

# On the Interaction of Bovine Pancreatic Trypsin Inhibitor with Maxi Ca<sup>2+</sup>-activated K<sup>+</sup> Channels

## *A Model System for Analysis of Peptide-induced Subconductance States*

KATHRYN J. LUCCHESI and EDWARD MOCZYDLOWSKI

From the Department of Pharmacology and the Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510

**ABSTRACT** Bovine pancreatic trypsin inhibitor (BPTI) is a 58-residue basic peptide that is a representative member of a widely distributed class of serine protease inhibitors known as Kunitz inhibitors. BPTI is also homologous to dendrotoxin peptides from mamba snake venom that have been characterized as inhibitors of various types of voltage-dependent K<sup>+</sup> channels. In this study we compared the effect of DTX-I, a dendrotoxin peptide, and BPTI on large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels from rat skeletal muscle using planar bilayer methodology. As previously found for DTX-I (1990. *Neuron*. 2:141–148), BPTI induces the appearance of distinct subconductance events when present on the internal side of maxi K(Ca) channels. The single channel kinetics of substate formation follow the predictions of reversible binding of the peptide to a single site or class of sites with a  $K_d$  of 4.6  $\mu$ M at 0 mV and 50 mM symmetrical KCl. The apparent association rate of BPTI binding decreases  $\sim$ 1,000-fold per 10-fold increase in ionic strength, suggestive of a strong electrostatic interaction between the basic peptide and negative surface charge in the vicinity of the binding site. The equilibrium  $K_d$  for BPTI and DTX-I is also voltage dependent, decreasing  $e$ -fold per 30 mV of depolarization. The unitary subconductance current produced by BPTI binding exhibits strong inward rectification in the presence of symmetrical KCl, corresponding to 15% of open channel current at +60 mV and 70% of open state at  $-40$  mV. In competition experiments, the internal pore-blocking ions, Ba<sup>2+</sup> and TEA<sup>+</sup>, readily block the substate with the same affinity as that for blocking the normal open state. These results suggest that BPTI does not bind near the inner mouth of the channel so as to directly interfere with cation entry to the channel. Rather, the mechanism of substate production appears to involve a conformational change that affects the energetics of K<sup>+</sup> permeation.

Address reprint requests to Dr. Edward Moczydlowski, Department of Pharmacology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510.

## INTRODUCTION

At the unitary level, most ion channels exhibit fluctuations between two well-defined current levels referred to as the closed and open states. The closed state is generally equivalent to zero current and the open state is the largest and most frequent conductance level corresponding to the open pore conformation. Many channels have also been found to exhibit discrete intermediate levels of current between the closed and open state that are called substates or subconductance events (Fox, 1987). In some cases, subconductance behavior is quite frequent and recognized as a characteristic signature of the channel's behavior; e.g., channels directly activated by  $\gamma$ -aminobutyric acid, glycine, or glutamate (Bormann et al., 1987; Jahr and Stevens, 1987). However, for other channels the appearance of substates is relatively uncommon. An example of this latter situation is that of large conductance (or maxi)  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (Blatz and Magleby, 1987; Latorre et al., 1989). Several groups have previously noted the appearance of brief substates in maxi K(Ca) channels but have not characterized such states in detail because of their rarity (Barrett et al., 1982; Moczydlowski and Latorre, 1983; Richards et al., 1989). Recently, Stockbridge and French (1989) documented four distinct substate levels of 51, 97, 132, and 178 pS by patch clamp recording of maxi K(Ca) channels in cultured human fibroblasts, which exhibited an open state conductance of 321 pS in symmetrical 140 mM KCl.

In studying the effect of dendrotoxin peptides on maxi K(Ca) channels from rat skeletal muscle, we previously observed that addition of Toxin I (or DTX-I) to the intracellular side induced a long-lived subconductance state at 65% of open channel current at +20 mV (Lucchesi and Moczydlowski, 1990). The kinetics of substate formation corresponded to reversible binding of DTX-I to a single site or a single class of sites. Our results led us to conclude that binding of DTX-I converts the channel to an inwardly rectifying substate conformation that relaxes back to normal conductance behavior when the toxin dissociates. DTX-I thus provides a pharmacological means of inducing a particular substate of maxi K(Ca) channels and can be used to probe the mechanism by which  $\text{K}^+$  permeation is altered in the substate conformation.

In the neurochemical literature, the black mamba snake peptide, DTX-I, has been primarily described as a potent electroconvulsant when injected into the central nervous system and a presynaptic facilitator of transmitter release at peripheral nerve endings (Harvey and Anderson, 1985). However, DTX-I is also a member of a larger family of homologous peptides which includes neurotoxins found in mamba snake venom and a certain class of protease inhibitor peptides called Kunitz inhibitors (Kunitz and Northrop, 1936) that are widely distributed in the animal kingdom (Dufton, 1985; Creighton and Charles, 1987). Members of this ~60-residue peptide family can be recognized by the presence of six cysteine groups at invariant positions which participate in three disulfide bonds, an abundance of basic lysine and arginine residues, and a number of other highly conserved residues at various positions. To date, three different biological activities have been described for such dendrotoxin/Kunitz inhibitor peptides at the molecular level: (a) inhibition of various voltage-dependent  $\text{K}^+$  channels at an external site (reviewed by Dolly, 1988; Moczydlowski et

al., 1988; Castle et al., 1989; Strong, 1990); (b) inhibition of serine proteases (such as trypsin, chymotrypsin, and kallikrein) at the active site for proteolytic cleavage (reviewed by Laskowski and Kato, 1980); and (c) induction of substate events in K(Ca) channels at an internal site (Lucchesi and Moczydlowski, 1990; this paper). The different peptide homologues of this family appear to be preferentially effective either as neurotoxins acting on K<sup>+</sup> channels (e.g., DTX-I) or as protease inhibitors (e.g., bovine pancreatic trypsin inhibitor [BPTI]); however, the structural requirements that determine specificity for these two activities have not been fully identified. While the evolutionary history and biological role of dendrotoxin peptides have been the subject of considerable speculation (Dufton, 1985; Creighton and Charles, 1987), the significance of K<sup>+</sup> channels and proteases as preferential targets of various types of these peptides remains an enigma.

Despite a vague understanding of their potentially diverse physiological roles, Kunitz inhibitor peptides have been intensively studied as a model system for analysis of protein structure, protein folding, and peptide inhibitor–protease complexes at the three-dimensional level using x-ray crystallography (Creighton, 1983). In particular, use of site-specific mutants of the 58-residue peptide, BPTI, has permitted detailed analysis of the contribution of various residues to the folding energetics and stability of this peptide (Goldenberg et al., 1989). The availability of such structural information and mutant peptides is potentially useful in studies of the interaction of these peptides with ion channel proteins.

In this paper we evaluated the use of BPTI as a probe for studying the mechanism of peptide-induced substates in maxi K(Ca) channels by comparing its behavior with that of DTX-I. When compared under the same conditions, we find that BPTI exhibits weaker affinity than DTX-I for the K(Ca) channel; however, binding of BPTI induces a lower conductance substate with more pronounced inward rectification than that induced by DTX-I. In experiments designed to establish the location of the peptide binding site with respect to the mouth of the channel, we found a lack of binding competition between BPTI and the internal pore blocker, Ba<sup>2+</sup>. Similarly for DTX-I, we found that internal tetraethylammonium (TEA<sup>+</sup>) is equally effective in blocking the DTX-I-occupied substate as the fully open state. These results further support the idea that dendrotoxin peptides induce substates via a conformational change of the channel that affects K<sup>+</sup> permeation, as opposed to a direct blocking mechanism involving partial occlusion of the ion permeation path.

## METHODS

### *Incorporation of K(Ca) Channels into Planar Bilayers*

Plasma membrane vesicles were prepared from rat skeletal muscle as described previously (Guo et al., 1987). Planar bilayers were cast from a solution of 25 mg/ml phospholipids in decane on 200- $\mu$ m-diam holes drilled in a polystyrene partition. Lipids were a mixture of 4:1 bovine brain phosphatidylethanolamine:1,2-diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL). The solution on both sides of the bilayer was 10 mM Mops-KOH, pH 7.4, and KCl ranging from 50 to 500 mM in various experiments. In some experiments KCl was replaced with NaCl or *N*-methyl-D-glucamine as indicated.

K(Ca) channel incorporation was accomplished by addition of rat muscle plasma membrane vesicles (1–10  $\mu$ g protein/ml) to the stirred side (*cis*) of a preformed bilayer in the presence of a

KCl gradient, higher on the vesicle-containing side (*cis*). Orientation of the channel was guaranteed by adding 50  $\mu\text{M}$   $\text{CaCl}_2$  to the *cis* or internal side as required for channel activation. The chelating agent, EDTA (100  $\mu\text{M}$ ), was added to the external or *trans* side to prevent activation of rare channels incorporated in the reverse orientation. Once a single channel had incorporated, the KCl gradient was equalized by addition of KCl to the *trans* side. This procedure decreased the probability of additional channel incorporation and often permitted long experiments (3–4 h) to be conducted on a single channel. Solution changes were performed by perfusing the chamber with 10–20 vol of new solution. The  $\text{Ca}^{2+}$ - and voltage-activated gating probability was maintained at an open state probability greater than 0.6. Low  $\text{CaCl}_2$  concentrations (50–150  $\mu\text{M}$ ) were sufficient to maintain high  $P_{\text{open}}$  in the positive voltage range (*trans* or external side = ground); however, for recording at more negative voltages internal  $\text{CaCl}_2$  was gradually increased to a maximum of 1–2 mM to ensure activation.

