Biochemical Pharmacology, Vol. 43, No. 1, pp. 21–28, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
Pergamon Press plc

BOVINE PANCREATIC TRYPSIN INHIBITOR AS A PROBE OF LARGE CONDUCTANCE Ca^{2+}-ACTIVATED K^+ CHANNELS AT AN INTERNAL SITE OF INTERACTION

EDWARD MOCZYDLOWSKI,* GUY W. J. MOSS and KATHRYN J. LUCCHESI

Departments of Pharmacology and Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

Abstract—Bovine pancreatic trypsin inhibitor (BPTI) is a 58 residue protein whose binding to various serine proteases has been extensively studied by X-ray crystallography. We have found that BPTI also binds to an intracellular site associated with the large conductance Ca^{2+}-activated K^+ channel, as detected by the production of subconductance events in single channels incorporated into planar lipid bilayers. BPTI is highly homologous to a family of mamba snake dendrotoxin proteins that inhibit various K^+ channels at an extracellular site. BPTI thus provides a useful model system to explore basic mechanisms underlying protein–channel interactions.

Small protein toxins are recognized as an important class of molecules in the pharmacology of ion channels. Channel-specific toxins are employed for various purposes by biochemists who bind "ligands" to identify channel proteins, pharmacologists who prescribe "drugs" to dissect a particular channel's contribution to cellular behavior and biophysicists who require "probes" to deduce channel mechanisms. A number of examples of natural protein toxins directed against ion channels are listed in Table 1 where they are grouped according to their preferential channel target. Such toxins are ubiquitous, components of animal venoms produced by bees (apamin, mast cell degranulating peptide) [1–3]; scorpions (α- and β- Na^+ channel toxins, charybdotoxins) [1–5]; sea anemones (Na^+ channel toxins) [6]; marine molluscs (conotoxins) [7–9]; snakes (α-neurotoxins, dendrotoxins) [1–3, 10–13]; and presumably other venomous species.

Studies of the mechanism of action of various protein toxins have revealed that virtually every aspect of channel function is susceptible to modification. For example, α-bungarotoxin inhibits nicotinic acetylcholine receptor channels of skeletal muscle by preventing the binding of agonists to two sites, one on each of the two α- subunits of the receptor [14]. α- and β- scorpion toxins induce hyperexcitability of nerve and muscle by altering the gating of voltage-dependent Na^+ channels, slowing the kinetics of inactivation or shifting the voltage-dependence of activation to more negative voltages [8, 15]. μ-Conotoxin blocks Na^+ current through voltage-dependent Na^+ channels of mammalian skeletal muscle by binding to an external site at the mouth of the channel that overlaps the site for the guanidinium toxins, tetrodotoxin and saxitoxin [16]. Similarly, charybdotoxin appears to block K^+ current through large conductance Ca^{2+}-activated K^+ channels [maxi K(Ca) channel†] by occluding K^+ permeation at the outer mouth of this channel [17].

Since three-dimensional structures of protein toxins are ultimately required for understanding their detailed mechanism of action, it is encouraging that the crystal structures of several of them have been solved by X-ray diffraction [18, 19] and the solution structure of others has been obtained by two-dimensional nuclear magnetic resonance spectroscopy [20]. However, the hope of obtaining detailed structures of toxin–channel complexes depends upon technical improvements in the crystallization of large membrane-spanning proteins. While awaiting such progress, it may be instructive to apply lessons from model proteins and protein–protein interactions where the molecular details are available at high resolution. The purpose of this essay is to introduce BPTI as such a model protein and an inhibitor of maxi K(Ca) channels.

BPTI (also known as aprotinin or by the Bayer trade name of Trasylol) is a 58-residue polypeptide (Fig. 1) that is primarily known as an inhibitor of various serine proteases. The dissociation constants for the non-covalent BPTI complex with various proteases are: 6×10^{-14} M for bovine trypsin [21], 2×10^{-6} M for bovine trypsinogen [22], 9.1×10^{-9} M for chymotrypsin [23] and 8×10^{-10} M for pig pancreatic kallikrein [24]. The X-ray crystal structure of BPTI has been derived for three crystal forms: I [25], II [25] and III [26]. Despite its small size ($19 \times 29 \times 19$ A°), the structure of BPTI has a number of features of secondary structure that are characteristic of larger soluble proteins. These features include a twisted antiparallel β-sheet formed by residues 17–24 and 29–35, a short carboxy-terminal α-helix formed by residues 47–56, a small hydrophobic core comprised of residues phe4, cys5–

* Corresponding author: Dr Edward Moczydlowski, Department of Pharmacology, Yale University School of Medicine, 333 Cedar St, New Haven, CT 06510, U.S.A. Tel. 203-785 4552.

† Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; maxi K(Ca) channel, large conductance Ca^{2+}- activated K^+ channel; DTX-I, dendrotoxin-I; I–V, current–voltage; TEA^+, tetraethylammonium.

