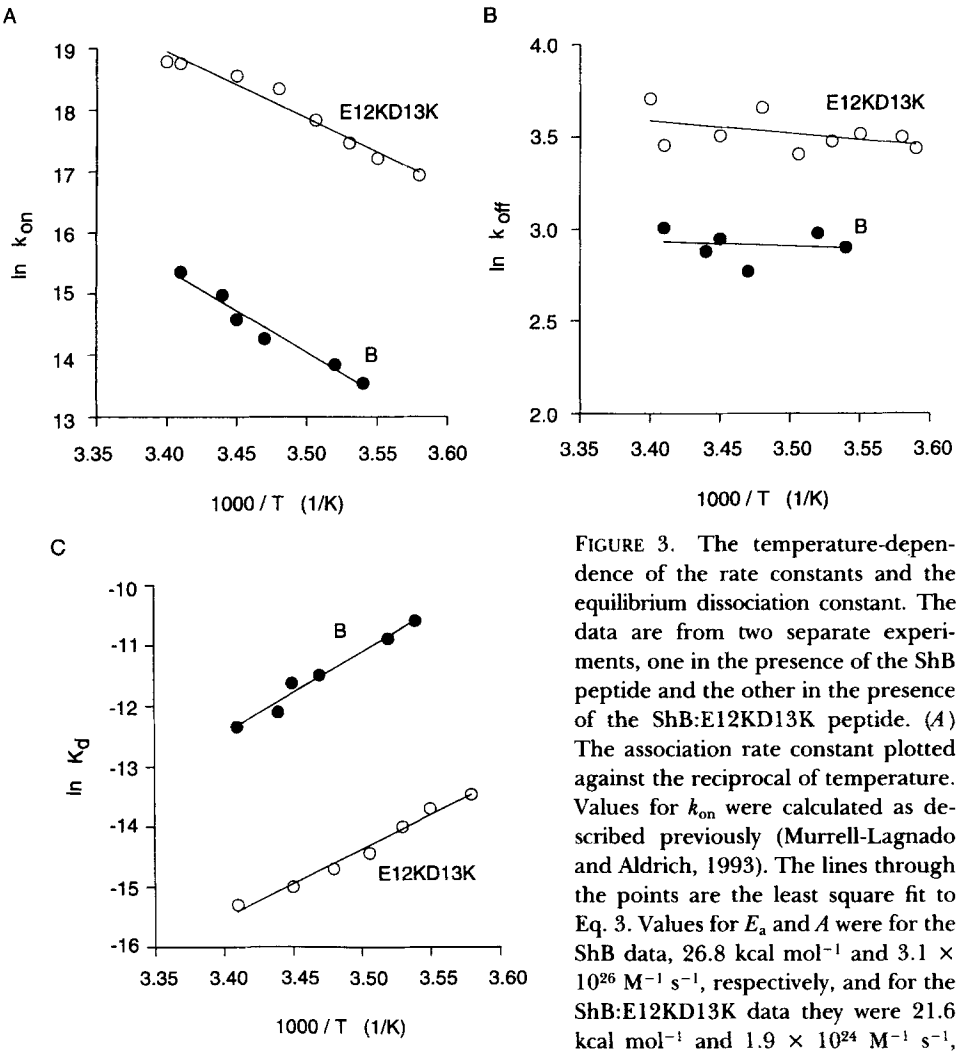

FIGURE 3. The temperature-dependence of the rate constants and the equilibrium dissociation constant. The data are from two separate experiments, one in the presence of the ShB peptide and the other in the presence of the ShB:E12KD13K peptide. (*A*) The association rate constant plotted against the reciprocal of temperature. Values for k_{on} were calculated as described previously (Murrell-Lagnado and Aldrich, 1993). The lines through the points are the least square fit to Eq. 3. Values for E_a and A were for the ShB data, 26.8 kcal mol^{-1} and 3.1 × 10^{26} M^{-1} s^{-1}, respectively, and for the ShB:E12KD13K data they were 21.6 kcal mol^{-1} and 1.9 × 10^{24} M^{-1} s^{-1}, respectively. (*B*). The dissociation rate plotted against the reciprocal of temperature. From the least squares fit to Eq. 3 values for E_a and A for the ShB peptide were 0.51 kcal mol^{-1} and 45 s^{-1}, respectively, and for the ShB:E12KD13K peptide they were 1.4 kcal mol^{-1} and 370 s^{-1}, respectively. (*C*) The values for K_d were calculated from the rate constants (k_{off}/k_{on}) and plotted against 1,000/temperature. The line fitted to the points corresponds to the van't Hoff Eq. 4. Values for ΔH and ΔS for the binding of the ShB peptide were +27 kcal mol^{-1} and +0.12 kcal mol^{-1} K^{-1}, respectively and for the ShB:E12KD13K peptide were +23 kcal mol^{-1} and 0.11 kcal mol^{-1} K^{-1}, respectively.

voltage-dependence to peptide block of ShBΔ6-46 channels we used a two pulse protocol as shown in Fig. 4. The membrane potential was stepped from −100 to +20 mV for 100 ms and then to +80 for 100 ms. Macroscopic currents were recorded before and after application of the peptide. The current recorded before addition of peptide showed a step increase in amplitude in response to stepping the potential

