Hypothesis for a Serine Proteinase-like Domain at the COOH Terminus of *Slowpoke* Calcium-activated Potassium Channels

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ABSTRACT Bovine pancreatic trypsin inhibitor (BPTI) is a 58-residue protein with three disulfide bonds that belongs to the Kunitz family of serine proteinase inhibitors. BPTI is an extremely potent inhibitor of trypsin, but it also specifically binds to various active and inactive serine proteinase homologs with K_D values that range over eight orders of magnitude. We previously described an interaction of BPTI at an intracellular site that results in the production of discrete subconductance events in large conductance Ca^{2+} activated K^+ channels (Moss, G.W.J., and E. Moczydlowski. 1996. *J. Gen. Physiol.* 107:47–68). In this paper, we summarize a variety of accumulated evidence which suggests that BPTI binds to a site on the K_{Ca} channel protein that structurally resembles a serine proteinase. One line of evidence includes the finding that the complex of BPTI and trypsin, in which the inhibitory loop of BPTI is masked by interaction with trypsin, is completely ineffective in the production of substate events in the K_{Ca} channel. To further investigate this notion, we performed a sequence analysis of the α-subunit of cloned *slowpoke* K_{Ca} channels from *Drosophila* and mammals. This analysis suggests that a region of ~250 residues near the COOH terminus of the K_{Ca} channel is homologous to members of the serine proteinase family, but is catalytically inactive because of various substitutions of key catalytic residues. The sequence analysis also predicts the location of a Ca^{2+}-binding loop that is found in many serine proteinase enzymes. We hypothesize that this COOH-terminal domain of the *slowpoke* K_{Ca} channel adopts the characteristic double-barrel fold of serine proteinases, is involved in Ca^{2+}-activation of the channel, and may also bind other intracellular components that regulate K_{Ca} channel activity.

KEY WORDS: bovine pancreatic trypsin inhibitor • planar bilayer • sequence alignment • sequence homology • single channel recording

INTRODUCTION

Ca^{2+}-activated potassium channels (K_{Ca} channels) play an important balancing role in the membrane physiology of many types of eucaryotic cells, as reflected by the diverse phylogenetic and tissue distribution of this class of ion channels (Hinrichsen, 1993; Latorre, 1994). The opening probability of these highly K^+-selective channels increases steeply with intracellular Ca^{2+} concentration, resulting in membrane hyperpolarization, which generally stabilizes or suppresses cellular function. For example, the contractile state of smooth muscle cells is regulated by a negative feedback mechanism involving the contracting influence of initial Ca^{2+}-entry through voltage-sensitive Ca channels counterbalanced by the relaxing influence of K_{Ca} channels (Brayden and Nelson, 1992; Nelson et al., 1995).

At the structural level, a major class of K_{Ca} channels consists of a tetrameric complex (Shen et al., 1994) of α-subunits encoded by the *slowpoke* gene that has been identified in *Drosophila* (Atkinson et al., 1991) and mammals (Butler et al., 1993). *Slowpoke* K_{Ca} channels (also known as BK or maxi K_{Ca} channels) are characterized by their high unitary conductance (~150–250 pS), activation by positive voltage, and sensitivity to extracellular block by the charybdotoxin family of scorpion peptide toxins (Latorre, 1994; Miller, 1995).

In addition to these basic functional characteristics, *slowpoke* K_{Ca} channels from mammals and *Drosophila* are inhibited by certain protein inhibitors of serine proteinases at an intracellular binding site (Lucchesi and Moczydlowski, 1991; Moczydlowski et al., 1992; Moss and Moczydlowski, 1996; Moss et al., 1996). This interaction is characterized by the appearance of discrete subconductance events induced by binding of bovine pancreatic trypsin inhibitor (BPTI)[1] or chicken ovoinhibitor (Moss et al., 1996) on the intracellular side of K_{Ca} channels. The small proteins, BPTI and chicken ovoinhibitor, are members of the distinct Kunitz and Kazal families of serine proteinase inhibitors, respectively (Laskowski and Kato, 1980). They inhibit serine

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[1] *Abbreviations used in this paper:* BPTI, bovine pancreatic trypsin inhibitor; SerP, serine proteinase.

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proteinases (SerPs) such as trypsin by binding tightly to the substrate site near the catalytic center (Bode and Huber, 1992). Since numerous SerPs share this specific inhibitor sensitivity with *slowpoke* K_{Ca} channels, we hypothesized that the K_{Ca} channel protein might contain a domain that resembles the tertiary structure of SerP enzymes. This hypothesis predicts that the binding interaction between the K_{Ca} channel and BPTI is structurally similar to that between SerP enzymes and BPTI. It also implies that the primary sequence of the K_{Ca} channel may contain a region that is homologous to members of the SerP family.

To pursue this hypothesis, we tested whether the complex of BPTI and trypsin, with a shielded inhibitory loop, is a competent inhibitor of the K_{Ca} channel. Our finding that this complex is inactive supports the idea that the inhibitory loop region of BPTI, which is known to bind to serine proteinases, is the same contact region required for K_{Ca} channel inhibition. We also carried out a systematic search for sequence similarity between chymotrypsin, related proteinases, and the *slowpoke* K_{Ca} channel α-subunit. The search has led to the identification of a continuous ~250-residue sequence near the COOH terminus of mammalian and *Drosophila* K_{Ca} channels that exhibits similarity to the superfamily of SerP enzymes.

MATERIALS AND METHODS

Planar Bilayer Experiments

Planar lipid bilayers were formed after spontaneous thinning of a lipid solution in decane (Mueller et al., 1962; Hanke and Schlue, 1993) spread across a 200-μm diameter hole in a polystyrene cup with a small glass rod. The lipid solution consisted of a 4:1 mixture of bovine brain phosphatidylethanolamine/1,2-diphytanoylcholine (Avanti Polar Lipids, Alabaster, AL) at a final concentration of 25 mg/ml in decane. Maxi K_{Ca} channels from rat muscle plasma membranes prepared according to Guo et al. (1987) were incorporated into preformed bilayers as previously described (Moss and Moczydlowski, 1996). The two bilayer chambers initially contained 10 mM MOPS-KOH, 50 mM KCl, 500 μM $CaCl_2$, pH 7.2 (*cis* side), and 10 mM MOPS-KOH, 0.1 mM EDTA, pH 7.4 (*trans* side). Plasma membrane vesicles were added to the *cis* side with continuous stirring, and bilayer experiments were performed at room temperature (22–24°C). After channel incorporation, the KCl concentration was made 50 mM symmetrical by addition of a stock solution of 3 M KCl, 10 mM MOPS-KOH to the *trans* side, and channel activity was recorded at a constant voltage of +20 mV (*trans* side ground, *cis* intracellular). Purified BPTI and bovine trypsin (type III from bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO), and molar concentrations of these proteins were standardized by quantitative amino acid analysis performed by the Yale Protein Chemistry Facility. Single-channel activity was recorded with a List (Westbury, NY) EPC-7 patch clamp amplifier, and stored on video cassette tape via an Instrutech (Great Neck, NY) VR-10 digital interface. Single-channel analysis of the kinetics of BPTI-induced substate events was performed essentially as described by Lucchesi and Moczydlowski (1991) using the TAC program (HEKA-Instrutech, Great Neck, NY) to measure individual dwell times from channel records filtered at 0.1 kHz (8-pole Bessel filter, Frequency Devices, Haverhill, MA) and digitized at 1 kHz. Mean dwell times of substate and nonsubstate events were typically computed from populations of at least 100 BPTI-induced substate events.

