An Evolutionarily Conserved Binding Site for Serine Proteinase Inhibitors in Large Conductance Calcium-Activated Potassium Channels[†,‡]

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ABSTRACT: Complementary DNA coding for the channel-forming α-subunit of a large conductance Ca^{2+}-activated K^+ channel (maxi K_{Ca} channel) was cloned from bovine aortic smooth muscle cells. This cloned mammalian K_{Ca} channel (Bslo) and its homolog from *Drosophila* (Dslo) were expressed in the HEK293 human embryonic kidney cell line. Both Bslo and Dslo K_{Ca} channels were sensitive to inhibition by the internally applied serine proteinase inhibitors: bovine pancreatic trypsin inhibitor (BPTI, $K_D = 7.0$ μM for Bslo and 2.6 μM for Dslo) and chicken ovoinhibitor (OI, $K_D = 1.5$ μM for Bslo and 11.4 μM for Dslo). BPTI and OI are members of the Kunitz and Kazal families of proteinase inhibitors, respectively. The ∼60-residue inhibitory domains of these proteins have a different tertiary structure except in the region of a loop formed by ∼6 residues, in which the peptide backbone adopts a similar conformation complementary to the active site cleft of many serine proteinases. At the single-channel level, BPTI and OI were found to inhibit K_{Ca} channels by a similar mechanism involving the production of discrete low-conductance events. These two inhibitors also exhibited competitive behavior, suggesting that they bind to an overlapping site. Kinetic characterization revealed that the dissociation rate of BPTI from the bovine K_{Ca} channel is fast ($k_{off} = 0.41$ s^{-1}), whereas that from the *Drosophila* K_{Ca} channel is slow ($k_{off} = 9.0 \times 10^{-4}$ s^{-1}) and indicative of a strong molecular interaction. The stable complex of BPTI and trypsin was inactive as a K_{Ca} channel inhibitor, further supporting the idea that the trypsin inhibitory loop of BPTI recognizes a specific site on the channel protein. These results lead to the conclusion that the α-subunit of maxi K_{Ca} channels contains a conserved proteinase inhibitor binding site. We hypothesize that this site corresponds to a C-terminal domain of the channel protein that structurally resembles serine proteinases.

Large conductance Ca^{2+}-activated K^+ channels, also known as maxi K_{Ca} or BK channels, are structurally related but functionally distinct from the extensive family of voltage-sensitive K^+ channels (K_V channels). Maxi K_{Ca} channels[1] are located in the plasma membrane of many diverse cell types, and their opening probability is a complex function of intracellular Ca^{2+} and membrane depolarization (Latorre, 1994). Hydrophobicity analysis of the primary sequence of cloned maxi K_{Ca} channels from flies (Atkinson et al., 1991) and mammals (Butler et al., 1993) reveals a pattern of six putative transmembrane segments within the N-terminal region of the channel that is homologous to the S1−S6 motif of K_V channels. However, maxi K_{Ca} channels differ from K_V channels by a unique, ∼800-residue C-terminal domain, which has been proposed to contain an additional four membrane-spanning segments (Butler et al., 1993). This large C-terminal region appears to be involved in Ca^{2+}-dependent activation and channel gating, although its function is not yet fully understood (Wei et al., 1994).

Work at the single-channel level has previously shown that maxi K_{Ca} channels from rat skeletal muscle (Lucchesi & Moczydlowski, 1991) and bovine aortic smooth muscle cells (Moss & Moczydlowski, 1996) are inhibited at an intracellular site by BPTI (bovine pancreatic trypsin inhibitor), a 58-residue protein inhibitor of serine proteinases such as trypsin and chymotrypsin. At the single-channel level, BPTI inhibition of these K_{Ca} channels is characterized by the appearance of discrete, low-conductance events that correspond to residence times of BPTI on the channel protein (Lucchesi & Moczydlowski, 1991). The apparent reduced conductance of BPTI-induced events has been found to arise from rapid fluctuation of the channel between the open state and a nearly closed state (Moss & Moczydlowski, 1996). The unique nature of this channel−ligand interaction has

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; Bslo, bovine *slowpoke*-related K_{Ca} channel; Cslo, canine *slowpoke* K_{Ca} channel; Dslo, *Drosophila slowpoke* K_{Ca} channel; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; K_{Ca} channel, large conductance Ca^{2+}-activated K^+ channel; Mslo, mouse *slowpoke* K_{Ca} channel; Mops, 3-(*N*-morpholino)propanesulfonic acid; OI, chicken ovoinhibitor; SerP, serine proteinase.

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motivated our attempt in this paper to correlate the location of the BPTI-binding site with the K_{Ca} channel protein and investigate whether this site is structurally related to that of a serine proteinase.

We approached this problem by cloning the channel-forming α-subunit of a bovine K_{Ca} channel (Bslo) and expressing it in a cultured cell line. We found that the expressed Bslo channel was sensitive to BPTI inhibition, implying that the BPTI-binding site is located on the α-subunit. We next tested whether members of other families of serine proteinase inhibitors also inhibit the cloned Bslo channel. Protein inhibitors of proteinases comprise an extensive and diverse group of molecules (Laskowski & Kato, 1980). Many of these inhibitors work by a similar mechanism involving tight binding to the active site cleft of target proteinases (Bode & Huber, 1992). BPTI is a member of the Kunitz family of proteinase inhibitors containing a single inhibitory domain with specificity for trypsin-like enzymes. Chicken ovoinhibitor (OI) is a 449 amino acid protein containing seven tandemly linked domains of the Kazal inhibitor family, which is structurally distinct from the Kunitz-type family but shares an inhibitory loop of similar peptide backbone conformation (Scott et al., 1987; Read & James, 1986; Bode & Huber, 1992). The multidomain structure of OI accounts for its ability to inhibit diverse proteolytic enzymes such as trypsin, chymotrypsin, and subtilisin (Tomimatsu et al., 1966). We found that OI also inhibits mammalian K_{Ca} channels by a mechanism similar to that of BPTI. Furthermore, single-channel experiments suggest that BPTI and OI compete for binding to the K_{Ca} channel, as if these inhibitors bind to a common site.

In exploring the generality of this effect, we observed that BPTI and OI also inhibit an insect K_{Ca} channel cloned from *Drosophila*, indicating that the serine proteinase inhibitor binding site has been conserved in evolution. These results, together with experiments implicating the trypsin-inhibitory loop region of BPTI in the channel−ligand interaction, support the notion that K_{Ca} channels contain a site that structurally resembles a serine proteinase. This hypothesis is further supported by sequence analysis, which indicates substantial similarity between aligned members of the serine proteinase family and an ∼250-residue region near the C-terminus of *Drosophila* and mammalian K_{Ca} channels (Moss et al., 1996). A preliminary verion of these results was reported in abstract form (Moss et al., 1995).

MATERIALS AND METHODS

Materials. The following purified trypsin inhibitors were purchased from Sigma: BPTI, chicken ovomucoid inhibitor, chicken ovoinhibitor, and soybean Bowman−Birk inhibitor. Soybean inhibitor (Kunitz) was obtained from Boehringer Mannheim.