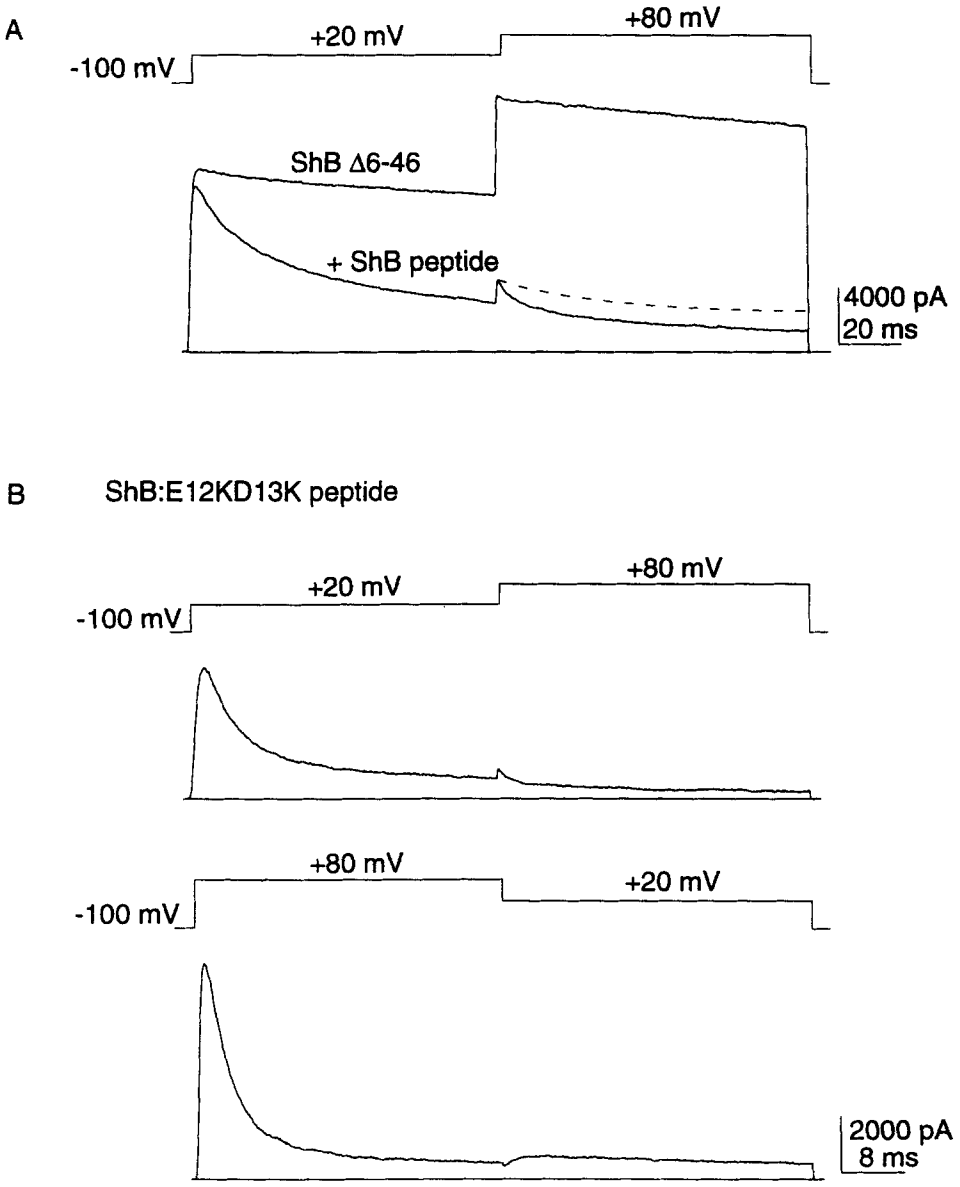

FIGURE 4. The blocking potency of the ShB peptides increases at more depolarized voltages (*A*) Macroscopic currents recorded from ShBΔ6-46 before and after application of ShB peptide to the bath. The pulse protocol is shown above the current traces. The dashed line indicates the predicted current amplitude if the affinity of the peptide was unchanged by stepping the voltage from +20 to +80 mV. (*B*) Macroscopic currents recorded from ShBΔ6-46 in the presence of ShB:E12KD13K peptide using the pulse protocol indicated. The records were from the same patch.

from +20 to +80 mV, in proportion to the increase in the electrical driving force for K^+. In the presence of the peptide there was also an initial increase in amplitude after a similar step in membrane potential, although the current then relaxed down to a new steady state level indicating that a greater fraction of the current was blocked at the more depolarized potential. A similar experiment but this time in the presence of the ShB:E12KD13K peptide is shown in Fig. 4 *B*. In the top trace the voltage was stepped initially to +20 mV and then to +80 mV and in the bottom trace the voltage was stepped to +80 mV and then back to +20 mV. Both traces show a relaxation of the current in the direction opposite to what would be predicted for the change in the driving force, indicating that the binding affinity of the peptide was higher at more depolarized potentials. Voltage-dependence was also seen with the binding of the ShBR17Q,K18Q,K19Q peptide which has zero net charge (not shown). Although we did not quantify the voltage-dependence of the binding affinities of the different peptides, by visual inspection of the traces they appeared to be similar and not dependent upon the net charge on the peptide. These results suggest that the peptides adopt a different bound conformation to that adopted by the NH_2-terminal domain in the inactivated state, and that one or more of the charged residues on the peptide enters some way into the pore.

The Peptide-Channel Complex is Destabilized by Increasing External K^+ Concentration

Demo and Yellen (1991) showed in the intact ShB channel that increasing the external K^+ concentration speeds recovery from inactivation in a voltage dependent manner, suggesting that the tethered NH_2-terminal domain physically blocks the channel pore and interacts with ions that are within the pore. Electrostatic repulsive forces are proposed to destabilize the bound inactivation domain, which suggests that one or more charges within this region face into the pore. As a way of comparing the structure of the bound peptide-channel complex with that of the inactivated state of ShB we looked at the effects of external K^+ concentration on peptide block. If the structures are similar then we would expect a similar relative change in k_{off} for a given increase in external K^+, for both the peptide and the tethered NH_2-terminal domain. We also compared the effects of external K^+ on block by the ShB:E12KD13K peptide to test whether altering the net charge within the COOH-terminal half of the peptide affected the amount by which a K^+ at the knockoff site destabilized the peptide-channel complex.

The rate of recovery from block was determined using a paired pulse protocol. A test pulse to +50 mV was given for 20 ms in the presence of a fairly high concentration of peptide, which blocked 85–95% of the current. The voltage was then stepped to −120 mV for a variable length of time before giving a second test pulse to +50 mV (Fig. 5 *A*). The fraction of the peak current blocked at the end of the first test pulse that had recovered by the second test pulse was plotted against the period of time between test pulses (Fig. 5 *B*). A correction was made for the proportion of channels that would have entered the C-type inactivated state during the first test pulse. The C-type inactivation process in ShB channels remains after removal of the NH_2-terminal domain (Hoshi et al., 1991) and has a time constant of ~200 ms in the presence of peptide. We calculated that ~10% of the channels would have entered