Sequence Analysis

Initial screening for sequence similarity between the *slowpoke* K_{Ca} channel α-subunit and various serine proteinases was performed with the use of GAP and BESTFIT sequence comparison programs of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI). These latter two programs produce optimized alignments between any two pairs of sequences and thus help to identify regions of possible similarity. In the early trials, the entire amino acid sequence of the *Drosophila* K_{Ca} channel (Dslo, Accession No. M96840) was directly compared to entire primary sequences of various members of the serine protease family (chymotrypsin, trypsin, elastase, and kallikrein) from diverse species. This approach yielded gapped alignments with an ambiguous level of sequence similarity (17–24% identity), but it was noted that most of the pairwise alignments fell within the COOH-terminal region of the K_{Ca} channel sequence. In the second phase of screening, a smaller test sequence consisting of only the COOH-terminal fragment of Dslo was aligned with SerP sequences using GAP and BESTFIT. This approach identified an interesting pattern of sequence similarity (30% identity over 43 residues of Dslo) that aligns directly the NH_2-terminal to the active site Ser195 residue of bovine chymotrypsin (see Fig. 2 *A*). When this latter 43-residue sequence of Dslo was used to search the whole protein database for similar sequences using the BLAST program (Altschul et al., 1990), no SerP-related sequences were found within the top 100 matches. However, when this query sequence was modified to reduce the loop length between the α1-β9 and β9-β10 regions defined in Fig. 2 *B* to match the corresponding length found in SerPs, then particular runs of the BLAST search scored as high as 46 SerP sequences within the top 100 similar sequences found in the database.

The possibility of homology to the COOH-terminal region of the K_{Ca} channel was further examined using various multiple sequence alignment techniques. This phase of analysis used seven test sequences of serine proteinases of known crystal structure (bovine chymotrypsin, bovine trypsin, pig elastase, pig kallikrein, rat mast cell protease, rat submaxillary gland tonin, and *Streptomyces griseus* trypsin) that have been previously aligned by Greer (1990) on the basis of superposition of tertiary structures. These latter proteinase sequences together with the COOH-terminal region of Dslo and mammalian *slowpoke* sequences (Mslo, mouse K_{Ca} channel, Butler et al., 1993; Hslo, human K_{Ca} channel, Tseng-Crank et al., 1994; Bslo, bovine K_{Ca} channel, Moss et al., 1995) were fully aligned with the aid of the MACAW program (Schuler et al., 1991; Lawrence et al., 1993) distributed by the National Center for Biotechnology Information and the PILEUP program (Feng and Doolittle, 1987) of the Wisconsin Package. The resulting multiple alignments were used to identify likely candidates for conserved and variable regions of the SerP domain, which respectively correspond to 14 conserved secondary structural elements (twelve β-strands, β1–β12; two α-helices, α1 and α2) and nonconserved connecting loops between these elements (Greer, 1990; Petersen et al., 1993). Manual adjustment was used to obtain the final alignment proposed in Fig. 2 *B* with gaps placed mostly in the nonconserved loop regions that connect the secondary structural elements.

To document overall similarity, 13 putatively identified structurally conserved regions of Dslo and Bslo predicted by the alignment of Fig. 2 *B* (boxes labeled β1–β12 plus α1) were scored against a test library of 109 known members of the SerP family.

This SerP library included all 35 sequences aligned by Greer (1990), 3 additional sequences (human and pig heparin binding proteins; crab collagenase) aligned by Petersen et al. (1990), and 71 additional nonredundant sequences taken from the current protein database that include 10 chymotrypsins, 40 trypsins, 11 elastases, and 10 kallikreins of diverse species. In Fig. 2 *B*, residues in Dslo and Bslo that occur in the aligned position at an arbitrarily chosen frequency $\geq$10% of the 109-member library are marked with a line (|) and those that occur at least once but at a frequency of <10% are marked with a colon (:). These same regions of Dslo and Bslo were also scored against a structural alignment of 10 distantly related SerP sequences (including microbial and viral SerPs) produced by Tong et al. (1993). In Fig. 2 *B*, residues of Dslo and Bslo that are identical to an equivalent position within 3.5 Å in at least one of these ten latter sequences are highlighted with yellow. Secondary structure predictions (α-helix, β-strand) of Dslo, Bslo and six of the SerP sequences of Fig. 2 *B* were performed using the program, PeptideStructure, of the Wisconsin Package, which compares methods of Chou and Fasman (1978) and Garnier et al. (1978).

A Technical Note on the Statistical Significance of the Proposed Alignment

As stated above, we found that automatically generated pairwise sequence alignments of the COOH-terminal region of Dslo or Bslo with various members of the SerP family indicated a low level of sequence similarity in the range of ~17–24% identity. This level of similarity falls into the "twilight zone" of protein evolution as defined by Doolittle (1986), so called because randomly chosen or scrambled amino acid sequences may often exhibit identity in the range of ~20% using gapped alignment techniques. Whereas it is difficult to establish statistical significance for a gapped alignment of <20% identity, there are numerous examples of structurally related proteins that have barely detectable sequence identity (Doolittle, 1990). If a functional relationship is known or suspected (as argued here for BPTI binding), then analysis of low-level sequence identity may have important predictive value. A typical example is the finding of 20% identity between a 113-residue fragment of the bovine rod cyclic nucleotide-gated channel and the cyclic nucleotide binding domain of the catabolite gene activator protein of *Escherichia coli* (Varnum et al., 1995).