Cloning. Degenerate oligonucleotides were designed according to six conserved regions of sequence in the *Drosophila* (Atkinson et al., 1991) and mouse (Butler et al., 1993) K_{Ca} channel. These synthetic oligonucletides were used in RT-PCR (reverse transcription polymerase chain reaction) to amplify large fragments of Bslo cDNA. One fragment encoded ∼550 bp of sequence on the 5′ side of the P-region (Figure 1) while a second fragment encoded ∼1250 bp near the 3′ end of the coding region. These PCR fragments were used to screen a cDNA library prepared from cultured bovine aortic smooth muscle cells. The library, in λgt10, was created through random priming and was constructed to contain inserts >500 bp. Screening at high stringency identified several overlapping cDNA fragments encoding ∼2.5 kb of sequence at the 3′ end of the coding region. The remaining ∼500 bp fragment was obtained by 5′RACE PCR (Frohman et al., 1988) using a kit from Gibco BRL. To minimize the possibility of PCR errors, RACE fragments of two different sizes were sequenced and found to be identical in the coding region. DNA sequencing was performed using the Sequenase 2.0 kit from USB. The derived sequence of our Bslo clone does not contain insertions at any of four possible sites of known alternative splicing in mammals labeled alt-1 to alt-4 in Figure 1.

Cell Culture and Expression. Stably transfected cell lines were obtained by calcium phosphate transfection of HEK293 cells (human embryonic kidney) with expression plasmids containing the *neo* antibiotic resistance gene and subsequent selection for colonies resistant to G418 (Geneticin, Gibco BRL). For this purpose the Bslo cDNA was subcloned into the pcDNA3 expression vector (Invitrogen). The Dslo cDNA, splice variant A1 C2 E1 G3 I0 of Adleman et al. (1992) in the pRc/CMV vector, was generously provided by Jing Wang and Irwin Levitan (Brandeis University). HEK293 cells were cultured at 37 °C in minimal essential medium containing Earle's salts and L-glutamine (Sigma). This medium was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin (Gibco BRL), and 0.9 mg/mL G418.

Membrane Preparation. Membranes from stably transfected HEK cells were prepared essentially as described by Sun et al. (1994). Briefly, ten 10 cm cell culture dishes of confluent, stably transfected HEK cells were scraped off using a rubber truncheon and resuspended in phosphate-buffered saline (PBS: 10 mM NaH_2PO_4 and 150 mM NaCl titrated to pH 7.4 with NaOH). The cells were collected by centrifugation at 2000 rpm for 3 min in a Sorvall TC6 bench top centrifuge containing an H400 rotor. The pellet was resuspended in 15 mL of solution containing 150 mM KCl, 2 mM $MgCl_2$, and 5 mM EGTA (titrated to pH 10.6 with ammonium hydroxide) and homogenized with a glass homogenizer. The homogenate was then layered onto a sucrose step gradient composed of 10 mL of 38% w/v sucrose and 10 mL of 20% w/v sucrose in 20 mM Mops−KOH buffer, pH 7.1. The gradients were centrifuged at 25 000 rpm in a Beckman SW28 rotor for 45 min at 4 °C. Membrane vesicles at the 38%/20% sucrose interface were collected (∼4 mL) and diluted to a final volume of 16 mL with water. The membranes were then pelleted by centrifugation at 40 000 rpm in a 50.2 Ti Beckman centrifuge rotor for 60 min at 4 °C. Finally, the membranes were resuspended in 1 mL of a solution containing 250 mM sucrose and 10 mM Mops−KOH, pH 7.0, and frozen at −70 °C in 50 μL aliquots. Native maxi K_{Ca} channels from rat skeletal muscle membranes were prepared as described previously (Guo et al., 1987).

Patch Recordings. Sylgard-coated, fire-polished patch pipets of 4−20 MΩ resistance were pulled from thin- or standard-walled borosilicate glass (WPI or Warner Instrument Co.). Gigaohm patch recordings were made at room temperature using the EPC-9 (HEKA-Instrutech) patch clamp amplifier and stored on video cassette tape at 10 kHz

bandwidth via a VR-10B digital interface (Instrutech). Patch pipets were filled with "low-Ca^{2+}" solution: 100 mM KCl, 3.8 mM $CaCl_2$, 5 mM EGTA (~0.2 μM free Ca^{2+}), and 10 mM Hepes−NaOH, pH 7.4. The standard solution ("high-Ca^{2+}") for bathing inside-out patches was 100 mM KCl, 100 μM $CaCl_2$, and 10 mM Hepes−NaOH, pH 7.4. To test for Ca^{2+} dependence and to monitor the kinetics of patch perfusion, the patch was transiently perfused with low-Ca^{2+} pipet solution.

For analysis the current records were refiltered either with an eight-pole Bessel filter (Frequency Devices) or with a digital Gaussian filter and digitized at 5−10 times the filter frequency. Results were analyzed using either PCLAMP6 (Axon Inst.), software kindly provided by Dr. F. J. Sigworth, or custom-written programs designed to run in the PowerMod programming environment (HEKA) on a MacIntosh Quadra 800 computer. Current responses to rapid perfusion of BPTI and OI were well described by fits to a single exponential function calculated using curve fitting routines in Igor (Wavemetrics) or SigmaPlot (Jandel Scientific) software.

Solutions bathing the intracellular face of the patch were changed by using a multibarrel perfusion system similar to that described by Yellen (1982) with the tubes arranged to obtain laminar flow in switching between any two solutions. Estimates of inhibitor rate constants from rapid perfusion experiments were made only on patches which showed fast-step responses to Ca^{2+} changes. The deadtime for solution exchange was 80 ± 13 ms (±SE, $n = 7$) as estimated from the current response to Ca^{2+}. By contrast, the fastest inhibitor assocation rate that we measured was 90% complete in 510 ms for the case of BPTI inhibition of Bslo (Figure 2B), indicating that these processes are reasonably well resolved. Reported rate constants are the average of measurements on two to five patches.

The extremely slow rate of dissociation of BPTI from Dslo made it difficult to perform similar kinetic measurements at +40 mV because significant potassium accumulation occurs in the patch pipet with large K_{Ca} currents. For this reason patches were held at 0 mV and current recovery was assessed by pulsing briefly to +40 mV. We occasionally observed patches which did not exhibit inhibition by BPTI or OI or which displayed a very slow response. Currents from such patches were also generally slower in response to calcium and were discarded from data analysis. We believe that this reflects a problem of occasional inaccessibility of protein ligands to the K_{Ca} channel binding site due to hindered diffusion in the inside-out patch configuration rather than a subset of insensitive channels. Other workers have previously reported that patch responses of K_{Ca} channels can be affected by access problems caused by a restricted patch configuration and/or by cytoskeletal components associated with the patch (Pallotta et al., 1992). Nonlinear current response associated with series resistance error was minimized by using patches that exhibited less than ~1 nA current.

Bilayer Recordings. Planar bilayer membranes were formed across a circular hole of 200 μm diameter in a polystyrene cup by painting a 25 mg/mL lipid solution using a thin glass rod. The lipid was a 4:1 mixture of bovine brain phosphatidylethanolamine/1,2-diphytanoylphosphatidylcholine (Avanti Polar Lipids) dissolved in decane. For experiments with native rat muscle membrane vesicles the bilayer chamber solutions contained 200 μM $CaCl_2$, 100 mM KCl, and 10 mM Mops−KOH, pH 7.2 (cis, "intracellular" chamber), or 50 mM KCl, 0.1 mM EDTA, and 10 mM Mops−KOH, pH 7.2 (trans chamber). OI and BPTI were added to the cis chamber at concentrations as high as 4.3 and 3.9 μM, respectively. For experiments with membrane vesicles prepared from HEK293 cells, the bilayer chambers contained solutions of 200 mM KCl, 1 mM EDTA, and 10 mM Mops−KOH, pH 7.2 (cis), and 100 mM KCl, 40 μM $CaCl_2$, and 10 mM Mops−KOH, pH 7.2 (trans, "intracellular" side), with 38 μM BPTI. [Note that these two membrane vesicle preparations exhibit opposite orientation of K_{Ca} channel incorporation (Sun et al., 1994).]