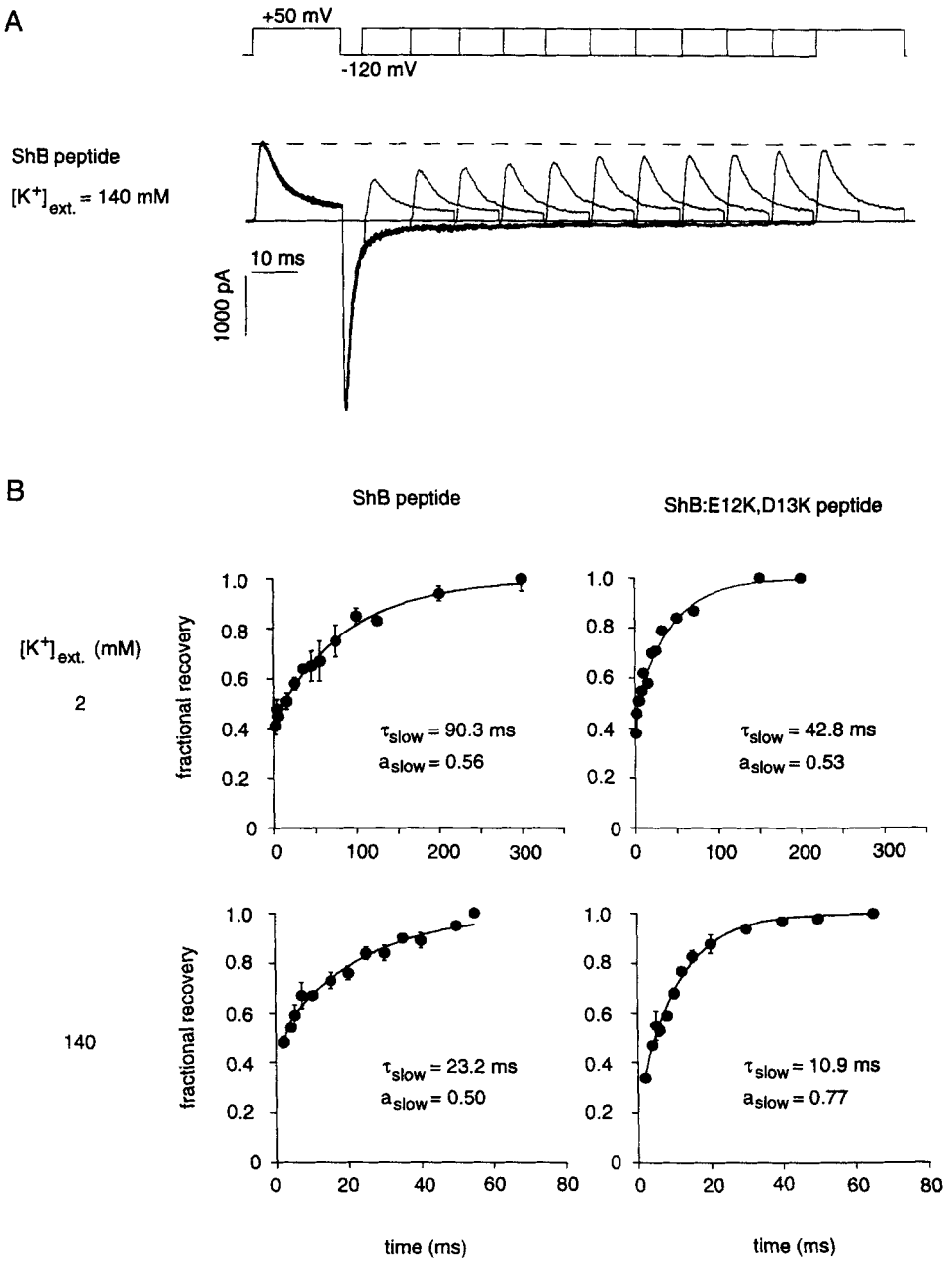

FIGURE 5. Time courses of recovery from peptide block. (*A*) Macroscopic currents from ShBΔ6-46 channels in an inside-out patch in the presence of 50 μM ShB peptide and with 140 mM symmetrical KCl. After a test pulse of 20 ms at +50 mV the voltage was stepped to −120 mV for a variable duration before a second test pulse to +50 mV. The shortest interpulse duration we used was 2 ms. (*B*) Fractional recovery is defined as the difference in amplitude between the peak current during the second test pulse and the current at the end of the first test pulse divided by the difference between the peak current during the first test pulse and the current at the end of this pulse. The points are averages from between 3–7 experiments. The sums of two exponential components were fitted to each of the recovery time courses and the values for the slow time constant (τ_{slow}) and the relative amplitude of this component are shown. At −120 mV the values for τ_{fast} were all less than 1 ms.

this state during a 20-ms test pulse, and we therefore normalized to recovery of 90% of the peak current. Recovery from C-type inactivation even at very negative voltages has a time constant on the order of seconds (Hoshi et al., 1991), which is considerably slower than the rate of recovery from peptide block under the conditions of our experiments.

The time courses of recovery from block by the ShB peptide and the ShB:E12KD13K peptide, at −120 mV and in the presence of either 2 or 140 mM external K^+ are shown in Fig. 5 *B*. Each plot shows the averaged data from between three to seven experiments. In each case there were clearly at least two components to the recovery time course. There was an extremely rapid component which had a time constant of < 1 ms at −120 mV, and a much slower component with a time constant of tens of milliseconds. The relative amplitudes of the two components were similar and appeared to be independent of the external K^+ concentration. For the ShB peptide the amplitude of the fast component (a_{fast}) was 0.50 ± 0.02 (SEM, n = 7), and for the ShB:E12KD13K peptide, a_{fast} was 0.37 ± 0.035 (SEM, n = 6).

The values for the slow time constant (τ_{slow}) are shown plotted against external K^+ in Fig. 6 *A*. There was a decrease in τ_{slow} with increasing external K^+ for both the ShB and ShB:E12KD13K peptides. For the ShB peptide there was a 3.9-fold decrease in τ_{slow} when external K^+ was raised from 2 to 140 mM. The corresponding rates ($1/\tau_{slow}$) were 11 s^{-1} in 2 mM K^+ and 43 s^{-1} in 140 mM K^+. For the ShB:E12KD13K peptide there was a 3.7-fold change from 23 s^{-1} in 2 mM K^+ to 86 s^{-1} in 140 mM K^+. This result suggests that a K ion bound at a site within the pore destabilizes the peptide-channel complex, similar to its effect on the inactivated state of ShB (Demo and Yellen, 1991). Comparing our findings with those obtained by Demo and Yellen (1991) for recovery from inactivation in the intact ShB channel, they showed that with 2 mM external K^+ and at −120 mV, the rate of recovery from inactivation was 20 s^{-1}, and this increased 3.3-fold to 65 s^{-1} with 160 mM external K^+. Thus, the rate of recovery from inactivation and the slow component of recovery from peptide block have a similar dependence on external K^+, which suggests that this slow component represents recovery from a blocked state that is similar to the inactivated state.

To test whether the correction that was made for C-type inactivation was valid we measured the time course of recovery from peptide block of a channel which has threonine at position 449 mutated to valine (ShBΔ6-46:T449V). This mutation appears to eliminate C-type inactivation (Lopez-Barneo, Hoshi, Heinemann and Aldrich, 1993). The value for τ_{slow} with 2 mM external K^+, was obtained by averaging data from four experiments and fitting the sum of two exponential components to the time course. It is represented in Fig. 6 *A* as the square symbol and the value is very close to the value obtained from ShBΔ6-46 channels after correcting for C-type inactivation. For the ShB:E12KD13K peptide, τ_{slow} was shorter at all external K^+ concentrations indicating that this peptide binds less tightly to the ShBΔ6-46 channel. This is consistent with the results from the previous paper (Murrell-Lagnado and Aldrich, 1993) where the value for k_{off} measured from macroscopic currents at +50 mV was 1.7-fold higher for the ShB:E12KD13K peptide compared with the parent ShB peptide.

The time course of recovery from peptide block of ShBΔ6-46 channels measured this way with macroscopic currents, will reflect not only the rate of dissociation of the

peptide from its binding site (k_{off}) but also the relative rates of channel deactivation [$\kappa(V)$] and channel blockade (k_{on}). This is represented by the following scheme:

$$C \underset{\kappa(V)}{\overset{\lambda(V)}{\rightleftarrows}} O \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} PO$$

SCHEME I

where the closed states on the activation pathway have been grouped together and are represented as C, the open state is O, and PO is the blocked channel. Here we are considering only the slow component of the recovery time course and assuming that the two components represent recovery from two separate blocked states. In this scheme, we have not included a pathway directly from the blocked state to the closed state although recovery may not always be via the open state (Demo and Yellen, 1991). During the slow phase of recovery the probability of a channel being in the open state is very low, as indicated by the very small slow component to the inward tail currents (e.g. Fig. 5 *A*). If we assume that the open probability remains constant, the recovery rate ($k_{rec} = 1/\tau_{slow}$) is given by the expression:

$$k_{rec} = k_{off}\left(\frac{\kappa(V)}{\kappa(V) + k_{on}^{app}}\right). \tag{5}$$

Therefore, when $\kappa(V)$ is much faster than k_{on}, the recovery rate will approximate k_{off}. The deactivation rate of ShBΔ6-46 was determined from the relaxation of inward tail currents after a test pulse to +50 mV to open the channels, and was estimated to be ~1,400 s^{-1} at −120 mV, and 300 s^{-1} at −80 mV (Hoshi et al., 1993). With 100 μM ShB peptide present and at +50 mV, k_{on} was about 400 s^{-1}. There is some indication from the time course of the tail currents, that at −120 mV in symmetrical 140 mM KCl, peptide block is slower (see below). Therefore, at −120 mV, $1/\tau_{slow}$ was a reasonable estimate of k_{off}, but at −80 mV it was probably significantly slower.