Table 1. Major classes of small protein toxins directed against ion channels

Channel target	Protein toxins	References
Acetylcholine receptor	Snake α-neurotoxins	[10–12]
	α-Conotoxins	[7–9]
Voltage-dependent Na^+ channels	α- and β- Scorpion toxins	[4, 5]
	Sea anemone toxins	[6]
	μ-Conotoxins	[7–9]
Voltage-dependent Ca^{2+} channels	ω-Conotoxins	[7–9]
Voltage-dependent K^+ channels	Mast cell degranulating peptide	[1–3]
	Dendrotoxins	[1–3, 13]
	Charybdotoxins	[1–3]
Ca^{2+}-activated K^+ channels	Apamin	[1–3]
	Charybdotoxins	[1–3]
	Leiurotoxin	[2, 3]

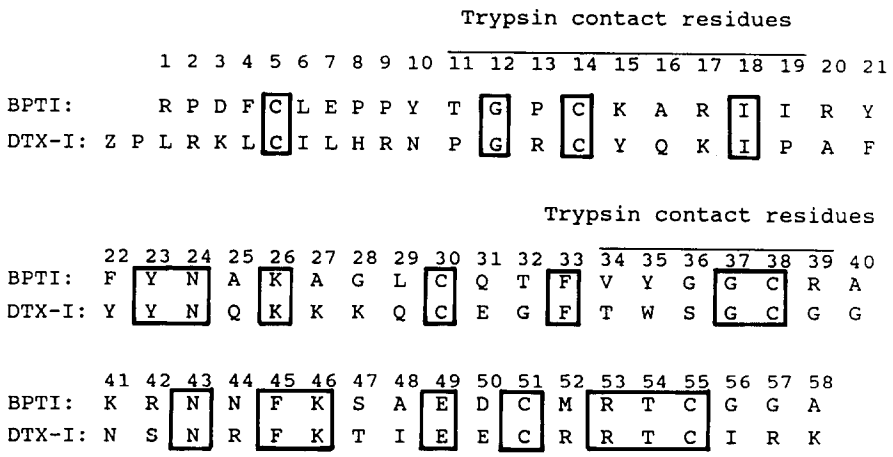

Fig. 1. Comparison of the amino acid sequences of BPTI and DTX-I. Identities at various positions are indicated by boxes. The residues of BPTI found to be in contact with trypsin in the BPTI–trypsin complex [46] are indicated by a solid line. The standard one letter amino acid code is used. Z indicates amino-terminal pyroglutamate.

cys55, phe22, tyr23, cys30–cys51, phe33, tyr35 and phe45 [27], and a number of polar surface residues with mobile side chains such as arg1, lys15, arg17, lys26, arg39, arg42, glu49 and asp50 [25]. The tertiary structure of BPTI is stabilized by three disulfide bonds between cysteine residues 5–55, 14–38 and 30–51. Disulfide bonds also form stable intermediates in the folding pathway of BPTI which has been characterized in great detail using techniques of protein chemistry and site-directed mutagenesis [28, 29].

The name bovine pancreatic trypsin inhibitor is misleading since it is present in many different bovine tissues. It is an intracellular protein that is a representative member of a large family of homologous proteins. Proteins belonging to this family are often referred to as Kunitz inhibitors after Kunitz and Northrop [30] but should not be confused with another family of inhibitors, including soybean trypsin inhibitor, that is also called Kunitz. Creighton and Charles [31] have catalogued an alignment of 29 homologous sequences of related Kunitz proteins found in species as diverse as silkworms and humans. Members of the BPTI/Kunitz inhibitor family can be recognized by the invariant positions of six cysteine residues and by low amino acid variability at a number of other positions [31].

From the standpoint of ion channels, interest in Kunitz inhibitor proteins derives from the fact that a group of neurotoxins called dendrotoxins are homologous members of this protein family. Dendrotoxins have been isolated from the venom of the black (*Dendroaspis polylepis*) and green (*D. angusticeps*) mamba snakes. These toxins have been characterized as potent inhibitors of a number of voltage-dependent K^+ channels when added extracellularly [10–13]. Figure 1 shows an alignment of the primary sequence of BPTI and DTX-I from the black mamba. These two proteins exhibit 32% sequence identity with conservative amino acid differences at a number of other positions. Spectroscopic studies indicate that the secondary structures and peptide backbone conformation of BPTI and DTX-I are quite similar [32]. Since BPTI

is a high affinity trypsin inhibitor and DTX-I is a high affinity blocker of certain K^+ channels, the evolution and nature of the molecular recognition that determines this target specificity is of intrinsic biochemical interest [33].

Our own interest in dendrotoxins began with experiments intended to explore the specificity of various K^+ channels for peptide neurotoxins. One universal characteristic of these toxins is their remarkable specificity for certain channel classes and subtypes. For example, μ-conotoxin blocks voltage-dependent Na^+ channels of mammalian skeletal muscle and *Electrophorus* electroplax but not neuronal or cardiac Na^+ channels of mammals [16]. To compare the toxin sensitivity of the maxi K(Ca) channel with that of other voltage-dependent K^+ channels we tested the effect of DTX-I on single maxi K(Ca) channels in the planar bilayer assay system [34]. We found that DTX-I had no effect on maxi K(Ca) channels when added to the external (extracellular) side but induced long-lived subconductance states ($\tau \simeq 20$ sec) of the channel from the internal side [35]. Since all of the other known examples of toxin–channel interactions occur on the extracellular side, it was rather surprising to find an intracellular site of action. Although such dendrotoxin-induced substates are probably irrelevant to the *in vivo* action of mamba snake venom, this phenomenon is nevertheless biophysically interesting, since it can be used to probe an internal site on the channel that is in some way coupled to ion permeation.