RESULTS

Cloning of a Bovine K_{Ca} Channel Subunit. At the outset of this work, inhibition of maxi K_{Ca} channels by proteinase inhibitors such as BPTI had previously been characterized for K_{Ca} channels from rat muscle membranes inserted into planar bilayers and for native maxi K_{Ca} channels in excised patches of bovine aortic smooth muscle cells (Lucchesi & Moczydlowski, 1991; Moss & Moczydlowski, 1996). In order to assess whether this sensitivity to BPTI is associated with the channel-forming subunit, we first cloned an α-subunit cDNA of a maxi K_{Ca} channel from a library prepared from cultured bovine aortic smooth muscle cells as described in Materials and Methods. We refer to this channel clone as Bslo (bovine slowpoke), as named after the first member of the K_{Ca} channel family cloned from *Drosophila* by identification of the defective gene underlying the *slowpoke* mutation (Atkinson et al., 1991). The Bslo sequence (Figure 1) is ~60% identical to Dslo and virtually identical (97.6%−99.7%) to similar clones reported from mouse (Butler et al., 1993), humans (McCobb et al., 1995; Tseng-Crank et al., 1994; Pallanck & Ganetzky, 1994; Dworetzky et al., 1994; Wallner et al., 1995), and dog (Genbank Accession No. U41001). Excluding possible splice site variations, the 1113-residue Bslo sequence (counting from Met-1) has only 3, 7, and 21 amino acid differences from Hslo, Mslo, and Cslo, respectively, indicating that this gene is highly conserved in mammals.

Comparison of Bslo and Dslo in Figure 1 shows that high similarity between mammalian and insect sequences is present in two distinct locations: the ~650-residue N-terminal region and the ~400-residue C-terminal region of the polypeptide. In contrast, there is little similarity in a middle connecting region of approximately 52 residues in Bslo and 115 residues in Dslo. The N-terminal region containing the S1−S6 K^+ channel motif has been termed the core domain, and the C-terminal region, previously implicated in the Ca^{2+}-sensing property of the channel, has been termed the tail domain (Wei et al., 1994). Figure 1 also summarizes currently available information on the extensive alternative mRNA splicing of this channel indicated by five reported splicing sites in *Drosophila* labeled alt-A, alt-C, alt-E, alt-G, and alt-I (Adelman et al., 1992) and four reported sites in mammals labeled here as alt-1 to alt-4 (Butler et al., 1993; Tseng-Crank et al., 1994). Since four different variations at the very end of the C-terminus have now been found in mammalian cDNA clones as cited above, it seems likely that this represents another site of alternative splicing, and we have accordingly labeled it alt-5.

Effect of BPTI and Ovoinhibitor on Cloned and Native Mammalian K_{Ca} Channels. Bslo K_{Ca} channels were ex-