We have used the values for τ_{slow} measured at −120 mV to calculate the increase in k_{off} caused by a K ion occupying the knockoff site, for the bound ShB peptide and ShB:E12KD13K peptide. The analysis is similar to that described by MacKinnon and Miller (1988) for the 'knockoff' of charybdotoxin from Ca^{2+}-activated K^+ channels. The dissociation rate is given by the expression:

$$k_{off} = P(occ.)k_{off}^{o} + [1 - P(occ.)]k_{off}^{v} \tag{6}$$

where $k_{off}^{o}$and k_{off}^{v} are the dissociation rates with the knockoff site occupied and vacant, respectively and $P(occ.)$ is the probability of the knockoff site being occupied. This assumes that K^+ at the knockoff site is in equilibrium with the external unbound K ions. The probability of the knockoff site being occupied is given by:

$$P(occ.) = \frac{1}{\left(1 + \frac{K_d}{a_K}\right)} \tag{7}$$

The K_d is the equilibrium dissociation constant for the K^+ binding to the knockoff site and is probably voltage-dependent (Demo and Yellen, 1991). The values are unlikely

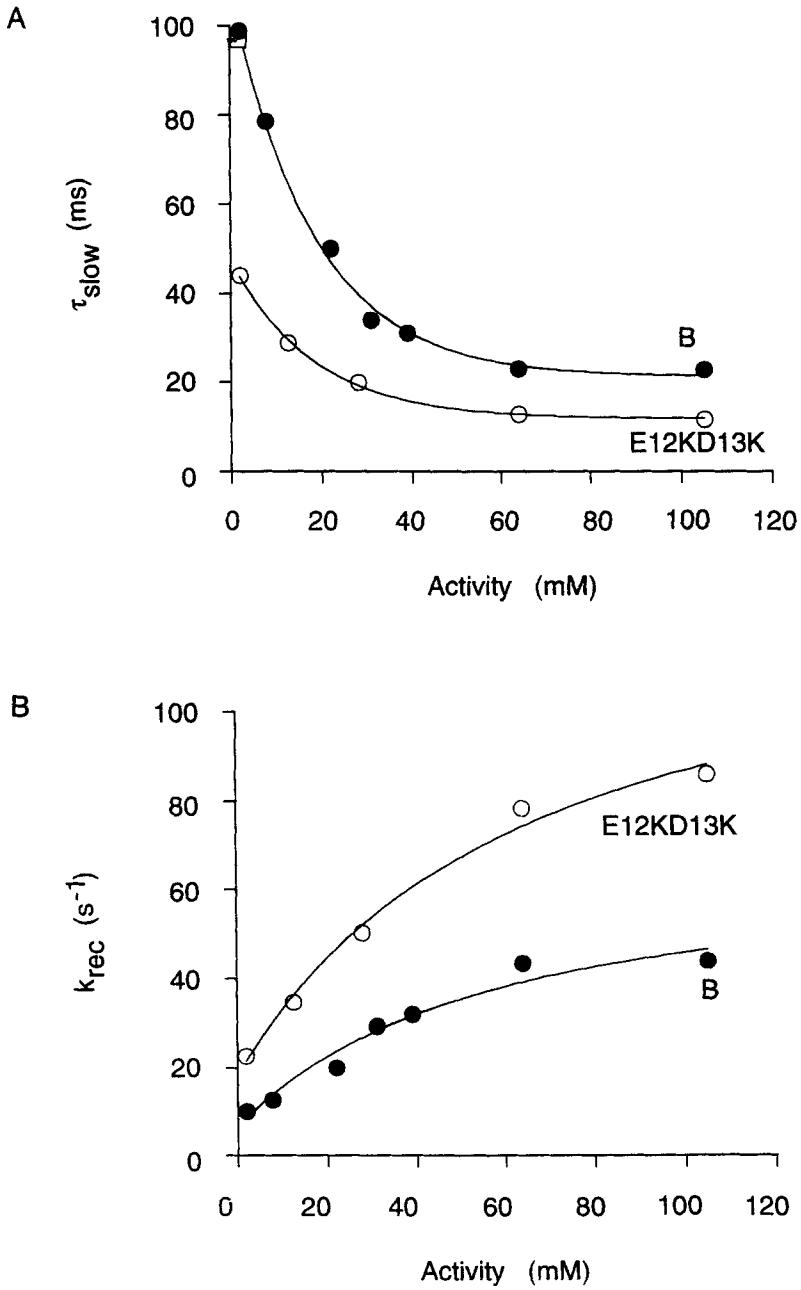

FIGURE 6. The dependence of the rate of recovery from peptide block on external K^+. (*A*) τ_{slow} from the time course of recovery from peptide block is shown at different external K^+ activities. Data from between 3–7 experiments was averaged and the time course fitted with the sum of two exponential components. The reason for averaging the data before fitting the time course was so that we could include data from experiments where there was not a complete range of recovery intervals. Because of the two components to recovery it was necessary to measure

to represent an intrinsic affinity because of K^+ occupancy of other sites within the pore. In Fig. 6 *B*, $1/\tau_{slow}$ is plotted against the external concentration of K^+ and the curves shown are the least square fits to Eq. 6 for the ShB and ShB:E12KD13K peptides. Values for k^v_{off} and k^o_{off} are 6.3 s^{-1} and 68 s^{-1}, respectively, for the ShB peptide and 18 s^{-1} and 132 s^{-1} for the ShB:E12KD13K peptide. For the ShB peptide-ShBΔ6-46 channel complex the increase in k_{off} with the 'knockoff' site occupied versus vacant was 10.8-fold, whereas for the more positively charged peptide, ShB:E12KD13K, the increase in k_{off} with the 'knockoff' site occupied versus vacant was only 7.3-fold. This suggests that for the more positively charged peptide the charge density on the surface facing into the pore may be actually less than for the parent ShB peptide.

The ShB Peptide Produces at Least Two Kinetically Distinct Blocked States

The two components to the time course of recovery from peptide block suggest that there are at least two blocked states. Whereas one of these states appears to be similar to the inactivated state of ShB, the other was extremely unstable at negative voltages. Upon stepping the voltage from +50 to −120 mV, approximately half of the blocked channels recovered within the shortest interpulse interval of 2 ms. However, at depolarized potentials there was no evidence for an unstable blocked state. At +50 mV and with 2 mM external K^+ the apparent k_{off} for the ShB peptide measured from macroscopic currents was 13.7 s^{-1} (Murrell-Lagnado and Aldrich, 1993) which is similar to $1/\tau_{slow}$ for recovery at −120 mV (11.1 s^{-1}).