The substate-inducing properties of dendrotoxins can be demonstrated by applying crude mamba snake venom to the internal side of single maxi K(Ca) channels. The venom of both the green and black mamba snake species result in single channel records that exhibit at least two distinct current levels besides the closed and open states of the channel. Such current records of maxi K(Ca) channels taken in the presence of internal mamba venom give the appearance of multi-channel records; however, it can easily be deduced that the record is derived from a single channel since the additive conductance states expected for independent openings of multiple channels of different unitary conductances are never observed. Thus, the experiments of Fig. 2 show that mamba snake venom induces various subconductance states which can be in the order of 1 min in duration.

Verification that dendrotoxin homologs in mamba snake venom are responsible for the production of substates comes from experiments with pure toxins such as DTX-I from *D. polylepis*. These studies revealed that binding of internal DTX-I induced the appearance of one predominant substate with 66% of the normal open state current at +20 mV in the presence of 50 mM symmetrical KCl [35]. By analysing the dwell-time distribution of substate durations and open-burst durations between substates as a function of DTX-I concentration, it can be shown that the kinetics of substate formation are consistent with reversible binding to a single site or homogeneous class of sites with an equilibrium K_d of 0.1 μM (at +20 mV, 50 mM KCl). As suggested by the records of Fig. 2, we also found that the maxi K(Ca) channel exhibits normal gating behavior in the DTX-I-induced substate. Detailed analysis showed that neither the voltage-dependence nor the Ca^{2+}-dependence of channel gating is altered in the substate (DTX-I-bound) mode [35]. Thus binding of DTX-I to an internal site exerts a specific effect on permeation of K^+ ions through the channel. Additional evidence for this conclusion comes from the fact that the I–V relationship of the DTX-I-induced substate exhibits a pronounced inward rectification as compared to the rather linear (ohmic) I–V relationship of the open channel in symmetrical KCl (e.g. Fig. 3).

Figure 2 shows that there are at least two different substates of comparable frequency in the presence of crude mamba venom, whereas experiments with the pure DTX-I homolog exhibit one predominantly occurring substate. This observation is explained by the fact that mamba snake venom contains a mixture of various dendrotoxin homologs [36, 37]. Apparently, different DTX homologs can induce different subconductance levels. Curiosity about the number of possible substates and the structure–activity basis for substate production provoked us to test dendrotoxin homologs and various members of the family of the BPTI/Kunitz inhibitor proteins.

Figure 4 shows that internal BPTI induces a single well-defined substate that is about 100-fold briefer than that induced by DTX-I. However, the BPTI-induced substate has a much smaller current level, about 16% of the open channel at +50 mV. Thus, despite the large structural divergence of BPTI and DTX-I indicated by the sequence comparison of Fig. 1, the BPTI molecule also contains the necessary structural requirements for substate production. Since the tertiary structure of BPTI is well characterized and its faster kinetics of substate production, as compared with DTX-I, permit large numbers of substate events to be efficiently analysed, BPTI is an ideal molecule for mechanistic studies of the substate effect [38]. Figure 5 shows current records of a single K(Ca) channel in the presence of 50 mM symmetrical KCl and 6.4 μM internal BPTI at a series of different holding voltages from +40 to −60 mV. Internal Ba^{2+} (1.7 μM) was also present in this experiment to permit clear definition of the closed/blocked current level. The experiment shown in Fig. 5 illustrates two characteristic features of the BPTI-induced substate phenomenon: the substate conductance is much smaller for outward versus inward K^+ current (inward rectification) and the kinetics of substate formation are strongly voltage-dependent with an e-fold increase in the substate lifetime per ~65 mV [38].

Figure 3 presents a comparison of the I–V curves of the open K(Ca) channel and the respective substates induced by BPTI and DTX-I under conditions of 50 mM symmetrical KCl. In view of the fact that the substates associated with binding of the two proteins exhibit such different I–V relations, one might ask whether their action is mediated by binding to the same site. Since the two proteins induce substates with different current levels and different mean durations, binding competition between DTX-I and BPTI can be examined by addition of BPTI to a single K(Ca) channel