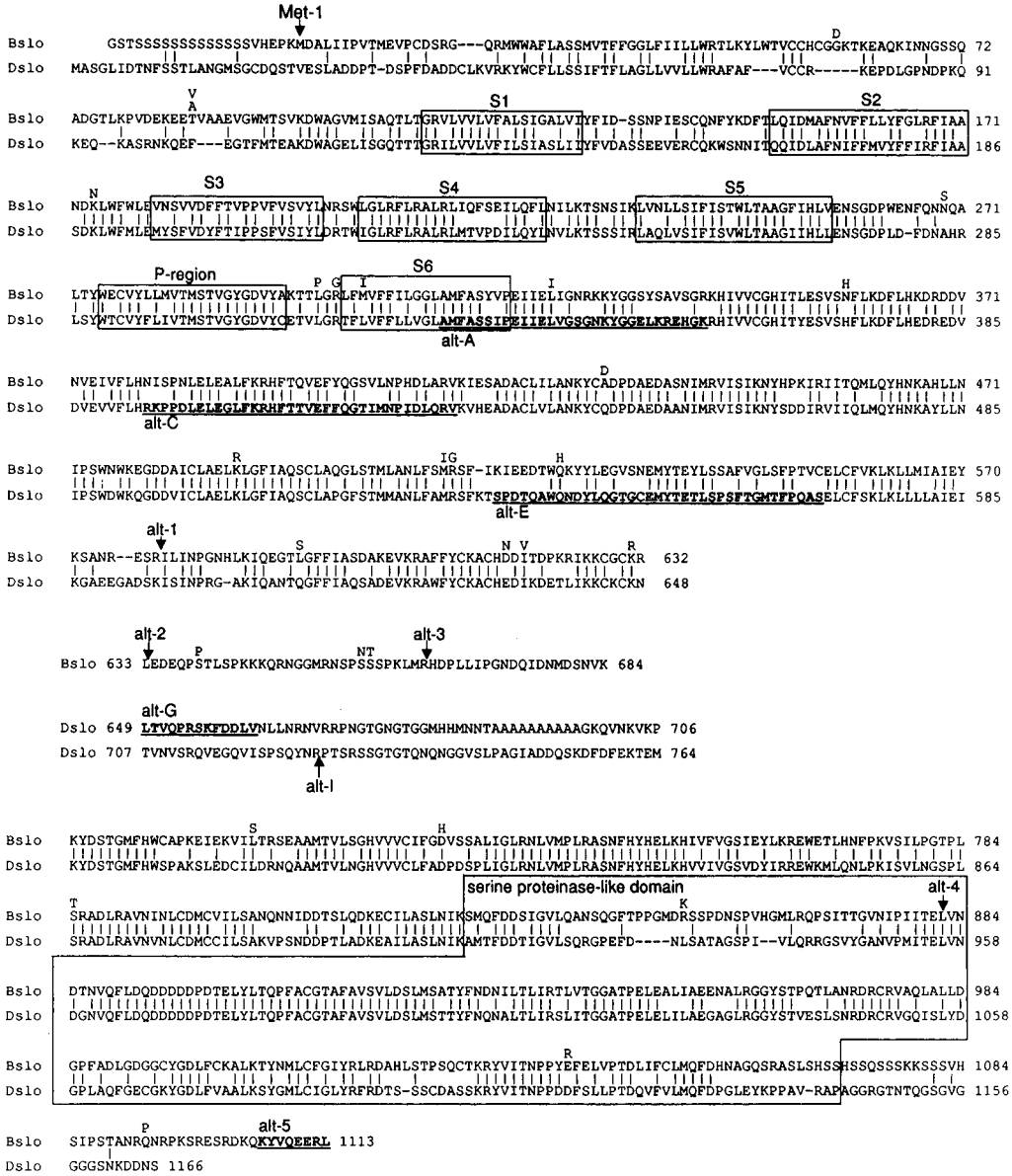

FIGURE 1: Sequence alignment of Bslo with the *Drosophila* K_{Ca} channel α-subunit. The primary sequence of Bslo was aligned pairwise with that of K_{Ca} channel homologs from human (Hslo, Genbank Accession No. U13913), mouse (Mslo, Accession No. A48206), dog (Cslo, Accession No. U41001), and *Drosophila* (Dslo, Accession No. M96840) using the GAP program of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI). Identities between the Bslo and Dslo sequences are indicated by a bar (|). Since all four mammalian homologs are very similar (>97% identity), any amino acid differences from Bslo that are present in Hslo, Mslo, or Cslo are shown at the corresponding position above the Bslo sequence. Bslo and Dslo sequences are numbered beginning at Met-1. Identification of the boxed S1−S6 motif is based on that of Adelman et al. (1992) using a multiple sequence alignment of *Drosophila* Shaker, Shab, Shal, and Shaw K^+ channels. The boxed P-region was assigned according to Durell and Guy (1992). Lack of significant identity in the connecting segment between the N-terminal core region and the C-terminal tail region is indicated by the indented segment of Bslo and Dslo shown without pairwise alignment. Five known locations of alternative splicing in Dslo are labeled alt-A, alt-C, alt-E, alt-G, and alt-I according to the nomenclature of Adelman et al. (1992). The particular splicing variant of Dslo used in this study and shown in the figure is A1-C2-E1-G3-I0 with the A, C, E, and G variant regions in bold underline. Five reported and apparent sites of alternative splicing in mammalian K_{Ca} channel clones are labeled alt-1 to alt-5, as discussed in the text. A region of ∼250 residues near the C-terminus of Bslo and Dslo that exhibits similarity to the family of serine proteinases by multiple sequence alignment techniques (Moss et al., 1996) is also boxed.

pressed in HEK293 cells either by transient transfection as described previously (Marshall et al., 1994) or by selection of a stably transfected cell line as described in Materials and Methods. Whole-cell and patch-recording voltage-clamp analysis showed that transiently and stably transfected HEK293 cells exhibited a large amplitude Ca^{2+}-dependent K^+ current that was not present in untransfected cells (not shown). The expressed Bslo channels have a characteristic unitary conductance of ∼250 pS and respond to voltage and internal Ca^{2+} in a manner very similar to that described for other cloned mammalian K_{Ca} channels expressed in *Xenopus* oocytes or cultured cells (Butler et al., 1993; Tseng-Crank et al., 1993; Dworetzky et al., 1994; McCobb et al., 1995; Wallner et al., 1995).

Figure 2 shows the effect of BPTI on an inside-out patch containing many Bslo channels that was held at +40 mV. The intracellular side of the patch in Figure 2A was first rapidly exposed to solutions containing high (100 μM) or low (∼0.2 μM) Ca^{2+}, to verify Ca^{2+} sensitivity of the K^+ current and unhindered accessibility of the patch to solution changes. The steplike current traces in response to the application of low-Ca^{2+} solution illustrate the rapid time

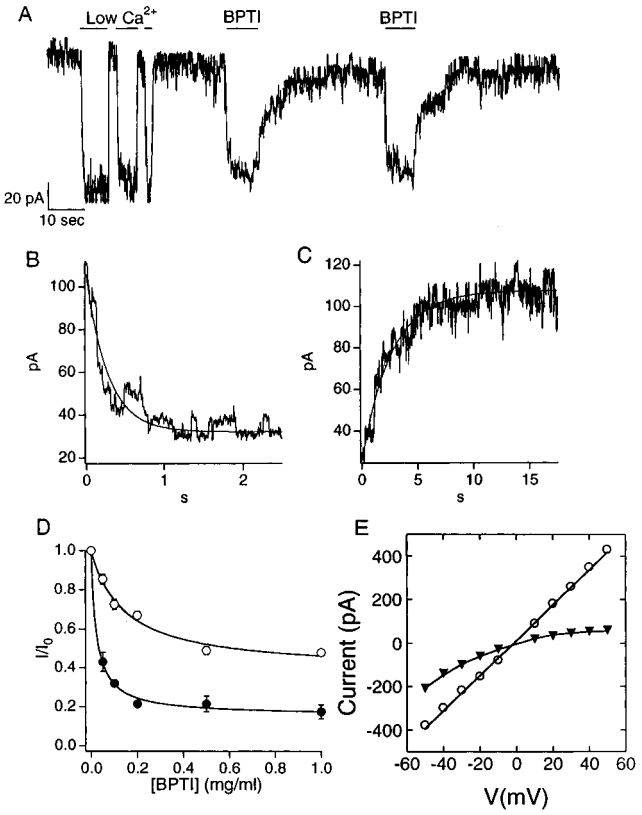

FIGURE 2: Inhibition of macroscopic Bslo current by BPTI. (A) Effect of rapid perfusion of low Ca^{2+} or BPTI on K^+ current recorded from inside-out patches pulled from HEK 293 cells expressing Bslo K_{Ca} channels. Outward K^+ current was recorded at +40 mV with pipet and bath solutions as described in Materials and Methods. During periods labeled low Ca^{2+}, the tip of the patch pipet was perfused with pipet solution, which transiently reduced free Ca^{2+} from 100 to ~0.2 μM. The record also shows two consecutive exposures to a bath solution containing 0.5 mg/mL BPTI (77 μM). The zero current level is indicated with a dashed line. (B, C) Expanded view of the time course of inhibition (B) and recovery (C) for BPTI as shown in panel A. Current traces are superimposed fits to a single exponential with time constants of 290 ms (B) and 2.9 s (C). (D) Inhibition of Bslo current at +40 mV (●) and −40 mV (○) as a function of BPTI concentration measured relative to the uninhibited control current, I_0. Data were fit to the equation $I/I_0 = 1 - \{(1 - I_{sub}/I_0)/(1 + K_D/[BPTI])\}$, where K_D is the equilibrium dissociation constant for binding of BPTI, I is the current at a given concentration of BPTI, and I_{sub} is the current flowing when all channels are in the BPTI-induced substate. Best fit values for K_D and I_{sub}/I_0 were 4.4 μM and 0.16 at +40 mV and 27.7 μM and 0.39 at −40 mV, respectively. Each data point represents the average of two patches. (E) Macroscopic steady-state $I-V$ curve for control and BPTI-inhibited channels. The control Bslo $I-V$ curve (○) was recorded for a multichannel patch at 100 μM Ca^{2+}, and the BPTI-inhibited $I-V$ curve was recorded from the same patch exposed to 154 μM BPTI in the bath solution. The control $I-V$ curve is fit with an ohmic linear function, and the BPTI-inhibited $I-V$ curve has been fit to $I = I_{control}K(V)/\{K(V) + 1\}$, with $K(V) = K(0)\exp(-zFV/RT)$ as described in Moss and Moczydlowski (1996). Best fit values were $K(0) = 0.42 \pm 0.01$ and $z = 0.51 \pm 0.02$. R, T, and F have their usual meaning.

course of patch perfusion (dead time = 80 ms). Brief application of BPTI (0.5 mg/mL, 77 μM) in the presence of high Ca^{2+} results in rapid inhibition of channel activity. Returning the patch to the BPTI-free solution results in a slow time course corresponding to the kinetics of BPTI dissociation. The derived time constant for exponential recovery (e.g., Figure 2C) from BPTI inhibition in such patches is 2.4 ± 0.1 s (±SE, $n = 4$) at +40 mV. This value is very similar to the mean dwell time of BPTI-induced low-conductance events in native K_{Ca} channels previously recorded from bovine aortic smooth muscle cells under similar conditions (Moss & Moczydlowski, 1996) and corresponds to a dissociation rate constant of $k_{off} = 0.41\ s^{-1}$. These observations confirm that the cloned Bslo channel α-subunit contains a functional binding site for BPTI.