At −120 mV, τ_{fast} was not properly resolved because it was <1 ms. However, we can obtain an estimate of the rate at which the peptide dissociates from this unstable blocked state at −120 mV, from the time course of the inward tail currents. Fig. 7 shows tail currents recorded from the same patch at −120 mV in symmetrical 140 mM K^+, before and after application of ShB peptide. Before applying the peptide the tail currents relaxed with a double-exponential time course, the major component having a time constant of 0.9 ms, corresponding to a deactivation rate of 1,100 s^{-1}. After applying peptide, 85% of the current was blocked by the end of the test pulse. However, upon stepping to −120 mV there was a large inward tail current, indicating that a significant proportion of the blocked channels had rapidly unblocked and were conducting. The tail currents following application of peptide were also fitted with two exponential components and the fast component had a time constant of 1.1 ms. In general the time constants varied from 0.7 to 1.5 ms. The rate of decay of the tail currents will have been determined by the relative rates of the

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recovery over a wide range of interpulse durations. The circles correspond to recovery from peptide block of ShBΔ6-46 channels and the square symbol corresponds to recovery from block of ShBΔ6-46:T449V channels by the ShB peptide. The point mutation at position T449 appears to eliminate C-type inactivation (Lopez-Barneo et al., 1993). The curves through the points do not represent actual fits to the data but are included to illustrate more clearly the relationship between τ_{slow} and external K^+ activity. (*B*) The rate of recovery ($k_{off} = 1/\tau_{slow}$) as a function of the external K^+ concentration. The curves correspond to equation 6. For the ShB peptide, the least square fit gives k^v_{off} = 6.3 s^{-1}, k^o_{off} = 68 s^{-1} and K_d = 56 mM. For the ShB:E12KD13K peptide, k^v_{off} = 18 s^{-1}, k^o_{off} = 132 s^{-1}, and K_d = 67 mM.

blocked channels opening, the open channels closing, and the rate at which the open channels are blocked. For the peak amplitude of the tail currents to be 40% of the peak amplitude in the absence of peptide, given that 85% of the channels were blocked and only ~50% of blocked channels recover rapidly, the blocked to open transition has to be significantly faster than the open to closed transition. We show later that to simulate currents with a similar time course to the traces in Fig. 7, before and after application of peptide, that 50% of blocked channels have to become

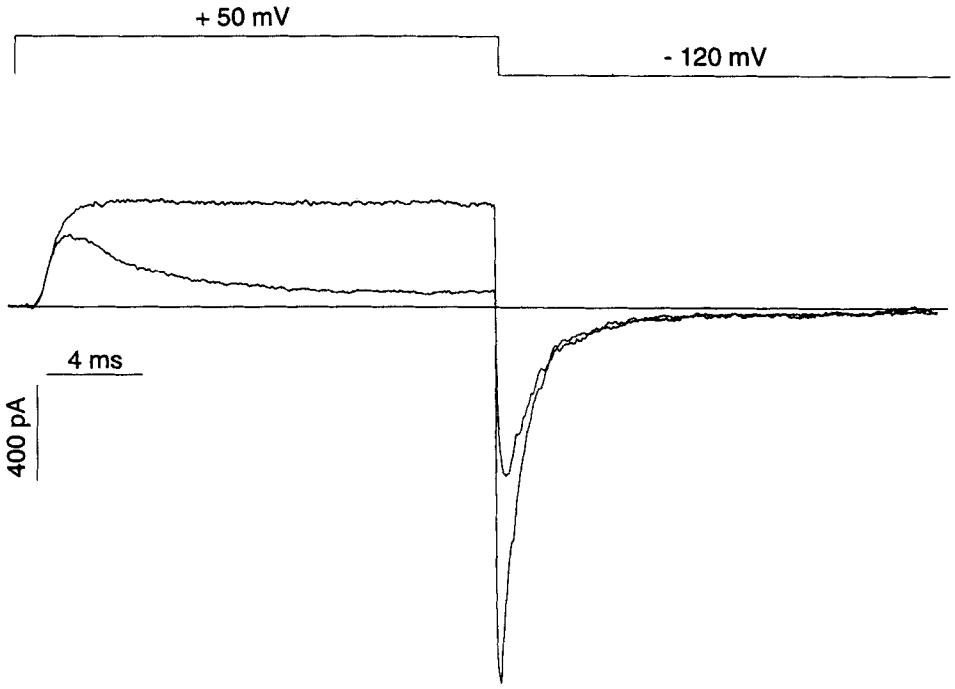

FIGURE 7. The time course of tail currents before and after application of ShB peptide. Macroscopic currents from ShBΔ6-46 channels in an inside-out patch recorded in symmetrical 140 mM KCl before and after application of 100 μM ShB peptide to the bath. The tail currents were fitted with the sum of two exponential components. In the absence of peptide the major fast component had a time constant of 0.9 ms and amplitude of 2,300 pA. In the presence of peptide the fit was: τ_{fast} = 1.1 ms, a_{fast} = 1,230 pA, τ_{slow} = 13 ms, a_{slow} = 93 pA. Currents were low pass filtered at 4 kHz and digitized at 25 μs.

unblocked at a rate of approximately 5,000 s^{-1}. If we make this rate slower, then the peak amplitude of the inward tail currents becomes too small. Consistent with k_{off} being relatively fast, the time course of the tail currents before and after applying peptide is similar, suggesting that the decay phase of the tail currents is largely determined by the rate of the open to closed transition. We have not determined whether the rate of dissociation of the peptide from the 'unstable' blocked state depends upon external K^+. At −120 mV, values for τ_{fast} from the two-exponential fits

to the recovery time courses, were less than 1 ms at all external K^+ concentrations (2–140 mM). However, a three–fourfold change in the rate of recovery would not be detected given the limited time resolution of these measurements.

The fraction of blocked channels that recovered rapidly versus slowly did not appear to be dependent on the voltage of the recovery pulse (V_2) however, it did depend upon the voltage of the initial test pulse. With V_2 equal to −80 mV the relative amplitude of the fast component of the recovery time course was 0.52 ± 0.013 (SEM, n = 8), and with V_2 = −120 mV it was 0.50 ± 0.02 (SEM, n = 7). Fig. 8 shows current traces from an experiment in which we varied the voltage of the test

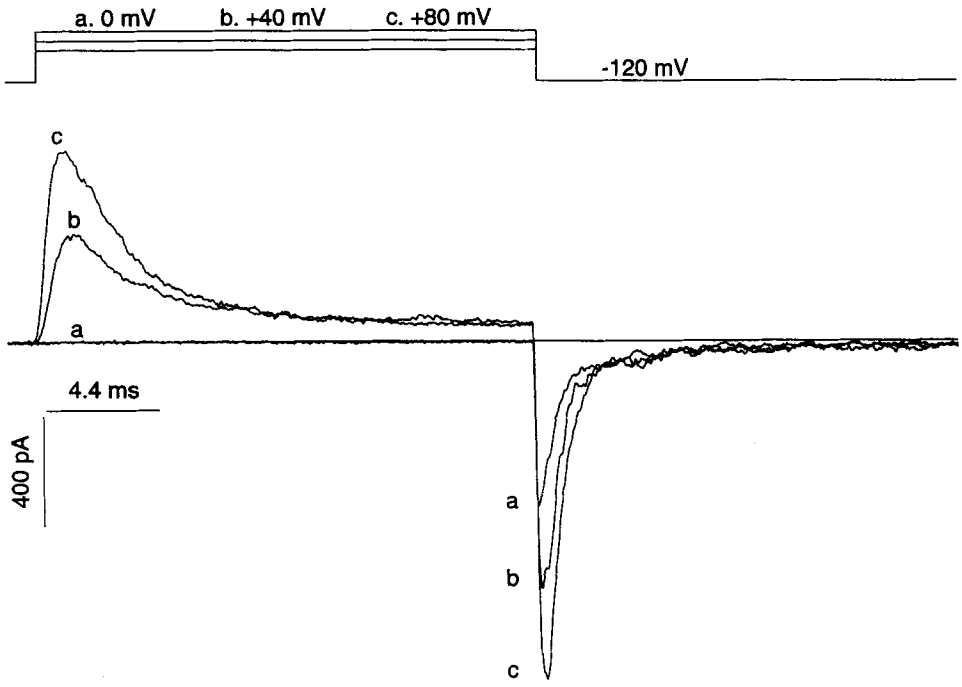

FIGURE 8. The effects of the test pulse voltage (V1) on the rate of recovery from peptide block. Macroscopic currents recorded from ShBΔ6-46 in the presence of 100 μM ShB peptide and in symmetrical 140 mM KCl. The voltage was stepped from a holding voltage of −120 mV to either 0, +40, or +80 mV for 20 ms, after which the voltage was stepped back to −120 mV.