Pure DTX-I was the generous gift of Prof. Alan Harvey (University of Strathclyde, Glasgow, UK). BPTI and tetraethylammonium chloride (TEA) were purchased from Sigma Chemical Co. (St. Louis, MO). Ultrapure KCl (Puratronic grade; Alfa Products, Ward Hill, MA) was used in bilayer solutions. Other chemicals were the purest available grade.

BPTI concentrations listed in this paper are based on the dry weight content of stock vials supplied by the manufacturer. Quantitative amino acid analyses indicate that concentrations reported in this manner may be overestimated by a factor of 2.0. Thus, the absolute  $k_{\text{on}}$  values for BPTI may be twofold higher and the absolute  $K_{\text{d}}$  for BPTI may be one-half of the values reported in the text. This problem does not apply to DTX-I since molar peptide concentrations are based on amino acid analysis.

#### *Data Acquisition and Analysis*

Single channel currents were measured with a patch clamp amplifier (model 8900; Dagan Corp., Minneapolis, MN). The headstage inputs were connected to the *cis* and *trans* chambers via Ag/AgCl electrodes and agar-KCl bridges (*trans* side ground). Data filtered at 1 kHz were recorded on FM tape for subsequent analysis. Single channel current amplitudes of open or substate events for construction of the current–voltage ( $I$ - $V$ ) curves in Fig. 6 or for blocking experiments with TEA in Fig. 13 were measured by either of two methods. In the first method, current amplitudes were taken as the difference of open-closed or substate-closed peaks of amplitude histograms constructed from current records digitized at 1 kHz and displayed using an LSI 1173 computer system (Indec Systems, Sunnyvale, CA). In the second method, current amplitudes were measured from chart records with the aid of a TG1017 digitizing tablet (Houston Instrument, Austin, TX). In this method, measurements were taken as the mean of three to five well-resolved transitions. Current amplitude measurements obtained by these two methods were in close agreement. Dwell times of long-lived substate events induced by DTX-I ( $\tau = 20$ –30 s at +20 mV) were measured from chart records with a digitizing tablet as described previously (Lucchesi et al., 1989; Lucchesi and Moczydlowski, 1990). Dwell times of shorter BPTI-induced substates ( $\tau = 0.3$ –0.4 s at +20 mV) were automatically measured from records digitized at 100 Hz using an Indec computer. Substate and open burst state events between adjacent substates were recognized using a 50% threshold criterion set between the substate current level and the open state. To exclude brief closures due to channel gating, a cutoff of 100 ms for the shortest acceptable substate closure was used. This cutoff limit excludes >95% of complete gating closures since under the present activation conditions of high  $\text{Ca}^{2+}$ , residual closing events have a lifetime less than 10 ms (Moczydlowski and Latorre, 1983). Substate or open burst state lifetimes were measured from populations of dwell time events ( $n = 100$ –500) fit to a single exponential by linear regression analysis of log cumulative probability vs. time plots as previously described for other examples of toxin-induced blocking events (Guo et al.,

1987). Measured open state lifetimes were corrected for the minimum cutoff limit of  $\alpha = 0.1$  s on substate events by the relation:  $\tau_{\text{corrected}} = \tau_{\text{observed}} \exp(-\alpha/\tau_{\text{closed}})$  as described previously (Guo et al., 1987).

## RESULTS

### *Kinetics of Substate Block by BPTI*

Fig. 1 shows a comparison of primary sequences of the 58-residue trypsin inhibitor, BPTI, and the 60-residue neurotoxin, DTX-I. These two peptides exhibit 32% homology based on identical residues or 50% homology including conservative substitutions. They also share identical disulfide linkages between cysteine residues 5-55, 14-38, and 30-51, resulting in similar solution structures as revealed by various spectroscopic techniques (Hollecker and Larcher, 1989). However, structural differences between BPTI and DTX-I are sufficient to affect the spectrum of biological

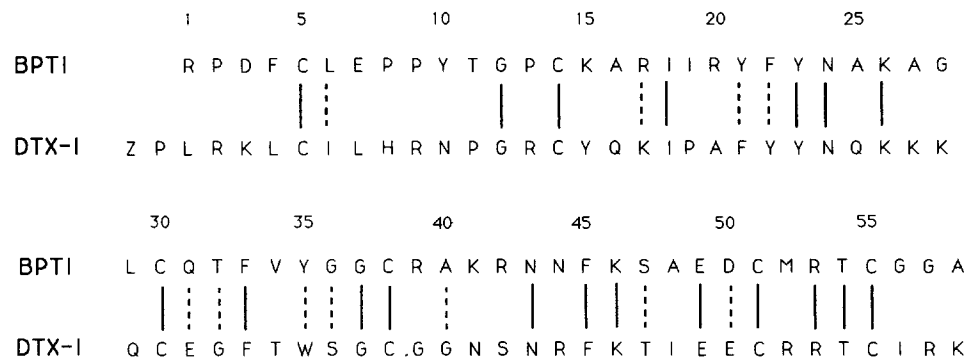


FIGURE 1. Amino acid sequence comparison of BPTI and DTX-I from *Dendroaspis polylepis*. The standard one-letter amino acid code is used. Identical residues are indicated by solid lines, while dotted lines indicate conservative substitutions according to Dayhoff et al. (1978). Sequence information on these and other homologues may be found in Dufton (1985) and Creighton and Charles (1987).

activity of these peptides. For example, intracerebral injection of DTX-I causes seizures in the rat, whereas BPTI and other Kunitz protease inhibitors are reportedly devoid of neurotoxic activity (Harvey and Karlsson, 1982; Harvey and Anderson, 1985). Because of this different specificity toward neuronal  $K^+$  channels, we did not expect BPTI to block maxi K(Ca) channels of skeletal muscle. We initially hoped to use BPTI as a negative control in our efforts to draw inferences about structure-activity relationships in the interaction of DTX-I with this channel. Surprisingly, we found that BPTI is active in the single K(Ca) channel assay.

As previously reported for DTX-I (Lucchesi and Moczydowski, 1990), BPTI tested at concentrations up to 100  $\mu\text{M}$  had no significant effect on the external side of single K(Ca) channels (not shown). In contrast, discrete interruptions in single channel activity were observed after addition of 1  $\mu\text{M}$  BPTI to the internal side as shown in Fig. 2 for a K(Ca) channel recorded in the presence of symmetrical 100 mM KCl. In the absence of BPTI, the "Control" record in Fig. 2 illustrates the high open

state probability and brief flickering closures characteristic of maxi K(Ca) channels recorded under these conditions (+20 mV, 50  $\mu$ M internal  $\text{CaCl}_2$ ). Increasing concentrations of internal BPTI result in a higher frequency of appearance of a distinct substate with a lifetime of 0.34 s and a current amplitude that is  $\sim 30\%$  of the

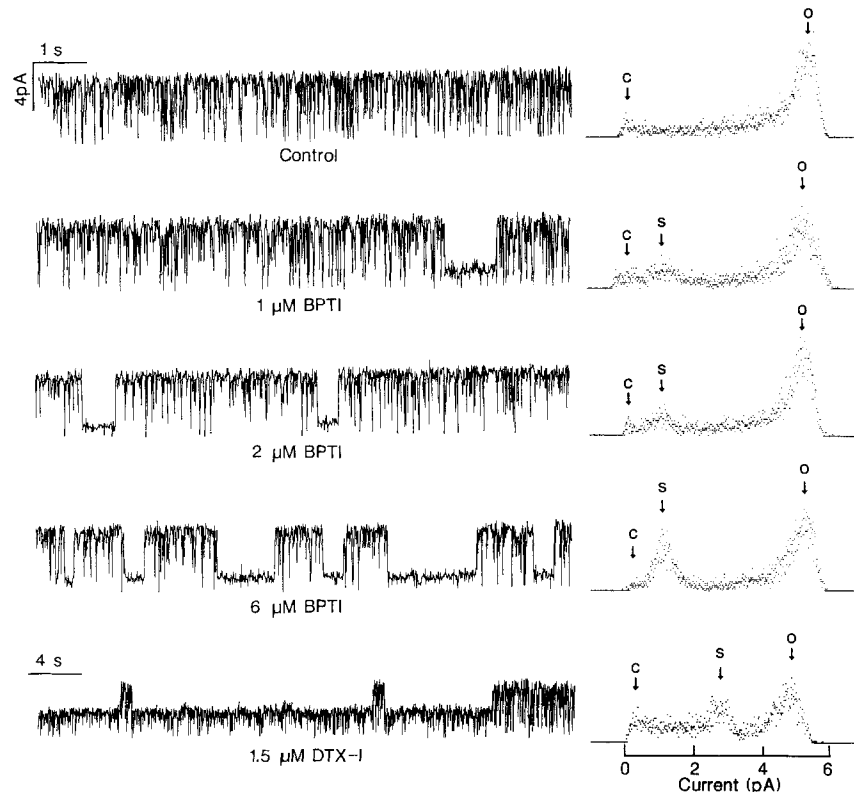
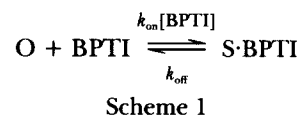