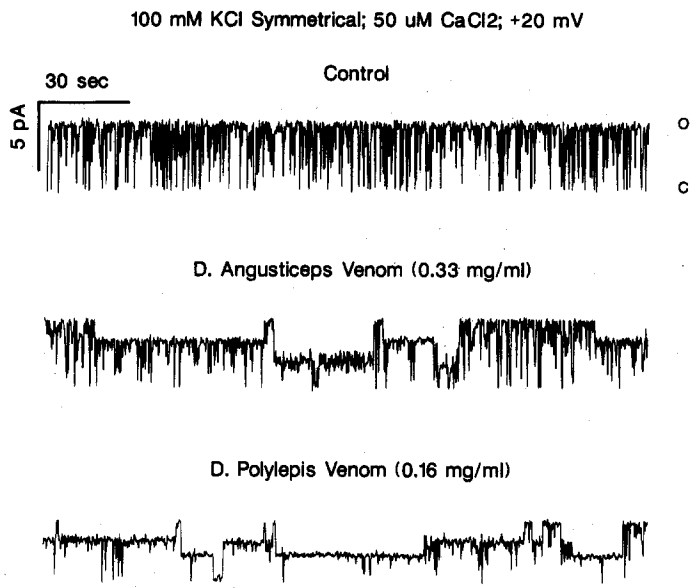

Fig. 2. Effect of crude mamba snake venom on single maxi K(Ca) channels. Single K(Ca) channels were incorporated into planar bilayers from plasma membrane vesicles of rat skeletal muscle and recorded as described previously [38]. A single channel recorded in the presence of 100 mM symmetrical KCl (control) is compared with single channels recorded under identical conditions after the addition of green mamba (*D. angusticeps*) or black mamba (*D. polylepis*) snake venom to the internal (intracellular) chamber.

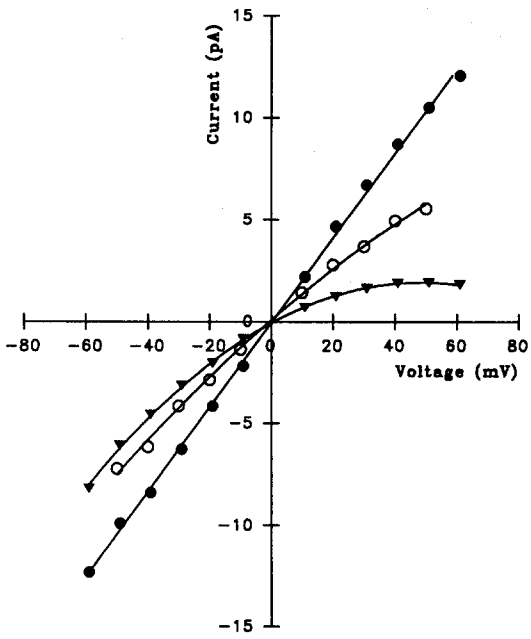

Fig. 3. Comparison of the I–V behavior of the open state with subconductance states induced by DTX-I and BPTI. Conditions: symmetrical 10 mM MOPS-KOH, pH 7.4, 50 mM KCl. Unitary I–V curves of the open state (●), DTX-I substate (○) and BPTI-substate (▼) were compiled for a single channel in experiments similar to that of Fig. 4. Solid lines through data points have no theoretical significance.

undergoing substate transitions induced by DTX-I. If the two proteins cannot bind simultaneously, then one would expect to observe an effect of BPTI only during intervals between DTX-I substates, when DTX-I is not bound. However, if there is simultaneous binding of the two proteins, one would expect to observe an effect of BPTI on the DTX-I occupied substate and *vice versa*.

The preliminary results of such experiments are consistent with the former possibility suggesting that the two proteins do bind to a common site and that simultaneous binding is greatly disfavored. An example of data supporting this interpretation is shown in Fig. 6. The third trace from the top in Fig. 6 shows that the DTX-I-substate is unperturbed by the presence of BPTI and is flanked by an entry from and an exit to the open state, indicating that DTX-I must bind to a channel that is not already occupied by BPTI. However, if the experiment shown in Fig. 6 is carried out at increasingly higher concentrations of BPTI, one does eventually observe the appearance of additional brief BPTI-induced substates emanating within the long-lived DTX-I-substates (Lucchesi and Moczydlowski, unpublished). This suggests that BPTI can bind with much lower affinity to a channel that is already occupied by DTX-I. Although there are many possible interpretations of this result, our current picture of the structure of other voltage-dependent K^+ channels offers an attractive hypothesis. If we assume that the maxi K(Ca) channel is formed by a rosette-like arrangement of four identical subunits as suspected for *Shaker*-like K^+ channels [39], then there could potentially be four identical binding sites

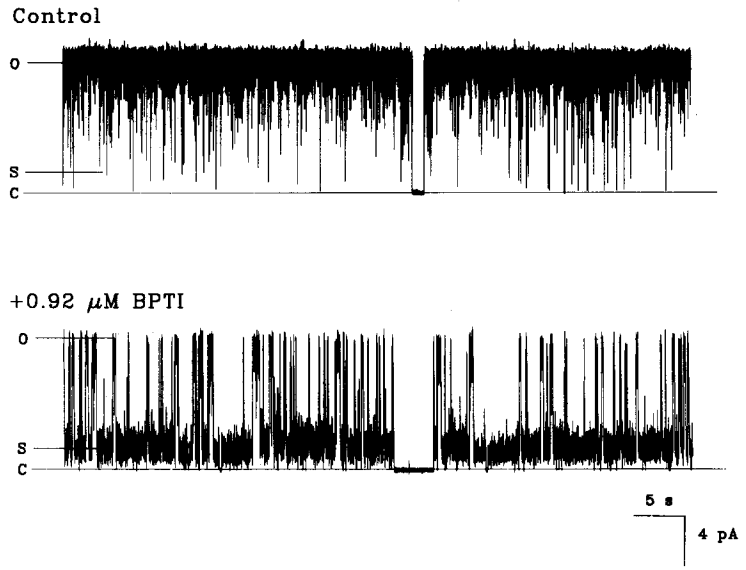

Fig. 4. Effect of BPTI on a single maxi K(Ca) channel. Conditions: symmetrical 10 mM MOPS-KOH, pH 7.4, 50 mM KCl, 0.2 mM internal $CaCl_2$, 0.1 mM external EDTA, holding voltage = +50 mV, filter 100 Hz, 23°. A record from the same channel is shown before (control) or after the additon of 0.92 μM internal BPTI. Various current levels are marked as o (open state), c (closed state), s (BPTI-induced substate).