pulse (V_1), stepping to either 0, +40, or +80 mV for 20 ms, followed by a step to −120 mV for 50 ms, in the presence of ShB peptide and symmetrical 140 mM K^+. The time constant of the fast decaying component of the inward tail currents was 0.7 ms in each case but the amplitudes of this component were 640, 1,400, and 2,000 pA after test pulses to 0, +40, and +80 mV, respectively. The simplest interpretation is that following a more depolarized voltage step a larger fraction of the blocked channels become unblocked rapidly, which would indicate that a larger fraction were in the relatively unstable blocked state at the end of the test pulse. In general there was quite a lot of variability in the time course of tail currents. However similar results

were obtained in three separate experiments. Accumulation of K^+ in the pipette during the test pulse is very unlikely to have contributed to the differences in the tail current amplitudes. Generally there were only signs of K^+ accumulation with noninactivating currents of 5–10-fold larger amplitude. The results suggest that there are at least two different ways that the peptide can bind to the channel and block conduction, and that at least one of the binding rates is voltage dependent.

Modeling Peptide Block of ShBΔ6-46 Channels in Terms of Two Kinetically Distinct Blocked States

The block of ShBΔ6-46 channels by ShB peptides can be modeled according to the following kinetic scheme:

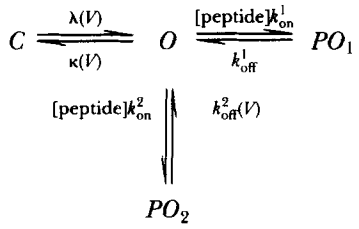

TABLE I

Rates for Scheme

	s^{-1}
λ	$200\ e^{(V/25.3)}$
κ	$12\ e^{(-V/25.3)}$
k^1_{on}	$7.5 \times 10^5\ e^{(V/50)}$
k^2_{on}	$7.5 \times 10^5\ e^{(V/50)}$
k^1_{off}	43
k^2_{off}	$160\ e^{(-V/35)}$

Values are based on measurements in 140 mM symmetrical K^+ and in the presence of ShB peptide. Current simulations are shown in Fig. 9. V is in mV.

SCHEME II

where C is the closed state of the channel, O is the open state, PO_1 is the stable blocked state and PO_2 is unstable at hyperpolarized potentials. A pathway from the blocked states to the closed state has not been included although a proportion of the blocked channels may recover without opening. We have also not included the possibility that the two blocked states communicate directly; for example, the peptide might initially bind in one conformation and then subsequently undergo a conformational rearrangement. However, the relative amplitudes of the fast and slow components of recovery did not appear to be dependent upon the duration of the first test pulse between 10 and 100 ms suggesting that if the two blocked states do communicate they equilibrate very rapidly.

Current simulations based on Scheme II using the rate constants given in Table I are shown in Fig. 9. The simulations are for the voltage protocols used to obtain the experimental records shown in Figs. 5 and 7, and the rate constants used are based

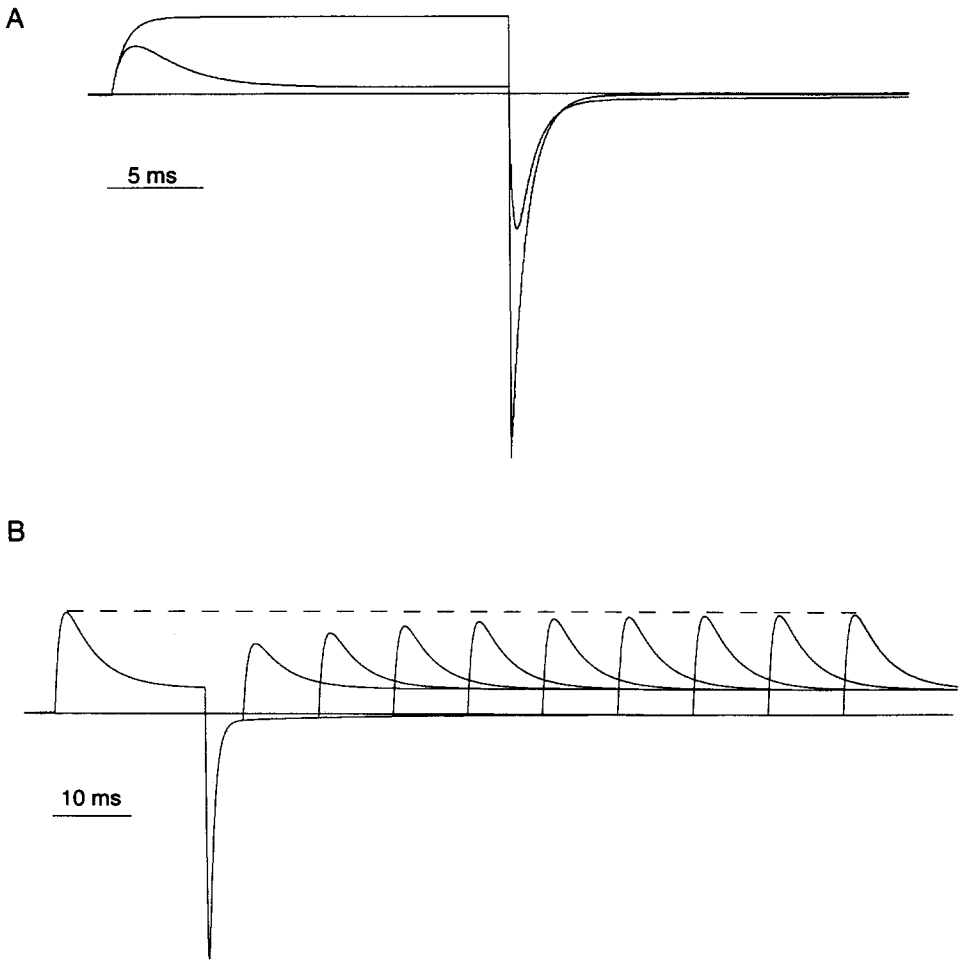

FIGURE 9. Simulated currents using Scheme 2 and the rates in Table I. (*A*) Simulations of current traces before and after application of 100 μM ShB peptide with the pulse protocol used to obtain the experimental records shown in Fig. 7. The C to O rate (λ) was set at 200 $e^{(V/25.3)}$ s^{-1}, to try and mimic the slow activation seen in the experimental traces, although it does not put in the delay seen in the activation of ShBΔ6-46. The channel conductance at −120 mV was set as being twice the conductance at +50 mV to account for the inward rectification seen in symmetrical 140 mM KCl. For the trace before application of peptide the rate constants k_{on}^{1} and k_{on}^{2} were set to zero. (*B*) Simulations of currents in the presence of 50 μM ShB peptide for a paired pulse protocol similar to that used to obtain the experimental traces shown in Fig. 5. Two voltage steps to +50 mV for 20 ms are separated by a hyperpolarizing step to −120 mV for between 5 to 95 ms in 10-ms increments.