Our sequence analysis takes advantage of the fact that secondary structural elements (β-strands) of the SerP family are more strongly conserved than variable loop regions. The most notable feature of the proposed alignment is the surprising number of correctly placed structural elements that can be identified by comparison techniques. The MACAW program for the interactive identification of blocks of similarity in a group of *n* multiple sequences (Schuler et al., 1990; Lawrence et al., 1993) calculates the statistical significance of an *n*-block of similarity according to a well-developed mathematical theory of protein sequences (Karlin and Altschul, 1990). To evaluate the suspected similarity by this approach, we used MACAW to search for local-similarity blocks constrained to include all seven of the Greer (1990) SerP sequences as shown in Fig. 2 *B* plus the candidate sequence of Dslo. This search identified five statistically significant and correctly ordered blocks of similarity in the eight sequences. These blocks include the following five regions of Fig. 2 *B* named according to the Dslo sequence: (*a*) QNALTLIRS, a nine-residue block overlapping the β6 region that surrounds the SerP catalytic residue Asp102; (*b*) GPLAQFGEC, a nine-residue block overlapping the β8-α1 region; (*c*) GMLCIG, a six-residue block coinciding with the β-9 region; (*d*) CDASSKRYVI, a ten-residue block overlapping the β10 region surrounding the SerP catalytic residue, Ser195; and (*e*) KPPAVRAPA, a nine-residue block overlapping the β12 region. The statistical significance of these five blocks as calculated by MACAW was: (*a*) $P = 3.0 \times 10^{-9}$; (*b*) $P = 6.6 \times 10^{-2}$; (*c*) $P = 3.7 \times 10^{-10}$; (*d*) $P \approx 0$; and (*e*) $P = 6.4 \times 10^{-11}$. As discussed by Schuler et al. (1991), these probabilities must be interpreted cautiously since a subset of related sequences can strongly bias the score for a particular block. In the final analysis, the significance of the proposed alignment rests on the number and placement of local similarities plus the functional evidence discussed in the text. Ultimate confirmation of the proposed homology depends on subsequent verification of specific structural predictions.

RESULTS AND DISCUSSION

Protein toxin inhibitors have often served as ligands to identify functional domains of channel proteins. One prominent example is the use of the scorpion toxin, charybdotoxin, to identify the extracellular pore-forming loop region of voltage-sensitive K^+ channels (MacKinnon et al., 1990; Miller, 1995). Since the initial finding of an unusual effect of dendrotoxin (Lucchesi and Moczydlowski, 1990) and the homologous protein, BPTI (Lucchesi and Moczydlowski, 1991), in producing discrete subconductance events when added to the intracellular side of maxi K_{Ca} channels, we have sought to identify the channel-associated binding site that mediates this inhibition. In this report, we present one line of experimentation and summarize accumulated evidence which suggests that the BPTI-binding site may be a domain of the K_{Ca} channel that is structurally related to a serine proteinase. We then present a protein sequence analysis that supports this conclusion and leads to a hypothesis concerning the structure and function of this domain in regulating the activity of *slowpoke* K_{Ca} channels.

Experimental Evidence for a Serine Proteinase-like Domain

Fig. 1 *A* shows the effect of adding 2.9 μM BPTI to the internal side of a single K_{Ca} channel from rat skeletal muscle as recorded in a planar bilayer at +20 mV and filtered at 100 Hz. In previous experiments (Lucchesi and Moczydlowski, 1991), the single-channel kinetics of BPTI inhibition were found to conform to a reversible binding reaction at a single site or a kinetically homogeneous class of sites (K_D = 2.3 μM at +20 mV, 50 mM symmetrical KCl). In other words, substate events correspond to individual residence times of a BPTI molecule on the channel and durations between substate events correspond to individual waiting times for the binding of BPTI. (We have also found that apparent subconductance events induced by BPTI binding are actually due to a change in the fluctuation rate of the open channel rather than physical obstruction of the pore by the ligand [Moss and Moczydlowski, 1996]. The present report is focused on the site of inhibitor binding, not its mechanism.)

Fig. 1, *C* and *D*, respectively, show a space-filling and backbone representation of the crystal structure (Rühl-