Results such as that in Figure 2A also allow determination of the bimolecular association rate constant, k_{on}, of BPTI binding to the Bslo channel according to the formula $k_p = [BPTI]k_{on} + k_{off}$, where k_p is the observed pseudo-first-order rate constant for BPTI inhibition. From exponential fits to the onset of inhibition (e.g., Figure 2B) and the derived value for k_{off}, we calculate $k_{on} \simeq (6.4 \pm 1.3) \times 10^4\ M^{-1}\ s^{-1}$ (±SE, $n = 4$). The equilibrium dissociation constant, K_D, for BPTI binding to the expressed Bslo channel can be estimated from the ratio of k_{off}/k_{on}, which gives a value of $K_D = 7.0 \pm 1.6$ μM. The equilibrium binding affinity of BPTI was also determined at +40 and −40 mV from titration of steady-state Bslo current as a function of BPTI concentration (Figure 2D). As expected from the mechanism of inhibition previously characterized in single-channel studies of maxi K_{Ca} channels in planar bilayers (Lucchesi & Moczydlowski, 1991; Moss & Moczydlowski, 1996), inhibition at the macroscopic level is well described by a simple saturation isotherm. The maximal extent of inhibition is incomplete and voltage dependent. The titration curves also indicate higher affinity for BPTI at positive voltage. Fitting the BPTI titration curves to an equation based on a one-site model with incomplete inhibition (see legend, Figure 2) gives a maximum level of ~84% inhibition at +40 mV and 62% at −40 mV. This difference in the maximal extent of inhibition at ±40 mV is expected from the known single-channel behavior, since the kinetics of rapid fluctuations characteristic of BPTI-bound K_{Ca} channels are voltage dependent and give rise to apparent inward inward rectification (Lucchesi & Moczydlowski, 1991; Moss & Moczydlowski, 1996). The derived K_D for BPTI is ~4.4 μM at +40 mV and ~27.7 μM at −40 mV. The K_D estimate at +40 mV from the steady-state titration is in reasonable agreement with the value of 7.0 ± 1.6 μM obtained from the ratio of reaction rates measured by rapid perfusion.

The apparent rectification that results from the voltage dependence of BPTI modulation is shown more directly in Figure 2E. A patch containing many Bslo channels exposed to high Ca^{2+} (100 μM) exhibits a nearly ohmic macroscopic $I-V$ curve over the range of ±50 mV, since this concentration of Ca^{2+} is sufficient to fully activate the channel in this voltage range. However, in the presence of saturating 1 mg/mL BPTI (154 μM), the residual current exhibits strong inward rectification.

Although the K_D that we derived for BPTI binding to the Bslo channel indicates much lower affinity than the BPTI interaction with trypsin ($K_D = 6 \times 10^{-14}$ M; Vincent & Lazdunski, 1972), it is similar in magnitude to that of the BPTI complex with trypsinogen ($K_D = 2 \times 10^{-6}$ M; Vincent & Lazdunski, 1976; Bode, 1979). BPTI is also known to specifically bind to various active and inactive homologs of the serine proteinase (SerP) family with wide variations in affinity (Vincent & Lazdunski, 1976; Antonini et al., 1983; Petersen et al., 1993). This led us to hypothesize that K_{Ca} channels may contain a BPTI-binding domain that is structurally homologous to the SerP fold. This hypothesis predicts that other classes of protein inhibitors of SerP's may

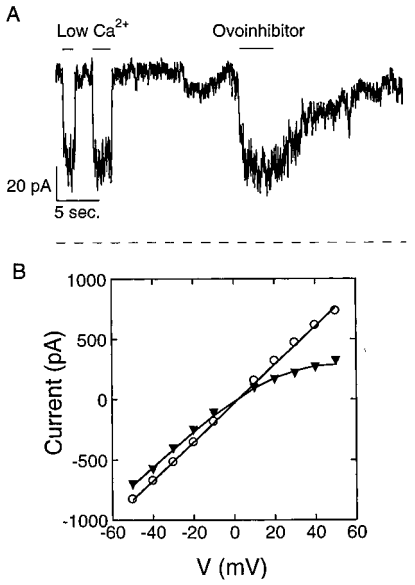

FIGURE 3: Inhibition of macroscopic Bslo current by ovoinhibitor (OI). (A) Effect of rapid perfusion (periods marked by solid lines) of low Ca^{2+} or OI on Bslo current recorded from a multichannel, inside-out patch at +40 mV. The protocol for this experiment was the same as described in Figure 2, with OI at a concentration of 1 mg/mL (22 μM). (B) Ohmic behavior of patch current in control conditions (m) is contrasted with the inwardly rectifying steady-state $I-V$ behavior (▼) in the presence of 1 mg/mL OI. The $I-V$ curve in the presence of OI has been fit with the same function given in the legend of Figure 2 using $K(0) = 2.0 \pm 0.1$ and $z = 0.57 \pm 0.04$.

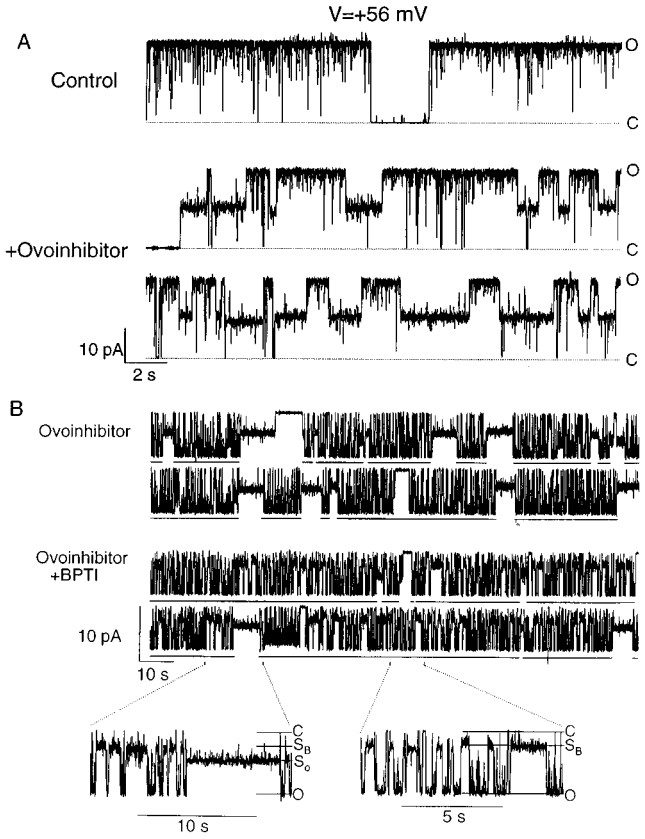

FIGURE 4: Production of discrete subconductance events by OI and apparent binding competition between OI and BPTI. (A) Representative behavior of a single rat muscle K_{Ca} channel recorded in planar bilayers before and after addition of 4.3 μM OI. The dashed line indicates the zero-current or closed level as marked by C; the open channel current level is marked by O. Bilayer bath solutions are described in Materials and Methods, and the holding voltage was +56 mV. (B) Test for binding competition between OI and BPTI. Bilayer solutions were 100 mM KCl, 200 μM $CaCl_2$, and 10 mM Mops−KOH, pH 7.2, on the cis side and 50 mM KCl, 0.1 mM EDTA, and 10 mM Mops−KOH, pH 7.2, on the trans side. The holding voltage was +20 mV. Channel opening is downward. The top two traces show a continuous record of a single K_{Ca} channel in the presence of 4.3 μM OI on the cis side. Durations of waiting times between adjacent OI substates at the major ~50% level have been marked with solid lines. The bottom two traces are a continuous record of the same channel after subsequent addition of 3.9 μM BPTI, showing an increase in the mean duration of waiting times between OI substates. The bottom two traces are expanded segments as indicated. The left-hand panel shows examples of two classes of substate levels: brief low-conductance substates (S_B) due to BPTI binding and a longer high-conductance substate (S_O) due to OI binding. The right-hand panel shows only BPTI-induced substates (S_B).