on measurements in 140 mM symmetrical K^+ and in the presence of ShB peptide. To reproduce the experimental traces it was necessary to give k^2_{off} sufficient voltage dependence so that at +50 mV it had a value similar to k^1_{off} but at −120 mV it had a value of about 5,000 s^{-1}. The voltage dependence of k^2_{off} is equivalent to 0.7 charges moving all the way across the membrane electric field. Some of this voltage-dependence is likely to arise from the voltage-dependence of K^+ occupancy of the knockoff site. Demo and Yellen (1991) reported that in ShB with high external K^+ this produced a voltage-dependence for recovery from inactivation of e-fold per 56 mV. However, our finding that there is voltage-dependence to peptide block with only 2 mM external K^+ strongly suggests that one or more charges on the bound peptide are within the membrane electric field. The apparent association rate constant for the ShB peptide in physiological solutions is 4.7×10^6 M^{-1} s^{-1} (Murrell-Lagnado and Aldrich, 1993). At +50 mV an equal fraction of the channels recover rapidly versus slowly and therefore the values of k^1_{on} and k^2_{on} have been set as being equal. Slightly better fits were obtained if the rate constants k^1_{on} and k^2_{on} were given some voltage dependence so that the rate of peptide block at +50 mV was 400 s^{-1} but at −120 mV was a factor or 10 or more slower. Otherwise the decay of the inward tail currents in the presence of peptide was too fast relative to the decay in the absence of peptide.

DISCUSSION

In this paper we have shown the following: (*a*). The rate of block of ShBΔ6-46 channels by the ShB peptide and the rate of inactivation in the intact ShB channel are both dependent upon ionic strength in a way that is consistent with long-range electrostatic interaction facilitating the diffusion of the blocking particle towards its binding site. (*b*). The dissociation rate constants for the peptide and the tethered NH_2-terminal domain are insensitive to ionic strength suggesting that the region between the bound state and the transition state is not accessible to ions in bulk solution. (*c*). Allowing for favorable electrostatic interactions the association rate constant is about four orders of magnitude lower than diffusion controlled. (*d*). The temperature dependence of the association rate of the peptide is very high, suggesting that with increasing temperature the proportion of time that either the peptide or channel spends in the correct conformation to bind is increased. (*e*). There are at least two kinetically distinct blocked states produced by the ShB peptide; one that has similar kinetics and dependence on external K^+ concentration as the inactivated state of the intact ShB channel (PO_1), and another that is stable at depolarized voltages but very unstable at hyperpolarized voltages. (*f*). Increasing the net positive charge within the COOH-terminal half of the peptide destabilizes the blocked state PO_1 by ~twofold, but makes it slightly less sensitive to changes in external K^+, suggesting that this region of the peptide faces away from the channel pore.

Our finding that the ShB peptide produces at least two blocked states whereas there appears to be only one kinetically distinct inactivated state of the intact ShB channel (Demo and Yellen, 1991; Zagotta et al., 1989) was surprising given that many of the properties of peptide block are very similar to those of inactivation. For example, both peptide block and inactivation are for the most part coupled to the

channel opening (Zagotta et al., 1989, 1990), and 1 mM internal TEA produces a similar decrease in the rate of peptide block and the rate of inactivation (Choi et al., 1991; Murrell-Lagnado and Aldrich, 1993). Substitutions within the peptide and within the NH_2-terminal domain of the ShB channel produced similar effects on the blocking and unblocking rates (Hoshi et al., 1990; Murrell-Lagnado and Aldrich, 1993; Zagotta et al., 1990). The apparent dissociation rate of the ShB peptide determined from macroscopic currents recorded at +50 mV was 13.7 s^{-1} (Murrell-Lagnado and Aldrich, 1993) whereas k_{off} for the tethered NH_2-terminal domain, determined from macroscopic currents from the intact ShB channel was 14.2 ± 2 s^{-1} (SEM, n = 2). One difference between peptide block and inactivation is the voltage-dependence. Inactivation has little voltage-dependence between −30 and +50 mV (Zagotta et al., 1989) whereas the blocking potency of the peptides clearly increases at increasingly depolarized potentials. The stable blocked state produced by the ShB peptide (PO_1) was similar to the inactivated state of the intact ShB channel in terms of its kinetics, dependence on external K^+ concentration and apparent lack of voltage dependence in physiological solutions (Demo and Yellen, 1991). The slow component of recovery at −120 mV and with 2 mM external K^+ ($1/\tau_{slow}$) was 11.1 s^{-1} which is close to the measured k_{off} at +50 mV. The stability of the other blocked state, PO_2, was very voltage dependent. However, at +50 mV ~ 50% of the channels were in state PO_2 and k_{off} was about 14 s^{-1}, which shows that at this voltage the stability of state PO_2 is similar to that of state PO_1. The simplest interpretation of the results is that the two blocked states represent the ShB peptide binding to a similar site at the mouth of the pore but in at least two different orientations. For example, in both cases the hydrophobic region of the peptide may contact the same nonpolar region of the channel but there may be a difference in the orientation of the charged COOH-terminal half of the peptide. This region is proposed to have an extended chain conformation (Lee, Murrell-Lagnado, Aldrich, and Gierasch, 1993) which would make it very flexible. In state PO_2, which is very unstable at hyperpolarized potentials, one or more of the charged residues might be further into the pore into the membrane electric field, whereas in the stable bound conformation the majority of the charges might stick out away from the pore. The NH_2-terminal domain being tethered at the charged end will have more constraints than the peptide and therefore might only bind in the second orientation.

THE ROLE OF ELECTROSTATIC INTERACTIONS IN PEPTIDE BLOCK AND *N*-TYPE INACTIVATION

In physiological solutions the predominant role of electrostatic interactions in the binding of the ShB peptide to the ShBΔ6-46 channel appears to be to enhance the rate of diffusion of the peptide towards the binding site and thus increase the encounter frequency. The evidence is as follows: (*a*). There is an approximate exponential relationship between peptide valence and k_{on} (Murrell-Lagnado and Aldrich, 1993). (*b*). Substituting uncharged polar residues for charged residues produced only small changes in k_{off} (Murrell-Lagnado and Aldrich, 1993). (*c*). The association rate constant decreased with increasing ionic strength. (*d*). The association rate constant for the more positively charged peptide was more steeply dependent upon ionic strength. (*e*). The estimated values for k_{on} at infinitely high

ionic strength for the ShB and ShB:E12KD13K peptides were similar to the values for k_{on} for the neutral peptide (ShB:R17Q,K18Q,K19Q) at physiological ionic strength (Murrell-Lagnado and Aldrich, 1993).