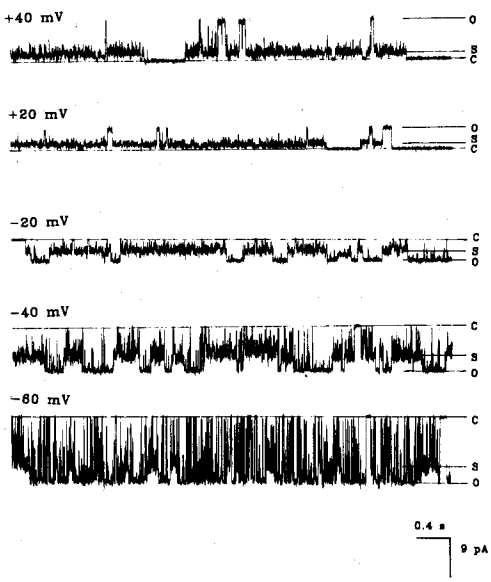

Fig. 5. Effect of voltage on BPTI-induced substate behavior. Conditions: symmetrical 10 mM MOPS-KOH, pH 7.4, 50 mM KCl, 0.2 mM internal $CaCl_2$, 6.4 μM BPTI, filter 200 Hz. Current records (5 sec) from a single K(Ca) channel at various holding voltages are shown. Current levels are labeled as in Fig. 3. $BaCl_2$ (1.7 μM) was also present on the internal side to facilitate accurate identification of the closed/blocked current level.

for DTX-I or BPTI, one on each subunit or near a subunit interface. In this case two possible mechanisms could make the simultaneous binding of more than one protein ligand improbable: electrostatic repulsion between positively charged protein ligands at neighboring sites (i.e. DTX-I has a net charge of +10 and BPTI has a net charge of +6) or a negative allosteric interaction that greatly lowers the affinity of unoccupied sites in the mono-liganded conformation.

Aside from the mechanism of binding to the maxi K(Ca) channel, how does binding of proteins belonging to the BPTI/dendrotoxin family result in a subconductance state? Some possible models to explain this effect are illustrated in Fig. 7. The "partial occlusion" idea of model A suggests that binding of the inhibitor directly interferes with the entry/exit of K^+ ions. The BPTI molecule might bind within the internal vestibule of the channel and sterically interfere with K^+ permeation, without completely occluding the pore. The reduced capture radius of ions entering the channel from the internal side would result in a discrete substate exhibiting a greatly reduced entry rate of internal K^+ and a somewhat lower rate of exit of K^+ ions flowing in the inward direction, leading to inward current–voltage rectification. One prediction of Model A is that organic blocking ions such as TEA^+ which appear to bind within the internal mouth of the maxi K(Ca) channel would not be able to block the substate induced by BPTI/DTX-I or would exhibit a lower blocking affinity for the substate versus the normal open state. However, in an experiment designed to test this prediction, we found that internal TEA^+ blocks the DTX-I-induced substate

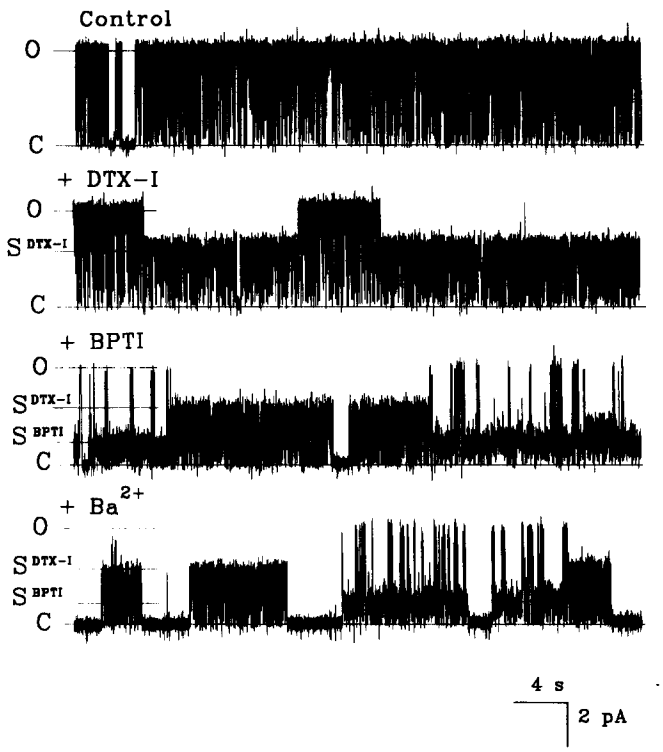

Fig. 6. A single-channel experiment testing for binding competition between DTX-I, BPTI and Ba^{2+}. Conditions: symmetrical 10 mM MOPS-KOH, pH 7.4, 51 mM KCl, 0.1 mM internal $CaCl_2$, 0.1 mM external EDTA, holding voltage = +20 mV throughout the experiment. Consecutive additions of 0.25 μM DTX-I, 3.8 μM BPTI and 1.7 μM $BaCl_2$ were made to the internal chamber of a bilayer containing a single K(Ca) channel and the current was recorded after each addition. Real time proceeds from right to left in all traces.