FIGURE 2. Effect of BPTI and DTX-I on single K(Ca) channels. Conditions: symmetrical 10 mM Mops-KOH, pH 7.4, 100 mM KCl, 50  $\mu$ M internal  $\text{CaCl}_2$ , 100  $\mu$ M external EDTA. Holding voltage = +20 mV. Channel opening is upward in all current traces. The top four traces show current records from the same single K(Ca) channel before (*Control*) and after addition of 1, 2, and 6  $\mu$ M BPTI to the internal chamber. The bottom record shows a current trace from a different K(Ca) channel displayed at a fourfold longer time scale and recorded under similar conditions in the presence of 1.5  $\mu$ M internal DTX-I. Amplitude histograms to the right of each trace were compiled from 40 s (BPTI) or 61.4 s (DTX-I) of current record digitized at 100 Hz. Amplitude peaks are identified as closed state (*c*) substate (*s*) or open state (*o*). The ordinate scale is the same for each histogram and corresponds to 57 points/bin for the open state in the control record.

open state current. Current amplitude histograms compiled under these conditions can be used to identify an amplitude peak corresponding to the substate, which increases in relative area but not in position with respect to the current axis at increasing BPTI concentrations (Fig. 2). For comparison, the bottom trace of Fig. 2

also shows an example of substate behavior induced by internal DTX-I under the same conditions. As described previously, the major substate induced by DTX-I occurs at  $\sim 65\%$  of the open state current at +20 mV and has a much longer lifetime in the range of 20–30 s (Lucchesi and Moczydlowski, 1990).

From a practical standpoint, the shorter lifetime of the BPTI-induced substate is advantageous since it facilitates kinetic analysis by allowing many more substate events to be recorded in a given time period. Since we previously found that the kinetics of substate formation induced by DTX-I followed a reversible, one-site binding reaction (Lucchesi and Moczydlowski, 1990), we carried out a similar analysis of the effect of BPTI. Specifically, we tested the applicability of the following scheme:



At the single channel level, this scheme implies that dwell times in the substate conformation, S·BPTI, correspond to residence times of individual BPTI molecules bound to the channel. Similarly, the uncomplexed O state refers to open-burst dwell times between adjacent substate events that correspond to individual waiting times of the normal channel to bind BPTI. Scheme 1 requires that populations of events belonging to the substate or normal conductance modes exhibit single exponential distributions. This is not a trivial assumption because there are many possible situations that could give rise to multi-exponential behavior. For example, if the commercial sample of BPTI was contaminated with a peptide homologue that binds with different affinity but induces the same subconductance level, a sum-of-two exponential distribution would be expected for the substate (Lucchesi et al., 1989). In another scenario, since some K<sup>+</sup> channels appear to be composed of oligomers of identical or homologous subunits (Isacoff et al., 1990; Ruppertsberg et al., 1990), one might expect that there could be more than one binding site for BPTI located on various subunits that would allow more than one BPTI molecule to be simultaneously bound. In this latter situation, if BPTI dissociated from any of the different occupancy states with different rates, then multi-exponential behavior would also be predicted.

Thus, as shown in Figs. 3 and 8, it is significant that dwell time populations of S·BPTI and O states exhibit single exponential behavior under a wide range of tested conditions that include variations in BPTI concentration, ionic strength, and voltage. Cumulative probability distributions of substate events are routinely linear down to 5% of total events when presented in a log-probability format (e.g., Fig. 3A), implying that >95% of the events reflect homogeneous binding to a single site or class of identical sites.

Fig. 3 also illustrates another prediction of Scheme 1, that the lifetime of the S·BPTI substate is essentially independent of BPTI concentration and that the lifetime of O mode or unblocked events is inversely proportional to BPTI concentration. Scheme 1 specifically requires that the reciprocal lifetime of the open-burst state is equal to the product of an association rate constant and BPTI concentration ( $k_{\text{on}}[\text{BPTI}]$ ), and that the reciprocal lifetime of substate events is equal to the

dissociation rate constant ( $k_{\text{off}}$ ). Fig. 4 illustrates this behavior for data compiled from many single K(Ca) channels recorded at +20 mV at different BPTI concentrations and for three concentrations of symmetrical KCl. Within experimental variation, the dependence of the data on [BPTI] in Fig. 3 is in accord with the predictions of the binding reaction of Scheme 1. The observed rate constants of the BPTI blocking reaction measured by the experiments of Fig. 4 may be directly compared with those previously reported for DTX-I under identical conditions (Lucchesi and Moczydlowski, 1990). From the linear fits of data in Fig. 4 A, at 50 mM symmetrical KCl and +20 mV,  $k_{\text{on}}$  for BPTI is  $7.6 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$  vs.  $3.0 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$  for DTX-I. In contrast to these rather similar values for the association rate constant of the two peptides, the mean value of  $k_{\text{off}}$  for BPTI is  $4.1 \pm 0.8 \text{ s}^{-1}$  vs.  $0.027 \pm 0.005 \text{ s}^{-1}$  for DTX-I. The ratio

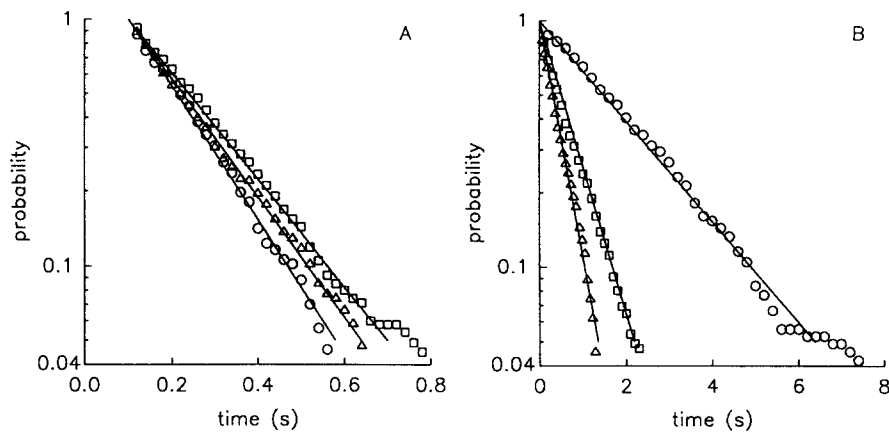


FIGURE 3. Cumulative probability histograms of substate or open state dwell times in the presence of various concentrations of BPTI. Populations of substate (A) and open-burst state (B) events were tabulated from a single K(Ca) channel as described in Methods. Bilayer conditions were the same as those of Fig. 2. The ordinate axis is the probability that an event is longer than a time on the abscissa. 2  $\mu\text{M}$  ( $\circ$ ), 6  $\mu\text{M}$  ( $\square$ ), and 10  $\mu\text{M}$  ( $\triangle$ ) internal BPTI. Distributions were fit to the following single exponential lifetimes: (A) 0.16 s ( $\circ$ ), 0.20 s ( $\square$ ), and 0.18 s ( $\triangle$ ). (B) 2.13 s ( $\circ$ ), 0.73 s ( $\square$ ), and 0.45 s ( $\triangle$ ). The number of events in each population ranged from 284 to 477.

of  $k_{\text{off}}/k_{\text{on}}$  gives equilibrium  $K_d$ 's of  $5.4 \times 10^{-6} \text{ M}$  for BPTI vs.  $9.0 \times 10^{-8} \text{ M}$  for DTX-I. From the results thus far, it is clear that structural differences between the two peptides greatly affect both the amplitude of the substate current and the peptide dissociation reaction, but have less influence on the association rate.

#### *Ionic Strength Dependence of BPTI Binding*

As previously observed for DTX-I (Lucchesi and Moczydlowski, 1990), the observed rate constants of BPTI binding are dependent on KCl concentration in the bulk solution. The most dramatic aspect of this effect is a large decrease in the association rate constant with increasing [KCl] illustrated by the data in Fig. 4 A. The linear fits



of the data in Fig. 4A correspond to the following association rate constants: 50 mM KCl,  $k_{on} = 7.6 \times 10^5 \text{ s}^{-1}\text{M}^{-1}$ ; 100 mM KCl,  $k_{on} = 1.7 \times 10^5 \text{ s}^{-1}\text{M}^{-1}$ ; 200 mM KCl,  $k_{on} = 1.2 \times 10^4 \text{ s}^{-1}\text{M}^{-1}$ . Fig. 4B also shows that there appears to be a systematic decrease in the dissociation rate constant as [KCl] is increased from 50 to 200 mM; however, this stabilizing effect on  $k_{off}$  is much weaker than the severe reduction of  $k_{on}$ .