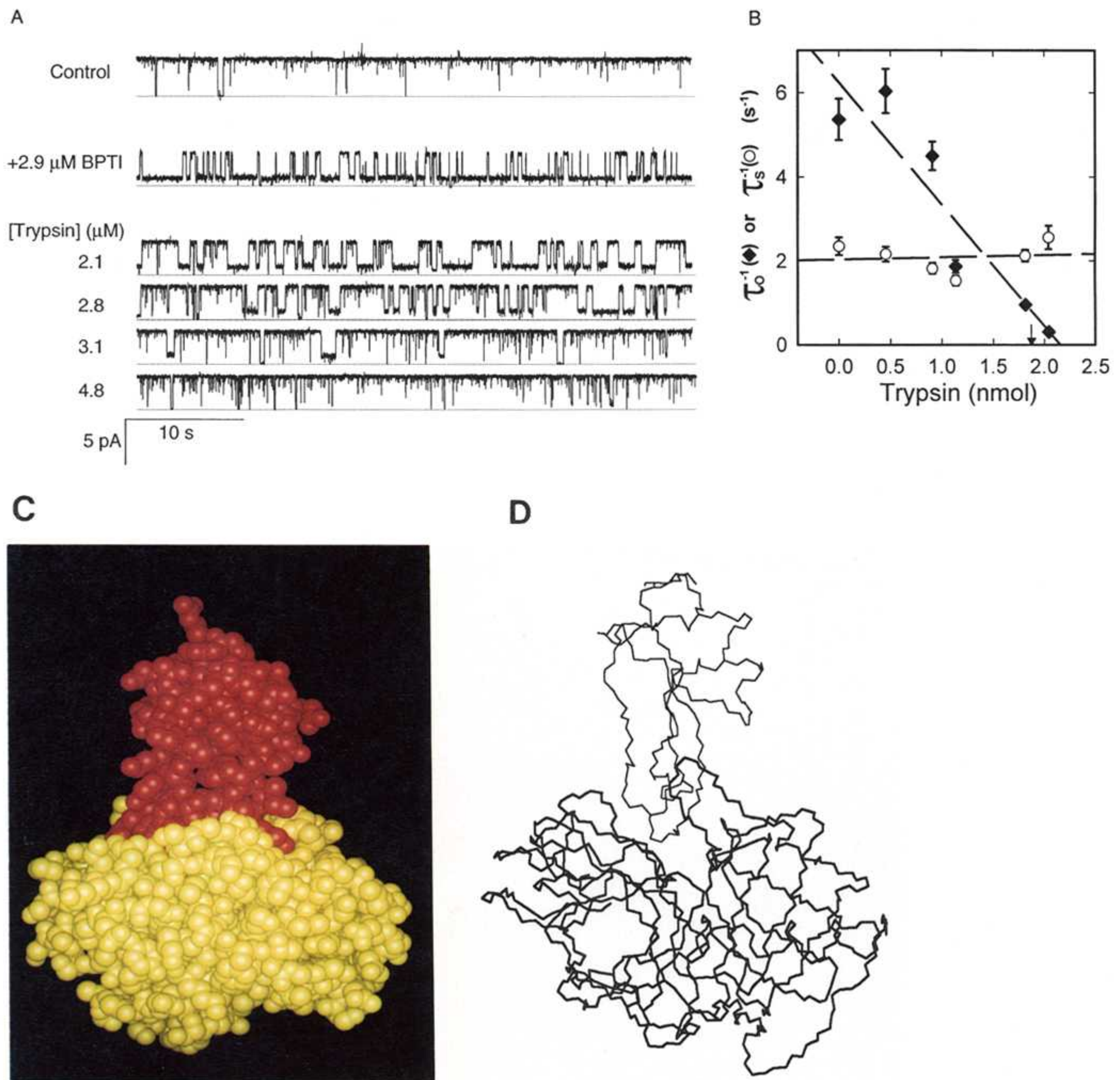

FIGURE 1. Demonstration that the BPTI-trypsin complex is inactive in the production of discrete substates. (*A*) Current records of a single K_{Ca} channel first exposed to 2.9 μM internal BPTI and then titrated with increasing amounts of trypsin to form trypsin-BPTI complex. Conditions: symmetrical 50 mM KCl, 10 mM MOPS-KOH, pH 7.2, 100 μM external EDTA, 500 μM internal $CaCl_2$, holding voltage = +20 mV. (*B*) Quantitative analysis of the trypsin-BPTI experiment in *A*. The reciprocal mean substate dwell time and the reciprocal mean dwell time between adjacent substates is plotted as a function of nmol trypsin added to the internal chamber. Long dashed lines indicate linear regression fits of the data and the arrow indicates the theoretical abscissa intercept as described in the text. (*C*) Space-filling (CPK) representation of the crystal structure of the complex of bovine trypsin (*yellow*) with BPTI (*red*) from the Brookhaven Protein Data Bank (2PTC entry, Marquart et al., 1983; Huber et al., 1974; Rühlmann et al., 1973). The molecular graphics image was produced with Insight software from Biosym (San Diego, CA). (*D*) Peptide backbone representation of the same complex of bovine trypsin (*thick line*):BPTI (*thin line*) produced with Hyperchem molecular graphics software (Hypercube, Waterloo, Ontario, CN).

mann et al., 1973; Huber et al., 1974; Marquart et al., 1983) of the 1:1 complex formed between bovine trypsin and BPTI. Bovine trypsin has very high affinity for BPTI. The equilibrium dissociation constant of the BPTI-trypsin complex has been determined to be 6×10^{-14} M with a half-life of dissociation estimated at ~17 wk (Vincent and Lazdunski, 1972; 1976). Thus, this interaction is practically irreversible. The contact region

between BPTI and trypsin is primarily formed by the so-called "inhibitory loop" of BPTI consisting of BPTI residues Pro-13 to Ile-19, which fits into a groove on the surface of trypsin that comprises the substrate recognition site of serine proteinases (Blow et al., 1972; Rühlmann et al., 1973; Janin and Chothia, 1976). In particular, the Lys-15 residue of BPTI fits into the substrate specificity pocket of trypsin which determines the site of proteolytic cleavage (Janin and Chothia, 1976). Structural analysis has shown that the conformation and packing of the inhibitory loop of BPTI is complementary to the substrate binding groove of trypsin and numerous other SerPs (Chothia and Janin, 1975; Janin and Chothia, 1990; Bode and Huber, 1992). This structural complementarity facilitates the formation of numerous molecular interactions between the enzyme and inhibitor, which greatly stabilizes this protein–protein interaction and reduces trypsin proteolytic action to a very small fraction of the normal turnover rate.

BPTI forms a similar complex with the inactive trypsin precursor, trypsinogen, albeit with much lower affinity, $K_D = 2 \times 10^{-6}$ M (Vincent and Lazdunski, 1976; Bode, 1979; Antonini et al., 1983). Structural analysis has shown that binding of BPTI to trypsinogen is accompanied by a large conformational change. The normally disordered state of ~15% of the peptide backbone in trypsinogen is converted to an ordered trypsin-like conformation upon the binding of BPTI (Bode et al., 1978; Bode, 1979). Other SerP enzymes also bind BPTI with lower affinity than trypsin: chymotrypsin, $K_D = 9.5 \times 10^{-9}$ M and kallikrein, $K_D = 9 \times 10^{-10}$ M (Vincent and Lazdunski, 1973; Antonini et al., 1983). In addition, a catalytically inactive homolog of elastase, known as human heparin binding protein, has been found to bind BPTI with a K_D in the range of $1–7 \times 10^{-7}$ M (Petersen et al., 1993). The wide variation in K_D values for BPTI binding to SerP homologs and the broad specificity of BPTI for binding to the substrate recognition cleft of active and inactive SerPs led us to consider whether the K_{Ca} channel site that binds BPTI may also be structurally related to this class of proteinases.

Several observations now appear to support this conjecture. First, in studies examining the interaction of BPTI mutants with single K_{Ca} channels in planar bilayers, we found that mutations within the inhibitory loop involving the trypsin specificity residue, Lys-15, reduce the characteristic rectification (Moss and Moczydlowski, 1996) of the substate I-V curve (Moss et al., 1991; Moss and Moczydlowski, unpublished results). This implies that the trypsin contact region of BPTI is also an important determinant of the BPTI-K_{Ca} channel interaction. Second, we have recently found that chicken ovoinhibitor (Scott et al., 1987), a member of the Kazal family of SerP inhibitors (Laskowski and Kato, 1980), also inhibits *slowpoke* K_{Ca} channels cloned from *Drosophila* and cow by inducing the production of discrete substates (Moss et al., 1996). Since Kazal domain proteins inhibit SerP enzymes by binding at the same site as BPTI (Bode and Huber, 1992), the coincidental finding of two different SerP inhibitors that also induce substates in the K_{Ca} channel, argues that some degree of structural similarity must exist between SerPs and the K_{Ca} channel. Third, we have found that cloned α-subunits of both *Drosophila* and bovine K_{Ca} channels expressed in a mammalian cell line are both sensitive to inhibition by internal BPTI. In particular, the cloned *Drosophila* K_{Ca} channel exhibits a slow time constant for dissociation of BPTI of 18.5 min (Moss et al., 1996). These results imply that the site of BPTI inhibition is located on the α-subunit of the K_{Ca} channel and that the interaction of BPTI with the *Drosophila* K_{Ca} channel is quite stable, in the fashion of SerP enzymes.