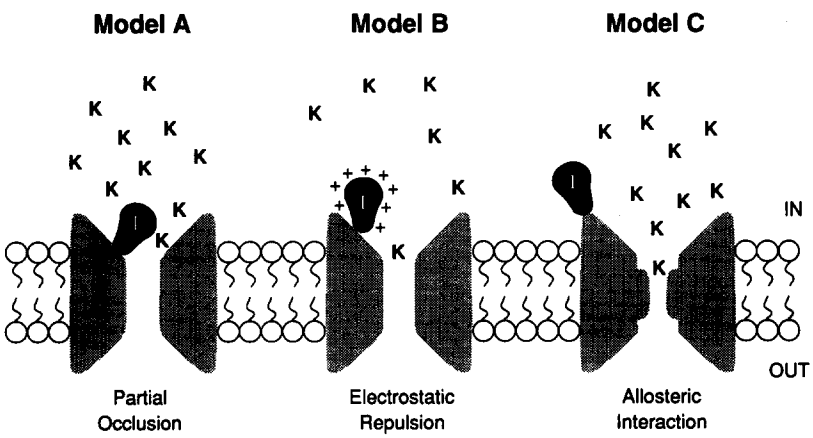

Fig. 7. Schematic illustration of three possible mechanisms for substate production by internal BPTI or dendrotoxin in maxi K(Ca) channels. The black pear-shaped molecule represents an inhibitor or toxin protein bound to the channel (grey stippled).

with exactly the same affinity as it does the open channel, with $K_d = 36$ mM for TEA^+ at +20 mV and 50 mM symmetrical KCl [38]. Similarly, we have observed that internally applied Ba^{2+} (a discrete pore blocker) readily induces blocking events within long-lived DTX-bound substates and titration of a single channel with increasing concentrations of BPTI does not affect the apparent k_{on} and k_{off} for Ba^{2+} block [38]. For example, the bottom record of Fig. 6 shows that addition of internal Ba^{2+} to a single

K(Ca) channel undergoing substate transitions in the presence of DTX-I and BPTI results in typical Ba^{2+}-blocked events that readily occur from either substate level. These latter results indicate that there is not a competitive binding interaction between internal DTX-I and TEA^+, or BPTI and Ba^{2+}, which implies that these small proteins do not bind within the inner channel vestibule. Thus, we presently believe that the partial occlusion idea of model A is unlikely as a mechanism for substate production.

Model B in Fig. 7 alternatively proposes that a bound BPTI/DTX-I molecule inhibits K^+ current via an electrostatic effect. Binding of these basic proteins could alter the electrostatic surface potential near the internal vestibule of the channel. The positive surface potential of the protein molecule repels cations and could result in a lower local concentration of K^+ ions. The lower substate current is then explained by a reduced K^+ concentration near the pore. This mechanism can be tested by examining the dependence of the substate current on ionic strength. At high ionic strength, positive surface potential is expected to be greatly decreased by screening of the fixed positive charges on the protein inhibitor [40]. One would therefore predict that the ratio of the substate current to the open state current should be increased dramatically by increasing internal KCl concentration. Our previous tests of model B in which [KCl] was varied from 50 to 500 mM, a range expected to substantially reduce the magnitude of the surface potential, did not significantly affect the substate/open state current ratio for BPTI-induced substates [38]. Thus, ionic strength does not relieve the substate current as expected for such a purely electrostatic effect. Thus model B also seems to be an unlikely mechanism.

A third possible mechanism that is consistent with the available data is illustrated by model C in Fig. 7. This model proposes that internal binding of an inhibitor protein at a site relatively distant from the pore induces a structural change in the channel protein, such that the energetics of K^+ binding within the channel are modified. Such an allosteric interaction between the BPTI/DTX-I site and the permeation site(s) for ions within the channel could change the rate-limiting steps of ion conduction. For example, an increased energy barrier for K^+ entry that is located closer to the internal side of the channel could result in the type of I–V rectification exhibited by the BPTI-substate.

Such an allosteric effect depicted by the cartoon of model C is somewhat analogous to that produced by the alkaloid toxins, batrachotoxin, veratridine and grayanotoxin, which bind competitively to voltage-dependent Na^+ channels. Aside from modifying the kinetics of Na^+ channel inactivation gating, binding of these toxins induce a lower unitary conductance for Na^+ and alter the permeability ratios for certain pairs of alkali cations [15, 41, 42]. Since experiments with a hydrophilic analogue of grayanotoxin indicate that this toxin binds to a site on the Na^+ channel that faces the internal solution, [43] the effect of BPTI and dendrotoxins might be mediated by a similar kind of binding site on the maxi K(Ca) channel.