We previously speculated that the similar large decrease in  $k_{on}$  of DTX-I that occurs at higher [KCl] may result from an electrostatic effect of negative surface charge in the vicinity of the binding site (Lucchesi and Moczydlowski, 1990). If there is a negative surface potential at the peptide binding site due to the presence of local negative charges on the protein, increasing [KCl] could act to reduce this surface potential via the screening effect of electrolyte solutions at a membrane/water

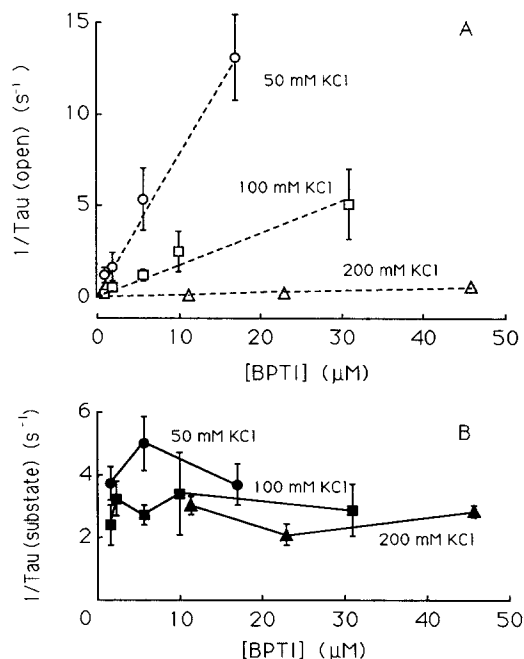


FIGURE 4. Dependence of open-burst state and substate lifetimes on BPTI concentration. Reciprocal lifetimes of open-burst (A) and substate (B) events at different concentrations of BPTI and symmetrical KCl (circles, 50 mM; squares, 100 mM; triangles, 200 mM) were determined from fits to cumulative probability histograms as in Fig. 3. The holding voltage was +20 mV. Dashed lines in A are linear fits for determination of bimolecular rate constants as given in the text. Data points and error bars correspond to the mean and standard error for 3–11 bilayers.

interface (McLaughlin, 1989). Since the DTX-I and BPTI peptides are highly basic, their effective local concentrations near the negatively charged surface at the binding site would be expected to increase at low ionic strength, resulting in a higher apparent  $k_{on}$ . This idea of electrostatic control of the association rate of a positively charged peptide is similar to that previously proposed for the binding of cationic guanidinium toxins to an external site on voltage-dependent Na<sup>+</sup> channels (Green et al., 1987; Ravindran and Moczydlowski, 1989; Cai and Jordan, 1990) and for the binding of basic charybdotoxin peptides to an external site on maxi K(Ca) channels (Anderson et al., 1988; MacKinnon et al., 1989). The following experiments examine whether this mechanism also applies to the case of BPTI binding to an internal site on maxi K(Ca) channels.

If the reduction in  $k_{on}$  at high  $[KCl]$  is the result of a surface charge effect, it would be expected to be dependent only on internal ionic strength and not on the ionic species of monovalent salts. Fig. 5 *A* compares measured  $k_{on}$  values for various ionic conditions. In these experiments, BPTI kinetics were measured at a constant external KCl concentration of 50 mM while the internal solution was increased from 50 to 100, 200, and 300 mM KCl. The 1,000-fold decrease in  $k_{on}$  observed over this range of internal KCl was also compared with  $k_{on}$  values measured by symmetrical KCl

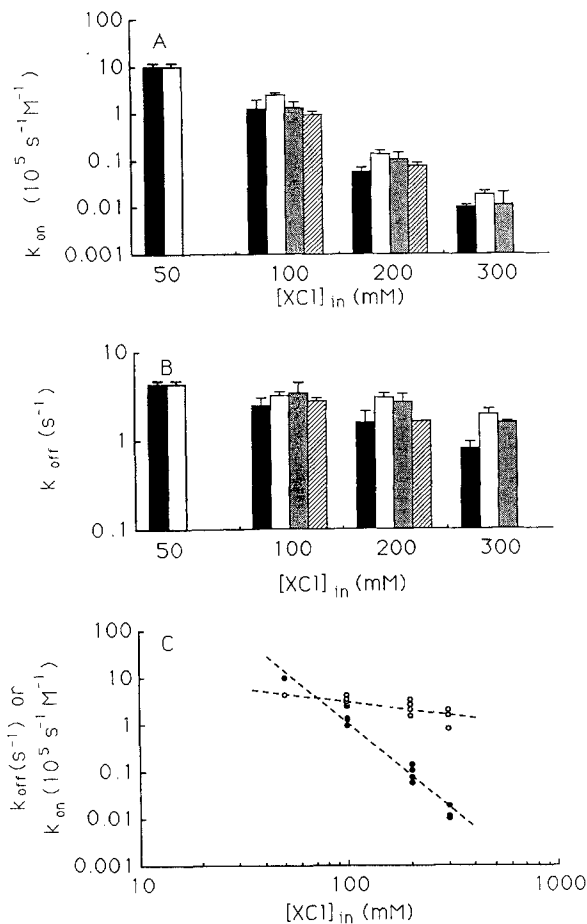


FIGURE 5. Dependence of BPTI kinetics on ionic strength. BPTI binding kinetics were measured at a holding voltage of +20 mV and a constant external KCl concentration of 50 mM. The ionic strength of the internal solution was varied from 50 mM KCl to 100, 200, and 300 mM with Cl<sup>-</sup> salts of X<sup>+</sup> = K<sup>+</sup> (solid bars), Na<sup>+</sup> (hatched bars), or N-methyl-D-glucamine (stippled bars). Solid white shading shows results obtained with symmetrical concentrations of KCl for comparison. Data are presented as a bar graph identifying different salt conditions for  $k_{on}$  in *A* and  $k_{off}$  in *B*. In *C*, the same data shown in *A* and *B* are plotted on a log-log scale for  $k_{on}$  (●) and  $k_{off}$  (○), with the abscissa referring only to the total internal ionic strength.

solutions and in asymmetrical solutions where changes in internal ionic strength were made with NaCl or the Cl<sup>-</sup> salt of the "inert" cation, N-methyl-D-glucamine<sup>+</sup> (NMDG-Cl). We first observe that results of Fig. 5 *A* indicate no significant effect of external KCl concentration in the tested range of 50–300 mM as shown by similar  $k_{on}$  values obtained in asymmetric vs. symmetric  $[KCl]$ . This suggests that the  $k_{on}$  for BPTI is independent of external K<sup>+</sup> ion concentration and ionic strength. The data of

Fig. 5 *A* also show that internal NaCl or NMDG-Cl is equally as effective as internal KCl in lowering  $k_{on}$ . These results are clearly consistent with a surface charge effect and also imply that binding of BPTI is not directly competitive with binding of the permeant ion,  $K^+$ , or with binding of the internal blocking ion,  $Na^+$ , which has been previously shown to produce a flickering block of maxi K(Ca) channels (Yellen, 1984).

The data of Fig. 5 *B* indicate that the various manipulations of ionic conditions do not have exceptional effects on the  $k_{off}$  of BPTI other than the previously noted tendency for  $k_{off}$  to decrease slightly as ionic strength is increased. The magnitude of these ionic strength effects can be better appreciated in the log-log plots of Figs. 5 *C*, where BPTI rate constants are plotted vs. internal salt concentration without regard to the specific ionic species. The slope of the data in Fig. 5 *C* indicates that the  $k_{on}$  for BPTI increases by a factor of 1,000 for a 10-fold decrease in internal ionic strength. The magnitude of this effect is approximately the same as that observed for the internal DTX-I binding reaction for this channel (Lucchesi and Moczydlowski, 1990). This effect on  $k_{on}$  is comparatively greater than that observed for binding of charybdotoxin peptides to a site on the external face of this channel, which shows a  $\sim 100$ -fold increase in  $k_{on}$  per 10-fold decrease in external ionic strength (Anderson et al., 1988; Lucchesi et al., 1989).

#### *Current–Voltage Rectification of Substates Induced by BPTI and DTX-I*

We previously observed that the substate induced by internal DTX-I exhibits inwardly rectifying *I-V* behavior in the presence of symmetrical KCl solutions (Lucchesi and Moczydlowski, 1990). Fig. 6 illustrates similar behavior for the BPTI-induced substate observed in the presence of symmetrical 50, 200, and 500 mM KCl. These experiments compare single channel *I-V* characteristics of the open state and the BPTI-induced substate. As previously described, the open state of maxi K(Ca) channels exhibits ohmic behavior in the voltage range of  $\pm 50$  mV over a wide range of symmetrical KCl concentrations (Moczydlowski et al., 1985). In contrast to the ohmic open state, the BPTI-induced substate rectifies more strongly than the DTX-induced substate. This is shown in Fig. 7 where the ratio of the unitary substate current to the unitary open state current is plotted as a function of voltage. The data for the two peptides indicate that the substate current increasingly diminishes as a fraction of the open state current as voltage increases in the positive direction. Comparison of the substate/open state current ratio at two different KCl concentrations (Fig. 7) also shows that increasing [KCl] does not significantly affect this ratio over the tested voltage range. We previously argued that the lack of relief of the substate current by increasing KCl concentration rules out a surface charge model for the mechanism of substate production, which envisions that peptide binds near the mouth of the channel (Lucchesi and Moczydlowski, 1990). In this model, positive surface charges of the bound peptide would impose a positive surface potential, which decreases the local  $K^+$  concentration available for permeation. The results of Fig. 7, showing no significant relief of the BPTI-induced substate current at 500 mM KCl vs. 50 mM KCl, provide additional evidence against this hypothesis.