Another piece of evidence is provided by the experiment of Fig. 1. If the inhibitory loop region of BPTI is also a principal contact region for the K_{Ca} channel interaction, then the trypsin-BPTI complex should be completely inactive with respect to channel inhibition. Fig. 1 *A* shows that addition of trypsin to a channel exposed to 2.9 μM BPTI reduces the frequency of BPTI-induced substate events in a dose-dependent manner. Since trypsin binds to BPTI stoichiometrically, one would predict that if the trypsin-BPTI complex cannot bind to the channel, then the apparent association rate constant for BPTI (reciprocal mean time of durations between successive substates, τ_O^{-1}) should decrease linearly with trypsin concentration ($\tau_O^{-1} = k_{on}\{[\text{BPTI}] - [\text{trypsin}]\}$) and the apparent dissociation rate constant (reciprocal mean time of substate events, τ_S^{-1}) should be independent of trypsin concentration ($\tau_S^{-1} = k_{off}$). The data of Fig. 1 *B*, which plots τ_O^{-1} and τ_S^{-1} vs. nmol of added trypsin, is consistent with this prediction. This model also predicts that the abscissa intercept of a plot of τ_O^{-1} vs. nmol trypsin should be equivalent to the amount of BPTI that is present in the assay. A linear regression fit of this data gives an intercept that is very close to the actual BPTI content of the assay, as indicated by the arrow in Fig. 1 *B*. Control experiments show that the stoichiometric relief of BPTI inhibition by trypsin observed in this experiment is not due to proteolysis. For example, once free BPTI has been fully complexed by trypsin in experiments such as Fig. 1 *A*, addition of excess BPTI over the trypsin equivalence point results in the resumption of typical BPTI substate activity (Moss et al., 1996).

The results of Fig. 1, *A* and *B*, are mechanistically significant, because of the particular structure of the BPTI-trypsin complex (Fig. 1, *C* and *D*). In this complex, only 20% of the surface area of BPTI forms a direct interface with trypsin (Chothia and Janin, 1975; Ja-