also inhibit maxi K_{Ca} channels, since many of these inhibitors recognize the same features of SerP structure (Bode & Huber, 1992).

To test this idea, we used Bslo channels expressed in HEK293 cells to screen several commercially available SerP inhibitors purified from diverse natural sources. Out of five proteinase inhibitors tested in this study the following three were inactive on both bovine and *Drosophila* K_{Ca} channels at 1 mg/mL inhibitor concentration: chicken ovomucoid inhibitor, soybean Bowman−Birk inhibitor, and soybean (Kunitz) inhibitor. However, in addition to BPTI, a protein found in chicken plasma and egg white called ovoinhibitor (OI) was also active. OI is a 449-residue polypeptide that contains seven tandemly linked Kazal domains (Scott et al., 1987). The Kazal domain is an ~60 residue motif with six conserved Cys residues that form three disulfide bonds (Read & James, 1986). The Kazal domain has a different tertiary fold than the Kunitz domain of BPTI but contains a very similar inhibitory loop that also recognizes the active site cleft of SerP's (Figure 8). Figure 3 shows the results of an experiment with a protocol similar to that of Figure 2A, but using OI instead of BPTI. Transient exposure of an inside-out patch containing Bslo channels to 1 mg/mL (23 μM) OI results in rapid inhibition. After exchange to a BPTI-free solution, there is a slow recovery phase with a time constant of ~4.1 s. This type of experiment provides estimates of the rate constants for OI binding and unbinding of $k_{on} \simeq (1.5 \pm 0.5) \times 10^5$ M^{-1} s^{-1} (±SE, $n = 4$) and $k_{off} \simeq 0.24 \pm 0.06$ s^{-1} (±SE, $n = 4$) at +40 mV, giving a K_D of 1.5 ± 0.5 μM. Figure 3B illustrates a typical effect of OI on the macrosopic steady-state Bslo $I-V$ curve taken with 100 μM Ca^{2+} on the intracellular side of the patch. The nearly ohmic control $I-V$ behavior is converted to an inwardly rectifying $I-V$ curve by the presence of 1 mg/mL internal OI. This rectification of the macroscopic current is qualitatively similar to that induced by BPTI, but the current is less strongly inhibited at any given voltage.

One explanation for the difference in the $I-V$ behavior in the presence of BPTI and OI would be that OI binding stabililizes a single-channel current that is larger and more weakly rectifying than that induced by BPTI. To examine this possibility, we studied the effect of OI on single K_{Ca} channels from native rat skeletal muscle channels inserted into planar bilayers. Figure 4A shows that OI does indeed induce discrete, BPTI-like "subconductance" events at a higher current level that those of BPTI at the same voltage (Moss & Moczydlowski, 1996). The average duration of the major type of low-conductance event at ~50% of the open channel current indicates a residence time for OI of

several seconds on the rat K_{Ca} channel, as would be expected from the off-rate of OI measured for Bslo channels (Figure 3A). Closer inspection of the record at a higher time resolution indicates that OI also induces another type of substate at a higher current level. Production of multiple classes of substates by OI may be expected from its multi-Kazal domain structure. Thus, despite their different tertiary structures, BPTI and OI both inhibit maxi K_{Ca} channels by the same fundamental mechanism: production of discrete, rectifying low-conductance events.

It is possible that these two inhibitors could exhibit a similar mechanism of action if the proteinase contact loop in at least one of the seven Kazal domains of OI recognizes the same site on the K_{Ca} channel as the analogous inhibitory loop of BPTI. To test this idea, experiments were performed to determine whether there is binding competition between OI and BPTI at the single-channel level. [A similar experimental paradigm has previously been used to establish binding competition between two different ligands in other channel families, e.g., Miller (1988) and Schild and Moczydlowski (1991).] Results from this type of experiment are shown in Figure 4B. The first two traces show a continuous record of a rat skeletal muscle channel incorporated into a planar bilayer, with 4.3 μM OI on the intracellular side. Frequent OI-induced events of low apparent conductance are observed. The mean duration of dwell times of "normal" channel behavior between adjacent OI events (at the major 40% sublevel) was measured to be 12.2 $\pm$ 1.3 s ($\pm$SE, n = 77 events), and the mean duration of OI events in this major sublevel was 5.7 $\pm$ 0.8 s. After addition of 3.9 μM BPTI, a new class of low-conductance events were induced, which are typical of BPTI. In Figure 4B (expanded trace, lower right) this BPTI sublevel is marked S_B, while the major OI sublevel is marked S_O (Figure 4B, lower left expanded trace). Three points are clear from this experiment. First, the average duration of dwell times between the OI-induced events is greatly increased by the addition of BPTI: mean duration = 41.8 $\pm$ 6.3 s (n = 53) after BPTI vs 12.2 $\pm$ 1.3 s before BPTI. The individual durations between OI substate events in Figure 4B have been marked with solid lines to illustrate the lengthening after BPTI addition. Second, the average duration of the OI-induced events remains unchanged (5.6 $\pm$ 0.6 s after BPTI vs 5.7 $\pm$ 0.8 s before BPTI). Third, close inspection of long-lived OI-induced low-conductance events reveals that BPTI-sublevel events are not observed within these OI-induced periods after addition of BPTI (expanded trace of Figure 4B, lower left panel). These three results are consistent with mutually exclusive binding competition between OI and BPTI. The durations between OI substates correspond to the waiting time for an unoccupied channel to become blocked by OI. Frequent binding and unbinding of BPTI during these periods would be expected to lengthen these intervals if the two ligands cannot bind simultaneously (Moczydlowski, 1992). The results of Figure 4B thus demonstrate that OI inhibits the Bslo channel by a mechanism similar to that of BPTI and that OI and BPTI bind competitively to maxi K_{Ca} channels.