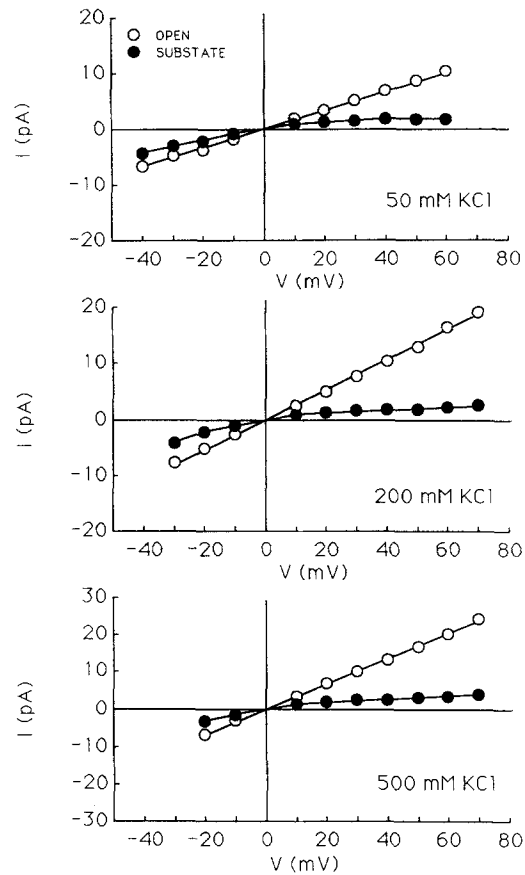


FIGURE 6.  $I$ - $V$  behavior of open and BPTI-induced conductance states.  $I$ - $V$  data were obtained from single K(Ca) channels in the presence of BPTI at various symmetrical KCl concentrations: 50 mM (top), 200 mM (middle), and 500 mM (bottom).  $\circ$ , open state;  $\bullet$ , BPTI-induced substate. Slope conductances of open state data are indicated by linear fits: 164 pS, 50 mM KCl; 264 pS, 200 mM KCl; 343 pS, 500 mM KCl. For the substate data, adjacent pairs of points are connected by straight lines to illustrate inward rectification. Data points are means of 4–11 bilayers with standard errors smaller than the symbol.

#### Voltage Dependence of BPTI and DTX-I Binding Kinetics

The binding affinity of many drugs and toxins to ion channel proteins is modulated by transmembrane voltage. In some cases, voltage-dependent binding appears to result from direct entry of the charged molecule into the transmembrane electric field (Miller, 1982a; Villarroel et al., 1988), while in other cases voltage dependence

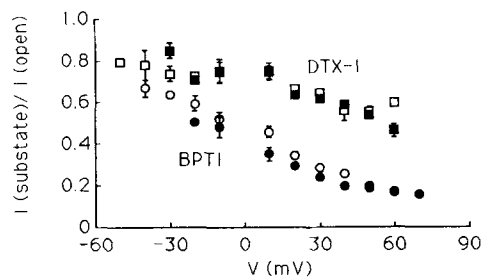


FIGURE 7. Comparison of  $I$ - $V$  rectification of BPTI- and DTX-I-induced substates at two different KCl concentrations. The ratio of unitary substate current to open state current is plotted as a function of voltage at symmetrical concentrations of 50 ( $\circ$ ) and 500 mM KCl ( $\bullet$ ) for BPTI and symmetrical 50 ( $\square$ ) and 100 mM KCl ( $\blacksquare$ ) for DTX-I. Data points and error bars represent means and standard errors for three to five bilayers.

may result indirectly from conformational changes at the binding site propagated from protein domains that are under direct influence of the field (Moczydlowski et al., 1984). Although binding of BPTI and DTX-I does not appear to occur near the internal mouth of the K(Ca) channel (as shown below in competition experiments with pore blocking ions), binding kinetics of the dendrotoxin peptides are nevertheless dependent on transmembrane voltage. This voltage dependence is illustrated in Fig. 8 by cumulative probability histograms of substate and unblocked events collected from a single K(Ca) channel in the presence of 1.9  $\mu\text{M}$  internal BPTI. The results indicate that the lifetime of the BPTI-induced substate progressively lengthens with increasing positive voltage (Fig. 8 A). Conversely, the lifetime of the unblocked state shortens with positive voltage (Fig. 8 B).

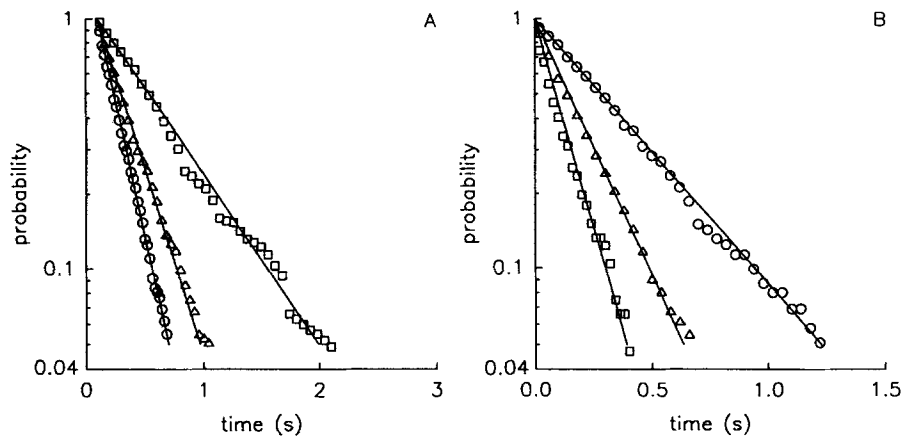


FIGURE 8. Cumulative probability histograms of BPTI-induced substate and open state dwell times as a function of voltage. (A) Substate; (B) open-burst state. Event populations were obtained as in Fig. 3 for a single K(Ca) channel in the presence of symmetrical 10 mM Mops-KOH, pH 7.4, 50 mM KCl, 100  $\mu\text{M}$  external EDTA, 50  $\mu\text{M}$  internal  $\text{CaCl}_2$ , and 1.9  $\mu\text{M}$  internal BPTI. Distributions were fit to the following single exponential lifetimes: (A) +10 mV, 0.20 s ( $\circ$ ); +30 mV, 0.29 s ( $\Delta$ ); +50 mV, 0.63 s ( $\square$ ); (B) +10 mV, 0.41 s ( $\circ$ ); +30 mV, 0.21 s ( $\Delta$ ); +50 mV, 0.13 s ( $\square$ ). The number of events in each population ranged from 106 to 471.

Lifetimes measured as in Fig. 8 were used to obtain dissociation and association rate constants for BPTI and DTX-I as functions of voltage. The results shown in Fig. 9 indicate that these rate constants can be fitted to the following exponential functions of voltage as previously shown for other examples of toxin blocking reactions (French et al., 1984; Cruz et al., 1985; Guo et al., 1987):

$$k_{\text{off}}(V) = k_{\text{off}}(0) \exp(-z'_{\text{off}}FV/RT) \quad (1)$$

$$k_{\text{on}}(V) = k_{\text{on}}(0) \exp(z'_{\text{on}}FV/RT) \quad (2)$$

In the above equations,  $V$  is the applied voltage,  $k_{\text{off}}(0)$  and  $k_{\text{on}}(0)$  are the respective dissociation and association rate constants at 0 mV, and  $z'_{\text{off}}$  and  $z'_{\text{on}}$  are equivalent valences that describe the voltage dependence of the respective processes and

$RT/F = 25.4$  mV at  $22^\circ\text{C}$ . Table I summarizes best-fit parameters for the voltage dependence of BPTI and DTX-I rate constants as measured for various concentrations of symmetrical KCl. Table I also lists respective parameters for the voltage dependence of the equilibrium dissociation constant as determined from the ratio of Eqs. 1 and 2,  $K_d(V) = k_{\text{off}}(V)/k_{\text{on}}(V)$ :

$$K_d(V) = K_d(0) \exp(-z'FV/RT) \quad (3)$$

Anderson et al. (1988) previously found that the binding of the scorpion peptide, charybdotoxin, to an external site on the maxi K(Ca) channel was dependent on both the opening probability of channel gating and voltage in a complex manner:  $k_{\text{on}}$  was sevenfold faster for binding to the open state vs. the closed state and  $k_{\text{off}}$  was independent of the closed/open conformation;  $k_{\text{on}}$  was intrinsically independent of

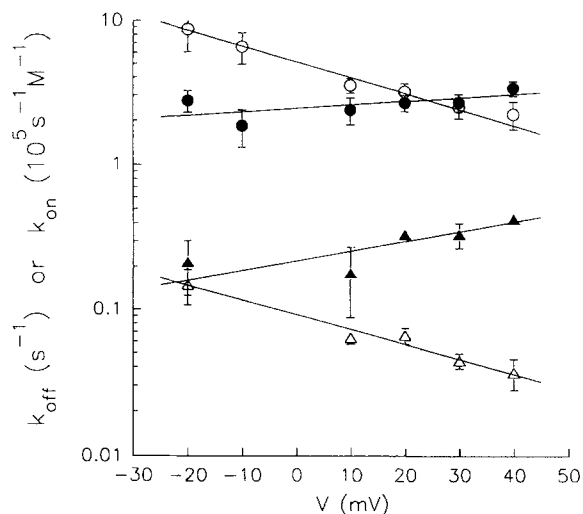


FIGURE 9. Voltage dependence of association and dissociation rate constants of BPTI and DTX-I as determined from single channel measurements. Symbols: (○)  $k_{\text{off}}$  BPTI; (●)  $k_{\text{on}}$  BPTI; (△)  $k_{\text{off}}$  DTX-I; (▲)  $k_{\text{on}}$  DTX-I. Dissociation and association rate constants were determined at various voltages from lifetime measurements of substate ( $k_{\text{off}}$ ) or open-burst state ( $k_{\text{on}}$ ) populations as in Fig. 8. Conditions: symmetrical 10 mM Mops-KOH, pH 7.4, 100 mM KCl, and 100  $\mu\text{M}$  external EDTA. Internal  $\text{CaCl}_2$  concentration was varied from 50  $\mu\text{M}$

to 1 mM as necessary to maintain voltage-dependent gating activity at  $P_{\text{open}} > 0.6$ . Data points represent means and standard errors of 3–11 bilayers. In some cases, error bars were smaller than the symbol. Solid lines are fits to exponential functions of voltage (Eqs. 4 and 5) with best-fit parameters listed in Table I.