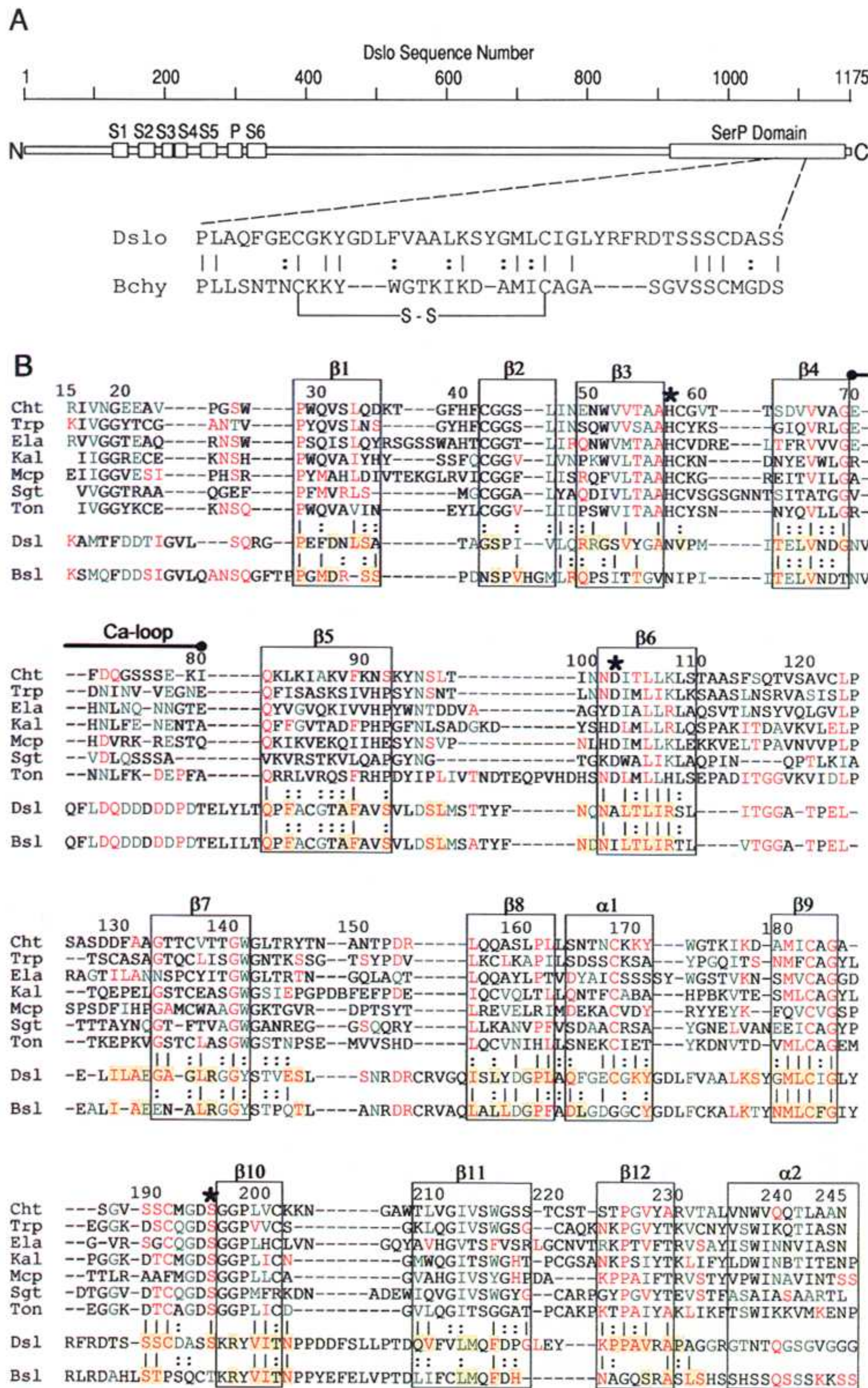

FIGURE 2. Identification of a COOH-terminal region of the *slowpoke* K_{Ca} channel that exhibits sequence similarity to serine proteinases. (*A*) Diagram of the linear sequence of the *Drosophila* K_{Ca} channel protein (*Dslo*). Boxes mark the location of the S1-S6 transmembrane spanning elements and the P-region motif shared in common with the family of voltage-gated K_V channels, as previously identified by Durell and Guy (1992). The box marked *SerP Domain* denotes the location of the serine proteinase-like domain of Dslo (residues 917-1167) that is fully aligned in *B*. The dotted lines mark the location of a candidate sequence (residues 1069–1111 of Dslo) that provided an initial clue to SerP homology. This Dslo sequence is aligned with residues 161–195 of bovine chymotrypsin (*Bchy*) showing identities (|), similarities (:) and gaps (−). The location of a highly conserved disulfide bond in the SerP family is noted (*S - S*). (*B*) Multiple sequence alignment of serine proteinases with the COOH-terminal region of *Drosophila* and bovine K_{Ca} channel proteins. The top seven sequences correspond to SerPs of known crystal structure previously aligned by structural superposition (Greer, 1990). The lower two sequences, Dslo and Bslo, respectively, correspond to residues 917–1167 of *Drosophila slowpoke* K_{Ca} channel (Accession No. M96840) and residues 827–1081 of bovine *slowpoke* K_{Ca} channel (Accession No. U60105; Moss et al., 1995; 1996). The sequence of Bslo is identical to that of mouse (Accession No. A48206) and human (Accession No. U13913) K_{Ca} channels in this region. Dashes correspond to gaps in the alignment generally placed in non-conserved loop regions. Sequence numbers correspond to the chymotrypsinogen numbering convention. The alignment begins at a conserved zymogen activation site of many SerPs (cleavage between Arg15 and Ile16 of Cht). The boxed regions approximately correspond to the locations of twelve β-strands (labeled *β1–β12*) and two α-helices of the SerP fold (labeled *α1* and *α2*) as previously identified (Gorbalenya et al., 1989; Petersen et al., 1993). Three asterisks (*) mark the location of the catalytic triad residues of SerPs (His57, Asp102, Ser195). The location of the Ca^{2+}-binding loop of trypsin (Bode and Schwager, 1975) is also noted. Red-colored residues indicate identity and green-colored residues indicate chemical similarity of residues in Dslo or Bslo with aligned residues of the seven SerPs. Chemical similarity is defined by the residue categories: (A, G); (D, E, N, Q); (F, Y, W); (M, L, I, V); (K, R); and, (S, T). As discussed in the text, thirteen predicted conserved regions of Dslo and Bslo (segments overlapping β1–β12 plus α1) were scored for identities against a test library of 109 known members of the SerP family as described in MATERIALS AND METHODS.

nin and Chothia, 1990). The remaining 80% of BPTI surface area is exposed in the complex and potentially available for interaction with the channel. Thus, this experiment suggests that the trypsin contact region of BPTI is specifically required for the K_{Ca} channel interaction. Other interpretations of this experiment are certainly possible, such as steric hinderance by trypsin in preventing interaction of an exposed site on BPTI with the channel. Nevertheless, as briefly outlined above, several lines of currently available evidence are consistent with the idea that BPTI binds to a region of the channel that structurally resembles a SerP domain.

Identification of a Region at the COOH Terminus of Slowpoke K_{Ca} Channels that Exhibits Sequence Similarity to Serine Proteinases

The 1175-residue sequence of the *Drosophila slowpoke* K_{Ca} channel protein (Dslo) (Atkinson et al., 1991; Adelman et al., 1992) contains a ~218-residue core domain (known as S1-S6) that is recognized as a basic structural motif of the voltage-gated family of K_V channels (Fig. 2 *A*). Dslo and representative mammalian homologs such as Bslo (bovine K_{Ca} channel, Moss et al., 1995) differ from the voltage-gated K_V channel family by a unique NH_2-terminal sequence (~125 residues) and an exceptionally long COOH-terminal tail (~833 residues). Catalytic SerP domains are typically about 250 residues. In a preliminary attempt to search for sequence similarity, we examined computer-generated alignments between the entire Dslo sequence and entire sequences of prototypical members of the SerP family (trypsin, chymotrypsin, elastase, and kallikrein) that are known to bind BPTI. Initial screening was carried out using the BESTFIT alignment program based on the algorithm of Smith and Waterman (1981) and the GAP alignment program based on the algorithm of Needleman and Wunsch (1970). The results of this pairwise sequence screening focused our attention on the COOH-terminal tail of Dslo and ultimately led to the identification of a 43-residue sequence near the COOH-terminus of Dslo that appeared to be a candidate for structural similarity. Fig. 2 *A* shows an alignment of this sequence with that of bovine chymotrypsin which exhibits 37% identity, excluding gaps. This level of similarity is comparable to that of different members of the SerP family in this same region (e.g., 40% identity between bovine chymotrypsin vs. porcine elastase). The aligned region in Fig. 2 *A* merits interest since the COOH-terminal residue of the chymotrypsin sequence is the catalytic Ser195 residue which would appear to be conserved in Dslo. It also includes three aligned Cys residues, two of which form the most highly conserved disulfide bond of the chymotrypsin-like SerP family (Cys168-Cys182, standard chymotrypsinogen numbering, Wang et al., 1985). (As discussed later, the three gaps placed in the alignment of Fig. 2 *A* happen to occur in variable, less-conserved regions of SerP sequences.)

Methods for sequence-based identification and homology modeling of divergent SerP domains are well established due to the fact that crystal structures of numerous members and close structural relatives of this family have been solved and compared by three-dimensional superposition (Greer, 1990; Tong et al., 1993). The SerP fold consists of 12 beta strands that form two orthogonally oriented, 6-stranded antiparallel beta-barrels (Richardson, 1981; Chothia and Janin, 1982; Branden and Tooze, 1991). From the amino end, the first six strands, β1–β6, form the first barrel domain and the following six strands, β7–β12, form the second barrel domain. In many SerPs two additional α-helical segments, α1 and α2, occur after β8 and β12, respectively. These 14 elements of secondary structure are conserved in nearly all chymotrypsin-like SerPs and provide the basis for identifying structurally conserved regions (SCRs) and intervening variable regions (VRs) that comprise the connecting loops within a candidate sequence (Greer, 1990; 1991). To assess whether the COOH-terminal region of the *slowpoke* K_{Ca} channel contains a SerP domain, we attempted to identify candidates for these 14 elements of secondary structure.

This analysis was carried out with the aid of multiple sequence alignment techniques that included the MACAW program for detection of regions of local similarity (Schuler et al., 1991; Lawrence et al., 1993) and the PILEUP program for optimizing global alignment (Feng and Doolittle, 1987). Fig. 2 *B* represents a possible alignment of a COOH-terminal region of Dslo and Bslo K_{Ca} channels with seven diverse members of the SerP family whose crystal structures have been solved. These seven SerPs were previously used by Greer (1990) to identify SCRs and align the sequences of 35 known members of

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The α2 region was not considered because of insignificant similarity and poor conservation between Dslo and Bslo. Residues in Dslo and Bslo that occur in the aligned position at a frequency ≥10% of the 109-member library are marked with a line (|) and those that occur at least once but at a frequency of <10% are marked with a colon (:). These same regions of Dslo and Bslo were also scored against a structural alignment of 10 distantly related SerP sequences (including microbial and viral SerPs) produced by Tong et al. (1993). Residues that are identical to an equivalent position within 3.5 Å in at least one of these ten latter sequences are highlighted with yellow. *Cht*, bovine chymotrypsin; *Trp*, bovine trypsin; *Ela*, pig elastase; *Kal*, pig kallikrein; *Mcp*, rat mast cell protease; *Sgt*, *Streptomyces griseus* trypsin; *Ton*, rat submaxillary gland tonin.

this family. Using these seven SerPs plus Dslo as test sequences, the MACAW program detected several statistically significant regions of similarity (see MATERIALS AND METHODS). Two of these regions closely correspond to the β6 and β9 strands of SerPs as shown in Fig. 2 *B*. These segments provided anchor points in the sequence alignment to search for candidates for the adjacent SCRs.