Effect of BPTI and Ovoinhibitor on a Drosophila K_{Ca} Channel. To examine whether a functional binding site for SerP inhibitors is also present in K_{Ca} channels from an invertebrate species, we examined the sensitivity to BPTI and OI of the previously cloned *Drosophila* K_{Ca} channel (Atkinson et al., 1991; Adelman et al., 1992). Figure 5

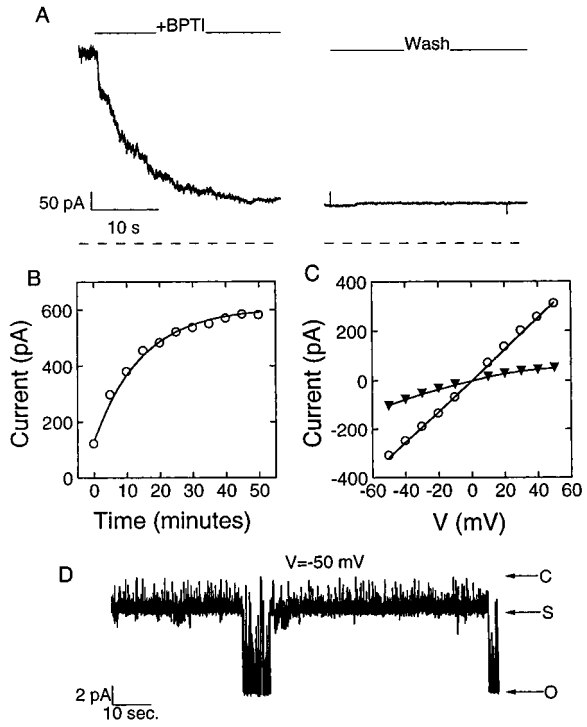

FIGURE 5: Effect of BPTI on Dslo K_{Ca} channels at the macroscopic and single-channel level. (A) Current record from a multichannel, inside-out patch containing Dslo K_{Ca} channels first perfused with bath solution containing BPTI (5 mg/mL) and then with the same solution minus BPTI (labeled wash). The holding voltage was +40 mV. (B) Slow time course of recovery of Dslo current following inhibition by BPTI and washout. The same patch shown in (A) was held at 0 mV after BPTI inhibition reached equilibrium. Recovery after perfusion with BPTI-free solution was assessed by briefly pulsing to +40 mV once every 5 min. The data (○) are fit to a single exponential assuming complete recovery with a time constant of 14.5 min. (C) Macroscopic $I-V$ relation of Dslo before (○) and after (▼) exposure to 5 mg/mL BPTI. $I-V$ data after BPTI inhibition have been fit to the equation given in the legend of Figure 2 with $K(0) = 0.31 \pm 0.01$ and $z = 0.23 \pm 0.01$. (D) Example of long-lived subconductance events exhibited by a single Dslo K_{Ca} channel recorded in a patch pipet at −50 mV in the presence of BPTI. Open, closed, and substate levels are indicated by O, C, and S, respectively.

shows results of an experiment on Dslo channels expressed in HEK293 cells which is analogous to the experiment with Bslo in Figure 2. An inside-out patch containing many Dslo channels was clamped at +40 mV in high-Ca^{2+} (100 μM) solution. When the patch was moved to a perfusion tube containing 5 mg/mL BPTI, slowly developing inhibition of Dslo K^+ current was observed (Figure 5A). Reversal of this inhibition did not occur rapidly when the patch was moved back to the BTPI-free, control solution as previously described for Bslo (Figure 2A). However, recovery of Dslo from BPTI inhibition developed slowly when the patch was kept in control solution and assayed by periodic voltage pulses from 0 to +40 mV (Figure 5B). The slow time course of recovery in Figure 5B is fit by an exponential time course with a time constant of 14.5 min. From this and similar experiments, the mean time constant for BPTI dissociation from Dslo channels is 18.5 $\pm$ 2.5 min ($\pm$SE, n = 5, measured at 0 mV; see Materials and Methods). This time constant corresponds to a rate constant of $k_{off} = (9.0 \pm 1.2) \times 10^{-4}$ s^{-1}, which is approximately 400-fold slower than that of Bslo channels. In comparison to the behavior of Bslo in Figure 2A, the association rate of BPTI with Dslo channels is also

exceedingly slow. The bimolecular association rate constant for Dslo estimated from BPTI perfusion experiments is (3.5 ± 1.3) × 10^2 M^{-1} s^{-1}, which is about 5 orders of magnitude slower than that of a typical channel−toxin interaction [e.g., Escobar et al. (1993)]. Despite the dramatic difference in kinetics, the combination of a slow association rate and a slow dissociation rate leads to a rather similar equilibrium $K_D \simeq 2.6 \pm 1.0$ μM for BPTI binding to Dslo, as compared to Bslo ($K_D = 7.0$ μM).

Given the kinetic difference, it was important to ascertain whether BPTI inhibition of Dslo channels occurs by the same type of substate mechanism observed for mammalian K_{Ca} channels. To investigate this question, single Dslo K_{Ca} channels expressed in HEK293 cells were recorded from excised inside-out patches in the presence of BPTI as shown in Figure 5D. These recordings demonstrated the presence of very long-lived subconductance events which are not observed in the absence of BPTI. An example of one complete, but unusually "brief" (∼1 min) BPTI-induced substate event is shown in Figure 5D, along with part of a second substate event. This observation confirms that BPTI inhibits Dslo channels by the same basic mechanism as that of Blso channels—by inducing discrete subconductance events. However, unitary BPTI-induced subconductance events for Dslo channels last for many minutes vs a few seconds for Bslo channels, consistent with the different macroscopic time course of recovery from BPTI inhibition. In Figure 5C, a multichannel $I-V$ curve for Dslo is compared before and after inhibition by BPTI. The effect of BPTI is to convert the near-ohmic control $I-V$ curve into an inwardly rectifying $I-V$ relation, similar to that described for Bslo (Figure 2C). However, BPTI induces a relatively lower substate current level in Dslo as reflected in the greater inhibition of macroscopic K^+ current, particularly at negative voltages (Figure 5C vs Figure 2E).

The results of Figure 5 lead to the conclusion that the BPTI-binding site is also present in the α-subunit of Dslo K_{Ca} channels but exhibits slower kinetics for the insect vs mammalian channel. Such kinetic differences, particularly the slow rate of ligand dissociation, may arise from stronger molecular interactions between BPTI and the *Drosophila* channel. However, the binding free energy as reflected by the equilbrium K_D for BPTI is similar for Bslo and Dslo because of the corresponding difference in association rate. Thus, the extremely slow reaction rates of BPTI with the Dslo protein appear to arise from the difficulty of reaching the transition state for BPTI binding/unbinding. This may occur when the protein conformation underlying this state is only rarely achieved (i.e., energetically unfavorable). It is interesting to note that the association rate of BTPI with trypsinogen (∼2 × 10^4 M^{-1} s^{-1}) is much slower than that of trypsin (∼1.1 × 10^6 M^{-1} s^{-1}) (Vincent & Lazdunski, 1976) and that trypsinogen undergoes a large conformational change upon binding of BPTI (Bode, 1979). It is tempting to suggest that the interaction of BPTI with the K_{Ca} channel is rather like that with trypsinogen, where a competent binding site is not preformed and is present only after the channel protein undergoes a significant alteration in structure.