voltage and  $k_{\text{off}}$  increased  $e$ -fold per 28 mV of depolarization. Our data on the effect of open state probability ( $P_{\text{open}}$ ) on the binding of BPTI at a fixed voltage of +20 mV do not reveal a significant dependence on channel gating. This is indicated by low correlation coefficients for plots of  $k_{\text{off}}$  vs.  $P_{\text{open}}$  ( $r = -0.33$ ) or  $k_{\text{on}}$  vs.  $P_{\text{open}}$  ( $r = -0.21$ ), as studied in the range of 0.6–1.0 by varying internal  $\text{Ca}^{2+}$  concentration (not shown). In contrast to the lack of dependence on  $P_{\text{open}}$ , Fig. 9 and Table I (100 mM KCl) indicate that the  $k_{\text{on}}$  of BPTI increases  $e$ -fold per 180 mV of depolarization and  $k_{\text{off}}$  decreases  $e$ -fold per 39 mV, resulting in an  $e$ -fold decrease per 32 mV for the equilibrium dissociation constant,  $K_d$ . The  $z'$  parameters summarized in Table I show a similar voltage dependence for DTX-I binding and relatively little effect of KCl concentration on the observed voltage dependence of the two peptide binding reactions. Peptide binding to the external charybdotoxin site and the internal

dendrotoxin site thus exhibits a similar equilibrium dependence on voltage in opposite directions: binding to the outside site is weakened with depolarization and binding to the inside site is enhanced with depolarization. However, charybdotoxin binding appears to sense the open/closed gating conformations of the channel, while dendrotoxin or BPTI binding does not. This latter observation is consistent with the finding that the channel gating equilibrium is not altered in the DTX-I-induced substate conformation as previously shown by the coincidence of  $P_{open}$  vs. voltage-activation curves for the normal channel compared with the DTX-I occupied substate (Lucchesi and Moczydlowski, 1990).

TABLE I  
*Parameters Derived from the Voltage Dependence of Peptide-induced Substates*

Peptide	[KCl]	$k_{off}(0)$	$z'_{off}$	$k_{on}(0)$	$z'_{on}$
	<i>mM</i>	$s^{-1}$		$10^3 s^{-1} M^{-1}$	
BPTI	50	$5.2 \pm 0.1$	$0.39 \pm 0.03$	$11.6 \pm 0.4$	$0.46 \pm 0.03$
	100	$5.2 \pm 0.1$	$0.65 \pm 0.04$	$2.4 \pm 0.2$	$0.14 \pm 0.08$
	200	$5.9 \pm 0.6$	$0.74 \pm 0.10$	$0.11 \pm 0.01$	$0.41 \pm 0.06$
DTX-I	50	$0.056 \pm 0.005$	$0.46 \pm 0.10$	$2.9 \pm 0.9$	$0.50 \pm 0.23$
	100	$0.092 \pm 0.004$	$0.60 \pm 0.05$	$0.22 \pm 0.01$	$0.40 \pm 0.14$
Peptide	[KCl]	$K_d(0)$	$z'$		
	<i>mM</i>	$\mu M$			
BPTI	50	$4.6 \pm 0.2$	$0.85 \pm 0.04$		
	100	$21 \pm 2$	$0.79 \pm 0.09$		
	200	$510 \pm 60$	$1.14 \pm 0.12$		
DTX-I	50	$0.19 \pm 0.06$	$0.96 \pm 0.25$		
	100	$4.2 \pm 0.2$	$1.00 \pm 0.15$		

Rate constants at 0 mV,  $k_{off}(0)$  and  $k_{on}(0)$ , and equivalent valences,  $z'_{off}$  and  $z'_{on}$ , for the kinetics of peptide-induced substate behavior were determined by fitting measured rate constants to exponential functions of voltage according to Eqs. 1 and 2 as illustrated in Fig. 9. Listed KCl concentrations are for symmetrical solutions on both sides of the bilayer. The uncertainty of the values refers to  $\pm$ SD derived from the nonlinear, least-squares fitting procedure. Values given for the equilibrium constant ( $K_d(0)$ ,  $z'$ ) were calculated from  $K_d(0) = k_{off}(0)/k_{on}(0)$  and  $z' = z'_{off} + z'_{on}$ .

#### *Tests for Binding Interactions between Pore Blockers and Substate-inducing Peptides*

Partial occlusion of the channel pore by dendrotoxin peptides bound within or near the internal mouth of the K(Ca) channel would be a possible mechanism of substate production. In this semi-occlusion model, steric or electrostatic interference between the bound peptide and permeant ions would lower the net ionic flux and result in a subconductance state. An expectation of this model would be the presence of binding interactions between dendrotoxins and small blocking ions such as  $Ba^{2+}$  or TEA that are known to enter the permeation path.

$Ba^{2+}$  has been extensively characterized as a pore blocker of maxi K(Ca) channels. Vergara and Latorre (1983) initially showed that  $Ba^{2+}$  produced discrete blocked states from both sides of the channel, but with much higher affinity from the internal side. Neyton and Miller (1988) later characterized various interactions between  $Ba^{2+}$

ions and  $K^+$  ions in the channel, providing strong evidence that the K(Ca) channel functions as a multi-ion channel capable of simultaneously binding two or more permeant ions within the conduction pathway.

If bound dendrotoxin occludes the internal mouth of the channel or occupies part of a  $Ba^{2+}$ -blocking site, then  $Ba^{2+}$  should not bind when the channel is in a peptide-induced substate. To directly test this idea, we examined the interaction between  $Ba^{2+}$  and BPTI. The top two records in Fig. 10 illustrate the effect of  $10 \mu M$   $BaCl_2$  on a single K(Ca) channel recorded at +20 mV in the presence of symmetrical 100 mM KCl. As previously described,  $Ba^{2+}$  induces the appearance of complete closures or blocked states of the channel, which have a lifetime of  $\sim 3.9$  s under these conditions (Vergara and Latorre, 1983). Analysis of such  $Ba^{2+}$ -blocked and unblocked events can be used to derive association and dissociation rate constants for the  $Ba^{2+}$  binding reaction as outlined previously (Vergara and Latorre, 1983; Neyton and

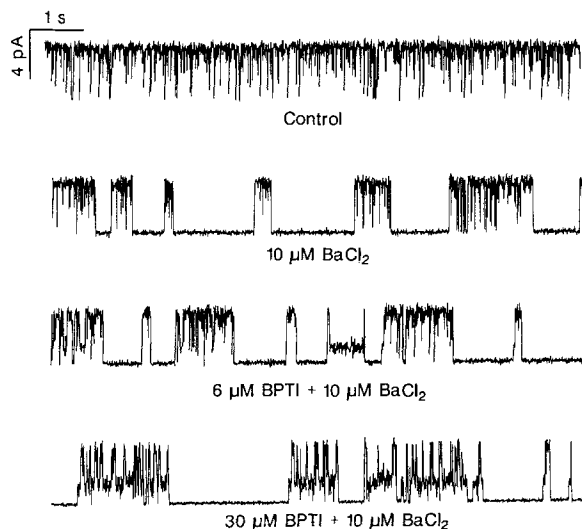


FIGURE 10. Effect of BPTI on  $Ba^{2+}$  block. Current records of a single K(Ca) channel in the absence of  $BaCl_2$  and BPTI (*Control*) and in the presence of  $10 \mu M$  internal  $BaCl_2$  plus 0, 6, or  $30 \mu M$  BPTI as indicated. Channel opening is upward. Conditions: symmetrical 10 mM Mops-KOH, pH 7.4, 100 mM KCl, 100  $\mu M$  external EDTA, and  $550 \mu M$  internal  $CaCl_2$ . Voltage, +20 mV.

Miller, 1988). If there are binding interactions between  $Ba^{2+}$  and BPTI, various types of competitive or noncompetitive interactions would predict a dependence of the  $Ba^{2+}$  rate constants on BPTI concentration. For example, simple competition or mutually exclusive binding of  $Ba^{2+}$  or BPTI to a common site would predict that the observed  $k_{off}$  for  $Ba^{2+}$  would be independent of [BPTI], while the apparent  $k_{on}$  of  $Ba^{2+}$  should follow the relation:

$$k_{on,apparent} = k_{on}[1 + [BPTI]/K_d]^{-1} \quad (4)$$

where  $k_{on}$  is the association rate constant of  $Ba^{2+}$  in the absence of BPTI and  $K_d$  is the equilibrium dissociation constant for BPTI binding. An example of such a competitive interaction that strictly follows Eq. 4 is the interaction between binding of charybdotoxin and TEA to a common or overlapping receptor site on the external side of the maxi K(Ca) channel as previously characterized by Miller (1988).