Fig. 2 *B* shows that Dslo and Bslo contain potential candidates for most of the conserved elements of SerP secondary structure. The weakest and most dubious regions of the alignment include the COOH-terminal α-helix (α2) and the β2 strand for both Bslo and Dslo plus the β12 strand for Bslo. For the rest of the known elements of SerP secondary structure, sequence similarity is supported by an analysis of naturally occurring substitutions in a test library of 109 readily aligned SerP sequences from diverse species as described in MATERIALS AND METHODS. Results of this analysis are indicated in Fig. 2 *B* by bar (|) and colon (:) symbols which mark the locations of Dslo and Bslo residues that are identical to those that frequently (≥10%) or rarely (<10%) occur at the same position in the test library of 109 SerP sequences. This comparison shows that there is a recognizable level of similarity between most of the regions of Dslo and Bslo that are proposed to align with the boxed secondary structure elements of the SerP family. Many of the residues identified by this approach correspond to those that have been previously noted by Greer (1990) as being highly conserved in the SerP family. The Dslo/Bslo sequences contain identities to the following highly conserved (almost invariant) residues as denoted by the chymotrypsinogen numbering convention: Pro28, Leu33, Val66, Gly69, Leu105, Gly140, Leu155, Cys182, Gly184, Cys191, Ser195, Pro225, Val227.

Color-coded residues in the 9-sequence alignment of Fig. 2 *B* are either identical (*red*) or chemically similar (*green*, defined in Fig. 2 *B*, legend) to those in Dslo and Bslo. This display method shows striking overall sequence similarity of the channel sequences to a structure-based alignment of seven SerP enzymes. In quantitative terms, there is 32% overall identity (53% chemical similarity) between the Dslo sequence and aligned residues in the profile of seven SerP sequences in Fig. 2 *B*. The corresponding numbers for Bslo are 29% identity and 49% similarity. Doolittle (1990) has suggested the criterion, that if two protein sequences of greater than 100 residues in length are >25% identical, common evolutionary ancestry can almost certainly be inferred. From our analysis and the uncertainty arising from the placement of gaps, the pairwise sequence identity to any particular member of the SerP family is not strong enough to permit a conclusive identification (see MATERIALS AND METHODS). However, from the standpoint of multiple sequence alignment, there is a strong basis for suspecting that the COOH-terminal region of Dslo and Bslo is distantly related to the SerP family of soluble proteins.

Structural analysis has previously shown that the SerP structure can tolerate many types of residue substitutions while still maintaining the same basic peptide backbone fold. For example, SerP core proteins of alphaviruses and cysteine proteases of picornoviruses have insignificant sequence identity with the chymotrypsin family yet share a remarkably similar backbone fold (Bazan and Fletterick, 1988; Gorbalenya et al., 1989; Tong et al., 1993; Allaire et al., 1994). Tong et al. (1993) have presented a structure-based alignment of six mammalian, two *Streptomyces*, one bacterial and one viral proteinase which identifies residues that are located within 3.5 Å of the equivalent Cα atom in chymotrypsin. In Fig. 2 *B* we have highlighted in yellow residues of Dslo and Bslo that are identical to an equivalent position (within 3.5 Å) in at least one of the ten SerP sequences aligned by Tong et al. (1993). This comparison also shows that nearly all of the proposed secondary structural elements in Dslo and Bslo score highly against an established structural alignment of rather divergent proteinases that share the SerP fold.

Compatibility of the candidate Dslo and Bslo sequences with a SerP structure was also investigated by the secondary structure prediction methods of Chou and Fasman (1978) and Garnier et al. (1978) as carried out using the program PeptideStructure of the Wisconsin Sequence Analysis Package. Results of these predictions must be taken cautiously since these methods have been found to be only ~55% accurate at predicting known β-sheet, α-helix and turn structure from linear sequences (Nishikawa and Noguchi, 1991). Based on actual structures, it is known that the typical fold of a SerP enzyme consists of ~40% β-strand and ~9% α-helical secondary structure. As a test of these algorithms using known serine proteinases, we found that the Chou-Fasman (1978) method predicted 34% β-strand and 21% α-helix, taken as the average for six of the SerP sequences of Fig. 2 *B*, whereas the corresponding prediction for the method of Garnier et al. (1978) was 29% β-strand and 15% α-helix. Thus, using only known SerP sequences, the two methods predict a structure that is primarily β-strand, but they underestimate the relative percentage of beta to alpha structure. Both algorithms predicted a similar secondary structure composition for the Dslo and Bslo sequence regions of Fig. 2 *B* as follows: Chou and Fasman (1978) method, 35% β-strand, 21% α-helix; Garnier et al. (1978) method, 28% β-strand; 21% α-helix, reported as the average of Dslo and Bslo. Discrete regions of Dslo and Bslo that were predicted to have a propensity for β-strand formation closely correspond to the β5, β6, β9, β10, and β11 segments (results not shown) that we have independently aligned with the same regions of the SerP do-

main on the basis of sequence similarity. Thus, the similar predicted α/β composition values for the SerP enzymes and the Dslo/Bslo COOH-terminus is further suggestive of structural similarity.

To summarize, the alignment of Fig. 2 *B* suggests that a continuous sequence near the COOH-terminus of *slowpoke* K_{Ca} channels may comprise a SerP-like domain based on sequence similarity and proper arrangement of expected elements of secondary structure. Comparison of the variable loop regions further suggests that most of the predicted loops of Dslo and Bslo are similar in length to those of known SerPs with a few notable exceptions (e.g., the loop between β10–β11 is probably lengthened in Dslo/Bslo). There are also interesting sequence similarities in some of the proposed loops.