Besides enhancing our understanding of channel substates, studies of the interaction of proteins of the BPTI/dendrotoxin family with maxi K(Ca) channels may provide insights into other types of important protein–channel interactions. For example, modulation of various voltage-dependent ion channels by some neurotransmitters is thought to occur via a direct interaction between ion channels and GTP-binding proteins on the internal side of the membrane [44]. Also, the inactivation process of certain voltage-dependent ion channels is mediated by binding of an amino-terminal "ball" domain of the channel to a receptor site on the internal side of the channel [45]. It is possible that these examples of protein–channel interactions share some structural principles in common with the binding interaction between BPTI and various serine proteases. Since the detailed molecular interaction between BPTI/trypsin [46], BPTI/trysinogen [47] and BPTI/kallikrein [24] is known from the X-ray crystal structure of these complexes, it should be possible to determine whether the same region of the BPTI molecule that interacts with these proteases is also involved in binding to the maxi K(Ca) channel. Site-directed mutagenesis of various residues within the trypsin contact region of BPTI, formed by residues 11–19 and 34–39 as indicated in Fig. 1, should provide a direct test of this possibility. Such an approach coupled with detailed functional resolution afforded at the single channel level should also help to distinguish further among the possible mechanisms of substate production envisioned in Fig. 7.

Acknowledgements—This work was supported by grants from the National Institutes of Health (AR38797 and HL38156) We thank Alan Harvey for the gift of DTX-I.

REFERENCES

1. Moczydlowski E, Lucchesi K and Ravindran A, An emerging pharmacology of peptide toxins targeted against potassium channels. *J Membr Biol* **105**: 95–111, 1988.
2. Strong PN, Potassium channel toxins. *Pharmac Ther* **46**: 137–162, 1990.
3. Dreyer F, Peptide toxins and potassium channels. *Rev Physiol Biochem Pharmacol* **115**: 93–136, 1990.
4. Watt DD and Simard JM, Neurotoxic proteins in scorpion venom. *J Toxicol Toxin Rev* **3**: 181–221, 1984.
5. Meves H, Simard JM and Watt DD, Interactions of scorpion toxins with the sodium channel. *Ann NY Acad Sci* **479**: 113–132, 1986.
6. Lazdunski M, Frelin C, Barhanin J, Lombet A, Meiri H, Pauron D, Romey G, Schmid A, Schweitz H, Vigne P and Vijverberg HPM, Polypeptide toxins as tools to study voltage-sensitive Na^+ channels. *Ann NY Acad Sci* **479**: 204–220, 1986.
7. Gray WR, Olivera BM and Cruz LJ, Peptide toxins from venomous *Conus* snails. *Annu Rev Biochem* **57**: 665–700, 1988.
8. Gray WR, Conotoxins as probes of channel subtypes. In: *Neurotoxins in Neurochemistry* (Ed. Dolly JO), pp. 151–161. Ellis Horwood, Chichester, U.K., 1988.
9. Olivera BM, Rivier J, Clark C, Ramilo CA, Corpuz GP, Abogadie FC, Mena EE, Woodward SR, Hillyard DR and Cruz LJ, Diversity of *Conus* neuropeptides. *Science* **249**: 257–263, 1990.
10. Dufton MJ and Hider RC, Conformational properties of the neurotoxins and cytotoxins isolated from elapid