The bottom two records of Fig. 10 show that when BPTI is added to a channel in the presence of  $\text{Ba}^{2+}$ , discrete substates are observed within opening bursts that separate consecutive  $\text{Ba}^{2+}$ -blocking events. Careful inspection of such records shows that long-duration  $\text{Ba}^{2+}$  closures can be entered while the channel is in the BPTI-substate; conversely, the channel can exit directly to a BPTI substate from the  $\text{Ba}^{2+}$ -blocked state. This qualitative observation implies that the two ligands can bind simultaneously. As discussed above, a quantitative measurement of interaction between the two ligands can be obtained from an analysis of  $\text{Ba}^{2+}$  rate constants as a function of [BPTI]. Visual comparison of the records in Fig. 10 suggests that the  $\text{Ba}^{2+}$  rate constants are not greatly affected by BPTI since the average duration of  $\text{Ba}^{2+}$ -blocked events and the burst length events between such long blocks are similar in the absence and presence of 30  $\mu\text{M}$  BPTI. The quantitative results in Fig. 11 verify this impression. Fig. 11 shows that the  $k_{\text{off}}$  of  $\text{Ba}^{2+}$  is unaffected by BPTI concentrations up to 100  $\mu\text{M}$ , which is approximately sixfold higher than the  $K_d$  for BPTI binding to the open channel. Fig. 11 also shows that the apparent association rate for

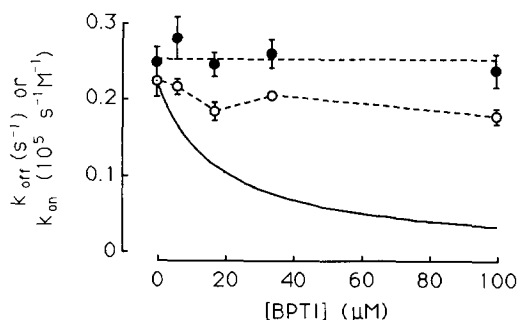


FIGURE 11. Observed rate constants for  $\text{Ba}^{2+}$  block as a function of BPTI concentration. Association ( $k_{\text{on}}$ , open circles) and dissociation ( $k_{\text{off}}$ , filled circles) rate constants for  $\text{Ba}^{2+}$  block were measured at various concentrations of BPTI. The lack of effect of BPTI on  $k_{\text{on}}$  for  $\text{Ba}^{2+}$  is illustrated by the horizontal dashed line through the

$k_{\text{off}}$  data, corresponding to a mean of  $0.26 \text{ s}^{-1}$ . The similar weak effect of BPTI on  $k_{\text{on}}$  for  $\text{Ba}^{2+}$  is illustrated by dashed lines connecting adjacent points. Solid line indicates the predicted behavior of  $k_{\text{on}}$  for a simple competition model as discussed in the text. Data points are means and standard errors for three bilayers.

$\text{Ba}^{2+}$  is also essentially independent of [BPTI] concentration, indicating that  $\text{Ba}^{2+}$  can bind at nearly equal rates to the open channel in the absence of BPTI and to the BPTI-occupied substate. If binding of BPTI and  $\text{Ba}^{2+}$  was mutually exclusive, the behavior predicted by the solid line in Fig. 11 would be expected, which is that of Eq. 4 using  $K_d = 17.5 \mu\text{M}$  for BPTI. Likewise, for a model in which there are negative or positive allosteric interactions between two different ligands that bind simultaneously, the  $k_{\text{off}}$  for  $\text{Ba}^{2+}$  might be expected to increase or decrease, respectively, as a saturating function of [BPTI]. The fact that neither of these possible effects was observed suggests that both  $\text{Ba}^{2+}$  and BPTI can bind simultaneously with practically no interaction between the sites. We also obtained similar results in experiments investigating possible interactions between  $\text{Ba}^{2+}$  and DTX-I. In such experiments  $\text{Ba}^{2+}$  can also clearly block the channel during long-lived substates induced by DTX-I, indicating that both ligands can bind simultaneously (data not shown).

Block of the maxi K(Ca) channel by TEA and various derivatives of TEA has been previously characterized in depth by Villarroel et al. (1988). These studies suggested

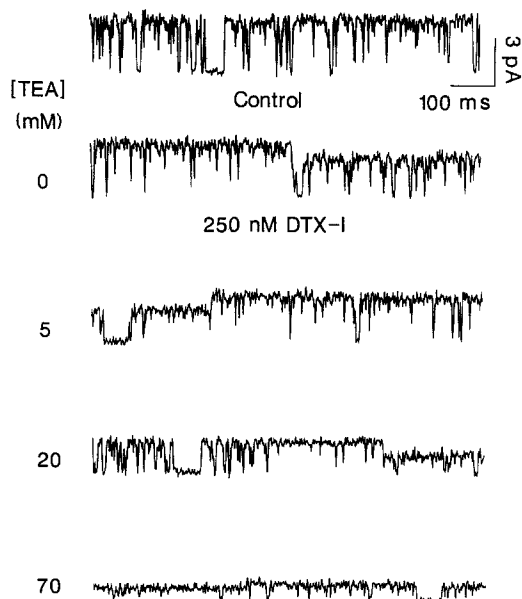


FIGURE 12. Effect of TEA on open state and DTX-I-induced substate current. Current records of a single K(Ca) channel in the absence of DTX-I and TEA (*Control*) and in the presence of 250 nM internal DTX-I plus 0, 5, 20, or 70 mM internal TEA are shown as indicated. Channel opening is upward. Conditions: symmetrical 10 mM Mops-KOH, pH 7.4, 50 mM KCl, 100  $\mu$ M external EDTA, and 50  $\mu$ M internal  $\text{CaCl}_2$ . Voltage, +20 mV.

that the internal receptor site for these cationic blocking drugs is within the conduction pathway because the block was competitive with  $\text{K}^+$  and the voltage dependence of the blocking reaction varied in an expected manner for monovalent and divalent TEA derivatives. To test for possible binding interactions between the dendrotoxin receptor and the TEA receptor, we compared the blocking affinity for TEA binding to the normal open channel and to the DTX-I-occupied substate. Current records from this experiment are shown in Fig. 12. Internal TEA produces a "fast block," which appears as a reduction in unitary conductance (Vergara et al., 1984). This effect is observed in cases where the dissociation rate of a blocker is faster than the resolution limit of the current amplifier, resulting in a lower mean current that is filtered by the recording system (Yellen, 1984). If TEA cannot bind to the

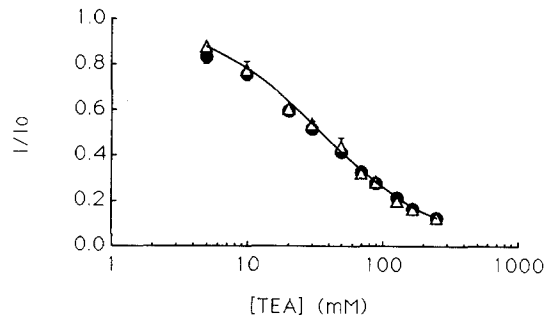


FIGURE 13. Titration of the reduction of unitary current by TEA. Symbols: (○) open state, (▲) DTX-I-induced substate. The ordinate axis is the ratio,  $I/I_0$ , of unitary current of the respective states in the presence of TEA to that measured in the absence of TEA. The solid line is a fit to a Langmuir isotherm (Eq. 4) with a  $K_d$  of 36 mM. In most cases the standard error of the mean (error bars) from three experiments was smaller than the symbol.

DTX-I-occupied substate, one would expect that the substate current would not be affected by TEA, while the normal open state would be progressively decreased by TEA titration. The records of Fig. 12 show that both the open state and substate current are each progressively reduced as internal TEA concentration is increased. Quantitative analysis of these results shows that the dose-response curve for TEA is identical for the open channel and the DTX-I-induced substate (Fig. 13). The data in Fig. 12 B can be fit to a single-site titration curve for TEA with a  $K_d$  of 36 mM at +20 mV, which is similar to that reported by Villarroel et al. (1988). This experiment indicates that binding of TEA to its internal receptor in the mouth of the channel is not affected by binding of DTX-I at the site that mediates substate production.

#### DISCUSSION

BPTI, a peptide that is present in all bovine tissues, is primarily known as a prototype of the Kunitz family of peptide inhibitors of serine proteases (Creighton and Charles, 1987). The present experiments describe a new activity of BPTI as substate-inducing inhibitor of large conductance K(Ca) channels. Our comparison of the activity of DTX-I and BPTI in this paper indicates that the structure of the peptide ligand greatly affects the shape of the  $I$ - $V$  curve of the induced substate as well as the peptide binding kinetics. DTX-I binds with 5- to 20-fold higher affinity than BPTI (Table I), but BPTI induces a lower substate current with more prominent rectification (Fig. 6). Because of the low affinity of BPTI for the K(Ca) channel that we observe in the physiological range of ionic strength (Table I), it seems unlikely that this activity could be of functional relevance in vivo. However, given the great diversity of  $K^+$  channels and Kunitz inhibitor/dendrotoxin peptides, it is possible that other structurally related  $K^+$  channels might express a binding site with higher affinity for a particular Kunitz inhibitor. Future studies examining the effect of BPTI and related homologues on the internal and external sides of other classes of  $K^+$  channels may shed further light on this possibility. Regardless of biological significance, our results establish the usefulness of BPTI as a molecular probe for the analysis of K(Ca) channel substates.