In particular, the connecting loop between the β4 and β5 strands of SerPs is typically rich in acidic residues (Sabharwal et al., 1995). In trypsin, chymotrypsin and their zymogens, this loop is known to form a single binding site for Ca^{2+} (K_D range 26-170 μM) (Chiancone et al., 1985). In trypsin, the Ca^{2+} ion is complexed in an octahedral coordination sphere with four coordinating residues (Glu70, Asn72, Val75, Glu80) located between positions 70–80 of Fig. 2 *B* (Bode and Schwager, 1975). In both *Drosophila* and mammalian *slowpoke* K_{Ca} channels, the region corresponding to this Ca-loop contains 7 Asp and 1 Glu residues (Fig. 2 *B*). If our alignment is correct, then this acidic stretch of the K_{Ca} channel sequence would be predicted to form a Ca^{2+} binding site. In the SerP enzymes, this Ca-binding loop is known to exhibit conformational changes as detected by comparison of different crystal structures (Cohen et al., 1981). Occupation of this site by Ca^{2+} stabilizes SerPs against denaturation and inhibits self-proteolysis (Chiancone et al., 1985). Ca^{2+} binding to this loop has also been shown to greatly enhance the interaction of blood coagulation Factor VIIa with an essential cofactor called tissue factor (Sabharwal et al., 1995).

In the K_{Ca} channel, we propose that this loop may be involved in Ca^{2+}-dependent gating of the channel. In previous work, we found that internal BPTI binds to the K_{Ca} channel in a voltage-dependent manner (Lucchesi and Moczydlowski, 1991) and that bound BPTI modulates a rapid opening and closing process of the channel (Moss and Moczydlowski, 1996). Thus, it is tempting to suggest that Ca^{2+} binding to this domain induces a conformational change that plays a role in the mechanism of K_{Ca} channel gating. Independent evidence for the involvement of this Asp-rich region in Ca^{2+}-activation of the K_{Ca} channel has recently been obtained by site-directed mutagenesis (Krause et al., 1996). This Asp-rich loop might also correspond to a regulatory binding site for divalent cations that has been inferred from a synergistic effect of Mg^{2+} to increase the Hill coefficient for channel activation by Ca^{2+} (Golowasch et al., 1986; Oberhauser et al., 1988).

Despite numerous similarities suggested by the alignment of Fig. 2 *B*, it is important to note that there are also significant sequence differences between the candidate domain of the K_{Ca} channel and the SerP family. For example, one of the most highly conserved sequence motifs of chymotrypsin-like SerPs is the sequence, GDSGGP, containing the active site residue, Ser195. The Dslo and Bslo sequences do not share this motif, although they do align at Ser195 with a Ser and Thr residue in Dslo and Bslo, respectively. However, the GDSGGP motif is also quite divergent in inactive mammalian SerP homologs such as protein Z (Højrup et al., 1985) and more distantly related bacterial or viral SerPs (Rawlings and Barret, 1994). While such variations do not necessarily rule out the possibility of a similar fold, the apparent lack of conservation of the other two residues of the SerP catalytic triad, His57 (aligned as Asn in Dslo and Bslo, Fig. 2) and Asp102 (aligned as Ala in Dslo, Ile in Bslo), implies that the identified Dslo/Bslo domains are not likely to be catalytically active as proteinases. There is a precedent for this situation in that a number of catalytically inactive SerP homologs with diverse functions have been previously described. These proteins include streptokinase (Jackson and Tang, 1982), haptoglobin (Kurosky et al., 1980), heparin binding protein/azurocidin (Petersen et al., 1993), protein Z (Højrup et al., 1985), hepatocyte growth factor (Nakamura et al., 1989), and hepatocyte growth factor-like protein (Han et al., 1991). Sequence analysis of these latter proteins clearly identifies each of them as SerP homologs, yet they exhibit a number of uncommon substitutions that are rarely found in active proteinases (Greer, 1990). The lack of a complete catalytic triad and the presence of unusual substitutions in the Dslo/Bslo sequence alignment is consistent with the observation of Greer (1990) that "deviation from the typical pattern of sequence conservation occurs most often in those members of the family that are no longer serine proteases in function."

What might be the function of a non-catalytic SerP domain at the COOH-terminus of *slowpoke* K_{Ca} channels? In addition to a possible role in channel gating mentioned above, it is likely that this domain is a locus of protein–protein interactions. In its multitudinous variations, the SerP domain specializes in binding interactions with other proteins at widely varying degrees of affinity and specificity. This is exemplified by the specific interaction of trypsin and BPTI with a K_D of 6×10^{-14} M (Vincent and Lazdunski, 1972) and low affinity interactions of SerP enzymes with an infinite number of peptide substrates that merely possess a preferred residue at the scissile peptide bond. Some SerP domains have evolved to function as specialized binding

proteins, as in the example of the avid binding of the αβ dimer of hemoglobin by the β chain of haptoglobin, a SerP homolog (Nagel and Gibson, 1971; Kurosky et al., 1980). Other SerP enzymes such as thrombin contain overlapping binding sites for a panoply of key substrates and thereby regulate a variety of cellular processes (Stubbs and Bode, 1995). Thus, it is likely that the SerP domain of *slowpoke* K_{Ca} channels serves as a binding domain for one or more cytoplasmic or membrane-associated components. These associations may involve cytoskeletal proteins or perhaps regulatory proteins such as protein kinases (Esguerra et al., 1994). Modulation of the K_{Ca} channel by the binding of endogenous SerP ligands such as Kunitz inhibitor proteins is also a possibility.

The SerP domain is a common element of mosaic proteins such as enterokinase and tissue plasminogen activator (Neurath, 1985). In such mosaic proteins, the SerP domain frequently occurs at the COOH-terminus (Rawlings and Barret, 1994), as in the region we have identified here in the K_{Ca} channel. Doolittle (1995) has summarized evidence indicating that the evolution of proteins occurred through extensive shuffling of modular domains. Other examples of channels or channel-related proteins that exhibit homology to domains of soluble proteins have been described. These include glutamate-receptor channels and the family of bacterial periplasmic binding proteins (O'Hara et al., 1993; Wo and Oswald, 1995); β subunits of K_V channels and a superfamily of oxidoreductases (McCormack and McCormack, 1994); the CFTR chloride channel and a superfamily of ATP-binding proteins (Gorbalenya and Koonin, 1990); and cyclic nucleotide-activated ion channels and cAMP-binding domains of the bacterial CAP protein (Varnum et al., 1995). The relationship proposed in this paper may represent yet another example of the modular design and evolution of channel proteins.

In conclusion, we have presented a hypothesis for the existence of an intracellular SerP domain at the COOH-terminal region of *slowpoke* K_{Ca} channels based on a functionally conserved interaction of the channel with specific SerP inhibitors (Moss et al., 1996) and sequence similarity recognizable at the level of multiple sequence alignment (Fig. 2 *B*). Our model predicts that the SerP-like sequence identified in Dslo and Bslo should comprise an intracellular domain of the K_{Ca} channel that does not contain membrane-spanning segments. It is also expected that this domain should bind certain SerP inhibitors such as BPTI and contain a Ca^{2+}-binding site located within the β4-β5 loop region. It should be feasible to test this hypothesis by various mutagenic approaches and direct structural analysis of the candidate channel domain.

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