The effects of ionic strength and net charge on the rate of inactivation in the intact ShB channel also strongly suggests that long-range electrostatic interactions speed the rate of association of the NH_2-terminal domain with the pore blocking site by increasing the frequency of encounters between the two regions of the channel protein (Hoshi et al., 1990; Murrell-Lagnado and Aldrich, 1993). This implies that a rate limiting step in the inactivation process is the diffusion of the NH_2-terminal domain towards the internal mouth of the pore. It supports the original proposal that the NH_2-terminal domain acts similarly to a blocking particle tethered some distance from its binding site at the mouth of the pore (Hoshi et al., 1990; Zagotta et al., 1990).

There are several reasons why the additional constraints on the tethered domain might tend to reduce the ionic strength dependence of the rate of inactivation compared to the association rate of the peptide. One possibility is that the effective valence of the tethered domain was less than that of the free peptide because of shielding of the charged residues within this domain by other regions of the channel protein. The orientation of the NH_2-terminal domain, relative to the binding site at the pore, will be restricted compared to the free peptide and the charged region of the domain (residues 12–20) may tend to face away from the binding site. The tethered domain may also fold differently so that on average less charge is exposed on the surface. Another possible explanation for the lower ionic strength dependence of inactivation, relates to the NH_2-terminal domain being restricted to be within a certain distance from the binding site. Using a simplified model in which the local electrostatic potential at the binding site decays exponentially with a certain length constant that depends upon the ionic strength of the bulk solution, one can easily predict how the potential felt at a point, distance x from the surface charges, will vary with the length constant. The shorter the distance x, the smaller the fold change in electrostatic potential for a given change in the length constant. Whether this contributes significantly to the lower ionic strength dependence of inactivation compared to peptide block will depend upon how close the NH_2-terminus is to the pore entrance which we cannot reasonably estimate. It would be interesting to change the length of the tether and correlate this with the dependence of the binding rate on net charge and ionic strength.

The strong ionic strength dependence of the binding rate and the weak ionic strength dependence of the dissociation rate are not compatible with a diffusion-limited binding reaction. This can be further elucidated by considering separately the electrostatic and nonelectrostatic components of the free energy of the transition state. The electrostatic energy of stabilization of the peptide in the immediate vicinity of its binding site versus out in bulk solution ($\Delta G^{\dagger}_{es}$) can be calculated for different ionic strengths from the expression:

$$\Delta G^{\dagger}_{es} = RT\{\ln k_{on} - \ln k_{on}(0)\} \tag{8}$$

where k_{on} (0) is the association rate at infinite ionic strength (1.3×10^5 M^{-1} s^{-1}, ShB peptide). At physiological ionic strength $\Delta G^{\dagger}_{es}$ for the ShB peptide was −2.1 kcal

mol^{-1}, whereas at low ionic strength it was -2.8 kcal mol^{-1}. The difference in free energy between the unbound state and the transition state (the Gibbs energy of activation, $\Delta G^{\dagger}$) will equal the sum of this term and a nonelectrostatic component ($\Delta G^{\dagger}_{nes}$), that can be calculated from k_{on} (0) from the expression:

$$\Delta G^{\dagger}_{nes} = -RT \ln \left(\frac{k_{on}(0)}{u} \right) \tag{9}$$

where u is the product of the transmission coefficient and the molecular vibration frequency and is assumed here to have a value of 10^{12} s^{-1}. For the ShB peptide this gives a value for $\Delta G^{\dagger}_{nes}$ of +9.2 kcal mol^{-1}. The lack of any apparent ionic strength dependence to the dissociation rate constant indicates that the transition state must be close to the bound state. There are two factors that could contribute to this. In the transition state the peptide molecule could be in contact with the channel protein so that the region between the transition state and the bound state was not accessible to ions in bulk solution. Also the peptide at the transition state and the bound state could be physically close enough so that the electrostatic potential was similar for both states.

The relatively high value for $\Delta G^{\dagger}_{nes}$, compared to a diffusion controlled reaction, indicates that when the peptide is at the binding site the fraction of time that it is in the correct conformation and orientation and has sufficient thermal energy to bind is low. If one were to represent crudely the association reaction in terms of free energy barriers and wells then there would be a large free energy barrier between the peptide in the immediate vicinity of the binding site but unbound, and the bound peptide-channel complex. The transition state is therefore likely to be within this region. In binding reactions where the association rate is close to diffusion controlled so that $\Delta G^{\dagger}_{nes}$ is very low, similar to the values for $\Delta G^{\dagger}_{es}$, then the transition state might not be close to the bound state. In this case one might expect an electrostatic influence on the dissociation rate, the magnitude of which would be affected by changes in ionic strength. Escobar, Root, and MacKinnon, (1993) reached a similar conclusion for the lack of ionic strength dependence to the dissociation rate of a peptide toxin from *Shaker* channels by analyzing the association and dissociation of the peptide in terms of a two step process with the intermediate state being the encounter state before the actual binding reaction.

Temperature Dependence of the Kinetics of Peptide Block

The temperature dependencies of k_{on} and k_{off} were unexpectedly high and low respectively. The value we obtained for the activation energy for association was too high to represent the true activation energy for the binding reaction and suggests that there is a shift in the conformational equilibria of either the peptide or the channel such that at higher temperatures the proportion of time spent in the correct conformation for binding increases. This would imply that the correct conformation is a relatively high energy state of the peptide or channel protein. For example, the tethered NH_2-terminal domain in the unbound state might prefer to fold with the hydrophobic residues partially buried rather than being completely exposed to the solvent. However, in order for the inactivation domain to bind to the pore-blocking site these residues probably have to be accessible to contact residues at the binding

site (Murrell-Lagnado and Aldrich, 1993). The small difference in the temperature-dependence of k_{on} for the parent ShB peptide and the ShB:E12KD13K peptide might reflect differences in their conformational equilibria in bulk solution. This is not the only possible explanation and our assumption that the conformational changes that the peptide and channel undergo are on a fast time scale relative to the binding-unbinding reaction may not be valid. For dissociation, the activation energy and the pre-exponential factor were extremely low. There are at least two possible explanations. Assuming that the peptide-channel complex is continuously undergoing small conformational rearrangements, it is possible that in one particular conformation the activation energy for dissociating is very low but the probability of being in this conformation is also extremely low. Alternatively there may be a temperature dependent shift in the conformational equilibria so that the probability of the complex being in a relatively unstable conformation decreases with increasing temperature and this compensates for the increase in thermal energy, thus giving an anomalous value for the activation energy and pre-exponential term.

Nobile et al. (1993) have reported that the rate of inactivation in the intact ShB channel is highly temperature dependent, similar to the rate of peptide block. Although we have no direct evidence as to whether the tethered NH_2-terminal domain of ShB is unstructured, this tends to suggest that it is similar to the free peptide (Lee et al., 1992). The effective concentration of the tethered inactivation domain is very low for an intramolecular reaction (95 μM) (Zagotta et al., 1990, Murrell-Lagnado and Aldrich, 1993). Our results suggest that those factors that are involved in determining the rate at which the ShB channel inactivates after it has opened are firstly, the electrostatically enhanced rate of diffusion of the tethered NH_2-terminal domain towards the pore blocking site and secondly, the fraction of time that both this region of the channel protein and the region at the binding site are in the correct conformation and orientation for the binding reaction. Given that the peptide of the NH_2-terminal domain does not adopt a stable structure we propose that the conformation of the tethered domain that is correct for binding is a relatively high energy state of this region of the channel protein and therefore the probability of being in this state is low, which contributes to the low effective concentration of the inactivation domain.

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