In a recent review of the literature concerning ion channel substates, Fox (1987) distinguished two types of subconductance behavior. The first type is termed an aggregation or subunit type of behavior that arises from the independent activity of multiple functional units. In some cases, such activity can be recognized by the appearance of identically spaced substate levels corresponding to independently operating pores that occur as well-defined oligomeric units. For example, the *Torpedo*  $Cl^-$  channel behaves as a dimer of identical protochannels (Miller, 1982b). As another example, Matsuda et al. (1989) described trimeric behavior of an inwardly rectifying  $K^+$  channel in the presence of certain blocking ions. In these examples, the key observation that identifies such subunit behavior is the presence of a binomial distribution of the various sublevels, as would be predicted for the operation of independently conducting pores. In other cases, such as that of the pore-forming peptide, alamethicin, substate behavior appears to be due to aggregation equilibria involved in forming pores of various sizes from a number of monomers, as in the manner of staves of a barrel (Boheim, 1974). In this latter case, the substate current levels are not necessarily equivalent and aggregation can be recognized by the

dependence of channel formation on high powers of monomer concentration. However, for ion channel proteins that are not known to function by free association and dissociation of subunits in the plane of the membrane, such an aggregation mechanism or subunit model cannot be easily invoked (however, see Schreibmayer et al., 1989).

Rather, a second type of subconductance behavior termed as a partial opening or partial closure appears to play an important role in the function of many types of ion channel proteins. Two kinds of mechanisms have been considered for the production of such partial openings: (a) semi-occlusion of the channel, or (b) a more generalized conformational change that affects ion permeation. In the case of substates induced by *d*-tubocurarine binding to acetylcholine-activated channels, Strecker and Jackson (1989) suggested that the blocking drug partially occludes the mouth of the channel, causing a partial reduction in current flow. In another example of ligand-induced substate behavior, external protons and deuterium ions produce brief discrete closures to 33% of the open state  $\text{Na}^+$  current through L-type  $\text{Ca}^{2+}$  channels (Prod'hom et al., 1987). Although this latter phenomenon was initially considered to result from a surface charge effect of external  $\text{H}^+$  bound to a negatively charged group near the outer mouth of the channel, more detailed mechanistic studies have since suggested that  $\text{H}^+$  binding to a site outside the pore induces a conformational change affecting permeation (Pietrobon et al., 1989). A similar type of substate behavior has also been recently observed by our laboratory in the case of block of cardiac  $\text{Na}^+$  channels by micromolar concentrations of external  $\text{Zn}^{2+}$  (Schild et al., 1991). In this example, a model based on a ligand-induced conformation change of the type used by Pietrobon et al. (1989) for the L-type  $\text{Ca}^{2+}$  channel was used to simulate the kinetics of the  $\text{Zn}^{2+}$ -induced substate process.

The dendrotoxin/BPTI-induced substates in K(Ca) channels studied here cannot be readily explained by a multi-barrel model of the channel based on independent channel subunits. Binding of each of the two tested peptides induces a different substate level with different degrees of *I-V* rectification. The substate levels that we observe do not correspond to any integer fraction of open state current but depend on the structure of the peptide and the voltage. In addition, we found that the pore blockers  $\text{Ba}^{2+}$  and  $\text{TEA}^+$  can block peptide-occupied substates and normal unoccupied channels with equal efficiency. These characteristics lead us to the working hypothesis that K(Ca) channel substates induced by these peptides are the result of conformational changes of channel structure that alter  $\text{K}^+$  permeation.

In our initial report of substate behavior induced by DTX-I, we noted that a twofold increase in ionic strength from 50 to 100 mM KCl resulted in a severe reduction of the association rate constant of toxin binding (Lucchesi and Moczydlowski, 1990). Since BPTI is commercially available and its residence time is much shorter than that of DTX-I, the trypsin inhibitor homologue facilitates a more detailed investigation of this electrostatic effect. The effect measured here, an approximately 1,000-fold increase in  $k_{\text{on}}$  per 10-fold decrease in ionic strength, is one of the strongest electrostatic effects yet documented for such a peptide-channel interaction. Since our experiments were carried out in bilayers formed from neutral phospholipids, the negative surface charge revealed in our experiments most probably results from aspartate and glutamate residues of the channel protein

located in the vicinity of the peptide binding site. This does not eliminate the possibility that negatively charged phospholipids may also enhance the binding of these basic peptides, a possibility that must be tested in future studies.

Despite the strong influence of ionic strength on the association rate of BPTI, we observed practically no increase in the relative substate current as KCl concentration was increased from 50 to 500 mM (Fig. 7). This experiment implies that the bound peptide does not reduce the unitary current by simple electrostatic repulsion of entering  $K^+$  ions. A 10-fold increase of ionic strength in this range would be expected to result in increased screening of such a positive surface potential and a relief of the substate effect. This evidence that the peptide does not bind near the internal mouth of the channel is also compatible with the finding that BPTI or DTX-I do not appear to compete with binding of the pore blockers,  $Ba^{2+}$  and  $TEA^+$ .

The binding kinetics of both DTX-I and BPTI exhibit voltage dependence similar to that of blockers that partly enter the channel and sense a portion of the transmembrane electric field. In the case of *d*-tubocurarine block of open acetylcholine-receptor channels, Strecker and Jackson (1989) cited the voltage dependence of binding as evidence for substate production by partial occlusion. However, for BPTI and DTX-I, the larger size of these molecules makes it unlikely that the peptide itself could enter very deeply into the vestibule of the channel and directly fall under the influence of the voltage drop across the membrane. Aside from this argument, the lack of dependence of the kinetics of the BPTI interaction on the open state probability of the maxi K(Ca) channel implies that this peptide can bind to either open or closed gating conformations of the channel without preference. This behavior is also in contrast to a pore blocker such as  $Ba^{2+}$ , which cannot enter a closed K(Ca) channel (Miller, 1987). Also, once  $Ba^{2+}$  has entered an open channel that is subsequently closed by hyperpolarization,  $Ba^{2+}$  cannot dissociate until the channel has reopened (Miller, 1987). Such contrasting behavior of blocking molecules that do enter channel pores and the substate-inducing peptides that we have studied further supports the view that binding of BPTI and DTX-I does not occur near the inner mouth of the channel.

Thus, if the mechanism for substate production does not involve direct obstruction of the channel mouth, a ligand-induced conformational change affecting permeation is an attractive possibility. A potential argument against this idea is the lack of an effect of BPTI on the  $Ba^{2+}$  dissociation rate. One might argue that if the energy profile of the channel is altered with respect to  $K^+$  ions, one would also expect this to be reflected in a change in the  $Ba^{2+}$  dissociation rate constant since  $Ba^{2+}$  is believed to bind deep within the channel. However, the lack of an effect on  $Ba^{2+}$  binding to its particular blocking site does not necessarily rule out a model based on conformational change, because the energy profile for  $K^+$  binding is undoubtedly quite different from that of  $Ba^{2+}$ .

In modeling studies conducted in collaboration with Osvaldo Alvarez and George Eisenman, we have been able to fit the rectifying *I-V* relations of the BPTI-induced substate with a three-barrier, two-site Eyring model for  $K^+$  permeation that includes double ion occupancy (Lucchesi, K., O. Alvarez, G. Eisenman, and E. Moczydlowski, unpublished observations). Our preliminary results indicate that the BPTI-induced conformational change can be modeled by conversion of the channel from a fairly

symmetric energy barrier profile to an asymmetric profile involving substantial changes in the energies of certain peaks and wells. Further application of such modeling techniques may help us to understand gross effects of the conformational changes that occur upon conversion to the substate.

The example of dendrotoxin/BPTI-induced substates in K(Ca) channels offers the possibility of gleaned much information about molecular details of the peptide-channel interaction, since several different natural homologues of the dendrotoxin family are available (Harvey and Anderson, 1985). Single amino acid mutations of BPTI are also available with the use of a bacterial expression system for this peptide (Goldenberg et al., 1989). In future studies, the role of surface charge in the increase of  $k_{on}$  at low ionic strength could be tested by the use of BPTI homologues with replacement of external positively charged residues with negatively charged residues. In the present comparison, DTX-I is predicted to bear a net positive charge of +10, while that of BPTI is +6 as calculated by simple addition of all ionized residues at neutral pH. For relatively small molecules approximating point charges, the apparent association rate of a ligand binding under the influence of surface charge is expected to vary as an exponential function of the valence (e.g., Ravindran and Moczydlowski, 1989). However, this relationship would not be expected to quantitatively describe the association rate of BPTI, which has an overall size of  $19 \text{ \AA} \times 29 \text{ \AA} \times 19 \text{ \AA}$  (Creighton, 1983). Knowledge of the three-dimensional structure of BPTI (Bode et al., 1978) may ultimately permit us to interpret the effect of specific charge substitutions on electrostatic interactions involved in the binding reaction. A review of the extensive literature on the interaction of BPTI with serine proteases also supports the idea that binding of this peptide can induce structural changes. X-ray crystallographic studies of trypsinogen and the BPTI-trypsinogen complex showed that binding of the peptide induced a conformational change in the structure of trypsinogen, causing it to assume a more trypsin-like conformation (Bode et al., 1978). The availability of such detailed structural data on the interaction of BPTI with soluble proteins enhances the attractiveness of the BPTI-K(Ca) channel interaction as a model system for the investigation of ion channel substates.

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