snake venoms. *CRC Crit Rev Biochem* **14**: 113–171, 1983.
11. Loring RH and Zigmond RE, Characterization of neuronal nicotinic receptors by snake venom neurotoxins. *Trends Neurosci* **11**: 73–78, 1988.
12. Mebs D, Snake venom toxins: structural aspects. In: *Neurotoxins in Neurochemistry* (Ed. Dolly JO), pp. 3–12. Ellis Horwood, Chichester, U.K., 1988.
13. Harvey AL and Anderson AJ, Dendrotoxins: snake toxins that block potassium channels and facilitate neurotransmitter release. *Pharmac Ther* **31**: 33–55, 1985.
14. Changeux J-P, Devillers-Thiery A and Chemouilli P, Acetylcholine receptor: an allosteric protein. *Science* **225**: 1335–1343, 1984.
15. Strichartz G, Rando T and Wang GK, An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annu Rev Neurosci* **10**: 237–267, 1987.
16. Moczydlowski E, Olivera BM, Gray WR and Strichartz GR, Discrimination of muscle and neuronal Na-channel subtypes by binding competition between [^{3}H] saxitoxin and μ-conotoxins. *Proc Natl Acad Sci USA* **83**: 5321–5325, 1986.
17. MacKinnon R and Miller C, Mechanism of charybdotoxin block of the high-conductance, Ca^{2+}-activated K^+ channel. *J Gen Physiol* **91**: 335–349, 1988.
18. Walkinshaw MD, Saenger W and Maelicke A, Three-dimensional structure of the "long" neurotoxin from cobra venom. *Proc Natl Acad Sci USA* **77**: 2400–2404, 1980.
19. Fontecilla-Camps JC, Almassy RJ, Ealick SE, Suddath FL, Watt DD, Feldman RJ and Bugg CE, Architecture of scorpion neurotoxins: a class of membrane-binding proteins. *Trends Biochem Sci* **6**: 291–296, 1981.
20. Bontems F, Roumestand C, Boyot P, Gilquin B, Doljansky Y, Menez A and Toma F, Three-dimensional structure of natural charybdotoxin in aqueous solution by ^{1}H-NMR. *Eur J Biochem* **196**: 19–28, 1991.
21. Vincent J-P and Lazdunski M, Trypsin–pancreatic trypsin inhibitor association. Dynamics of the interaction and role of disulfide bridges. *Biochemistry* **11**: 2967–2977, 1972.
22. Vincent J-P and Lazdunski M, Pre-existence of the active site in zymogens, the interaction of trypsinogen with the basic pancreatic trypsin inhibitor (Kunitz). *FEBS Lett* **63**: 240–244, 1976.
23. Vincent J-P and Lazdunski M, The interaction between α-chymotrypsin and pancreatic trypsin inhibitor (Kunitz inhibitor). *Eur J Biochem* **38**: 365–372, 1973.
24. Chen Z and Bode W, Refined 2.5 A X-ray crystal structure of the complex formed by porcine kallikrein A and the bovine pancreatic trypsin inbibitor. *J Mol Biol* **164**: 283–311, 1983.
25. Wlodawer A, Deisenhofer J and Huber R, Comparison of two highly refined structures of bovine pancreatic trypsin inhibitor. *J Mol Biol* **193**: 145–156, 1987.
26. Wlodawer A, Nachman J, Gilliland GL, Gallagher W and Woodward C, Structure of form III crystals of bovine pancreatic trypsin inhibitor. *J Mol Biol* **198**: 469–480, 1987.
27. Read RJ and James MNG, Introduction to the protein inhibitors: X-ray crystallography. In: *Proteinase Inhibitors* (Eds. Barrett AJ and Salvesen G), pp. 301–336. Elsevier Science Publishers, Amsterdam, 1986.
28. Creighton TE and Goldenberg DP, Kinetic role of a meta-stable native-like two-disulfide species in the folding transition of bovine pancreatic trypsin inhibitor. *J Mol Biol* **179**: 497–526, 1984.
29. Goldenberg DP, Frieden RW, Haack JA and Morrison TB, Mutational analysis of a protein-folding pathway. *Nature* **338**: 127–133, 1989.
30. Kunitz M and Northrop JH, Isolation from beef pancreas of crystalline trypsinogen, trypsin, a trypsin inhibitor, and an inhibitor-trypsin compound. *J Gen Physiol* **19**: 991–1007, 1936.
31. Creighton TE and Charles IG, Biosynthesis, processing, and evolution of bovine pancreatic trypsin inhibitor. *Cold Spring Harbor Symp Quant Biol* **52**: 511–519, 1987.
32. Hollecker M and Larcher D, Conformational forces affecting the folding pathways of dendrotoxins I and K from black mamba venom. *Eur J Biochem* **179**: 87–94, 1989.
33. Dufton MJ, Proteinase inhibitors and dendrotoxins. Sequence classification, structural prediction and structure/activity. *Eur J Biochem* **153**: 647–654, 1985.
34. Latorre R, The large calcium-activated potassium channel. In: *Ion Channel Reconstitution* (Ed. Miller C), pp. 431–467. Plenum Press, New York, 1986.
35. Lucchesi K and Moczydlowski E, Subconductance behavior in a maxi Ca^{2+}-activated K^+ channel induced by dendrotoxin-I. *Neuron* **2**: 141–148, 1990.
36. Benishin CG, Sorensen RG, Brown WE, Krueger BK and Blaustein MP, Four polypeptide components of green mamba venom selectively block certain potassium channels in rat brain synaptosome. *Mol Pharmacol* **34**: 152–159, 1988.
37. Schweitz H, Bidard J-N and Lazdunski M, Purification and pharmacological characterization of peptide toxins from the black mamba (*Dendroaspis polylepis*) venom. *Toxicon* **28**: 847–856, 1990.
38. Lucchesi KJ and Moczydlowski E, On the interaction of bovine pancreatic trypsin inhibitor with maxi Ca^{2+}-activated K^+ channels. A model system for analysis of peptide-induced subconductance states. *J Gen Physiol* **97**: 1295–1319, 1991.
39. Miller C, Annus mirabilis of potassium channels. *Science* **252**: 1092–1096, 1991.
40. McLaughlin S, The electrostatic properties of membranes. *Annu Rev Biophys Biophys Chem* **18**: 113–136, 1989.
41. Garber SS and Miller C, Single Na^+ channels activated by veratridine and batrachotoxin. *J Gen Physiol* **89**: 459–480, 1987.
42. Correa AM, Latorre R and Benzanilla F, Ion permeation in normal and batrachotoxin-modified Na^+ channels in the squid giant axon. *J Gen Physiol* **97**: 605–625, 1991.
43. Seyama I, Yamada K, Kato R, Masutani T and Hamada M, Grayanotoxin opens Na channels from inside the squid axonal membrane. *Biophys J* **53**: 271–274, 1988.
44. Brown AM and Birnbaumer L, Ionic channels and their regulation by G protein subunits. *Annu Rev Physiol* **52**: 197–213, 1990.
45. Hoshi T, Zagotta WN and Aldrich RW, Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science* **250**: 533–538, 1990.
46. Ruhlmann A, Kukla D, Schwager P, Bartels K and Huber R, Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. *J Mol Biol* **77**: 417–436, 1973.
47. Bode W, Schwager P and Huber R, The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding. *J Mol Biol* **118**: 99–112, 1978.