The finding that the Dslo channel is sensitive to BPTI implies that it may also be sensitive to other proteinase inhibitors. Figure 6A shows typical results of an experiment in which a patch containing many Dslo channels was exposed to OI. The fast responses to solution changes at the

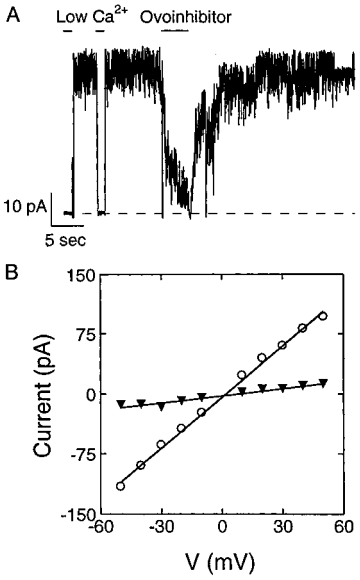

FIGURE 6: Inhibition of macroscopic Dslo current by OI. (A) Effect of rapid perfusion (marked by solid lines) of low Ca^{2+} (0.2 μM) and OI (1 mg/mL) on Dslo current recorded from a multichannel, inside-out patch at +40 mV. (B) $I-V$ data from one patch containing Dslo channels taken in the absence (○) and presence (▼) of 1 mg/ ml OI. Both sets of $I-V$ data are fit with a linear function.

intracellular face of the patch between high (100 μM) and low (∼0.2 μM) Ca^{2+} solution demonstrate the Ca^{2+} dependence of the expressed channels and the rapid time course of the perfusion system. Exposure to a brief pulse of solution containing 1 mg/mL OI resulted in rapid current inhibition, with recovery occurring within a few seconds after return to control solution. Kinetics analysis of this interaction yields values of $k_{on} \simeq (4.2 \pm 1.1) \times 10^4$ M^{-1} s^{-1} (±SE, $n = 7$) and $k_{off} \simeq 0.49 \pm 0.16$ s^{-1} (±SE, $n = 7$), corresponding to a K_D of 11.4 ± 4.8 μM. Figure 6B shows the effect of OI on the macroscopic $I-V$ relation of Dslo channels. The OI-inhibited $I-V$ curve is not rectifying (in contrast to the corresponding Bslo $I-V$ curve of Figure 3B) and appears to reflect a complete or nearly complete block of Dslo channel current in the range ±50 mV. This is not entirely unexpected because the production of different levels of substate current with varying rectification behavior by different protein ligands seems to be a hallmark feature of the structure−activity behavior of this channel−ligand interaction (Moczydlowski et al., 1992; Moss & Moczydlowski, 1996). The ability of OI to inhibit Dslo current is nevertheless consistent with the possibility that a proteinase inhibitor binding site also exists on the Dslo channel.

Since OI and BPTI have completely different structures, except in the conformation of the respective proteinase contact loops (Figure 8), we reasoned that this inhibitory loop may be the basis of their competitive binding to maxi K_{Ca} channels. This leads to the clear prediction that blockage or shielding of the proteinase contact loop should abolish the interaction with the K_{Ca} channel. One way to shield access to the inhibitory loop is to take advantage of the virtually irreversible binding of trypsin and BPTI ($K_D = 6 \times 10^{-14}$ M). In the trypsin−BPTI complex, 80% of the surface area of the BPTI molecule is exposed to solvent (Janin & Chothia, 1990), and the remaining 20%, including the inhibitory loop, forms a shielded interface with trypsin. If trypsin and the channel bind to the same loop region of

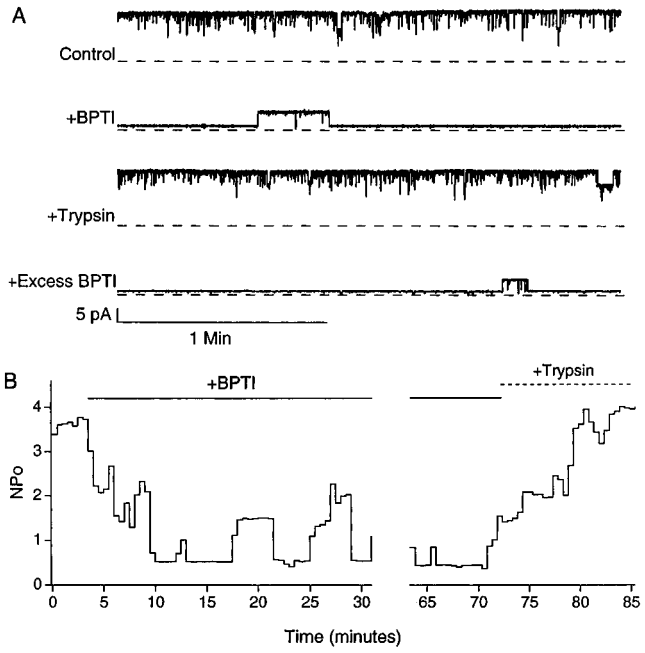

FIGURE 7: Demonstration that Dslo K_{Ca} channels are protected from BPTI inhibition by formation of the trypsin−BPTI complex. (A) Representative segments of a planar bilayer recording of four Dslo channels at selected periods after consecutive addition of BPTI, trypsin, and BPTI. Dslo channels were incorporated into a planar bilayer from membrane vesicles of stably transfected HEK293 cells. Bilayer solutions are described in Materials and Methods, and voltage was held constant at +40 mV. The zero current level is indicated with a dashed line. The top trace (control) shows activity of four K_{Ca} channels before addition of BPTI. The second trace taken after addition of 0.25 mg/mL BPTI (39 μM) to the intracellular side shows a period of nearly complete inhibition of channel activity. The third trace taken after addition of trypsin (42 μM) shows complete reversal of BPTI inhibition. The fourth trace taken after a second addition of excess BPTI (116 μM total) shows that the protective effect of trypsin is reversible. (B) NP_O representation of Dslo channel activity versus time. A segment of the bilayer experiment described in (A) is shown as a continuous record of NP_O (opening probability, P_O, times the number of channels, N) measured at consecutive intervals of 30 s. After the first 3.5 min of the control period, 39 μM BPTI was added to the intracellular side and channel activity was recorded for ~60 min (period marked by a solid line). At a time commencing with the dotted line, 42 μM trypsin was added. A period of low channel activity from 31 to 63 min is deleted to emphasize the slow time course of BPTI inhibition and its reversal by trypsin.

BPTI, then the trypsin−BPTI complex should be inactive. However, if the channel protein binds to different regions of the BPTI molecule, then the BPTI−trypsin complex could still be an active inhibitor. Figure 7 shows the results of such an experiment carried out on a planar bilayer containing four Dslo channels oriented in the same direction. In this experiment, BPTI inhibition of Dslo channels was reversed by "chelating" free BPTI with the trypsin. After addition of BPTI to the internal side of the bilayer, slow inhibition of the four channels gradually developed, reflecting the slow association rate for BPTI binding (Figure 7B). Bilayer recordings as well as patch recordings of Dslo channels confirm that individual BPTI-induced subconductance events last for many minutes as expected from the slow macroscopic dissociation rate of BPTI (Figure 6B). Addition of trypsin in excess of BPTI to the four-channel bilayer resulted in slow recovery of activity (Figure 7B) until BPTI inhibition of all four channels was completely reversed (Figure 7A, third trace). To test whether the trypsin effect may be due to irreversible proteolysis of the channel, another aliquot of BPTI was added to the bilayer chamber after trypsin reversal. In many experiments of this type, we found that trypsin-treated K_{Ca} channels are still sensitive to inhibition by excess free BPTI (Figure 7A, bottom trace), showing that trypsin is acting primarily as a chelator of an inhibitory site on BPTI and not via channel proteolysis. Analogous trypsin reversal experiments with the rat muscle K_{Ca} channels gave similar results (Moss et al., 1996).

Thus our results with BPTI, OI, and the BPTI−trypsin complex (using the cloned Bslo and Dslo α-subunit as an assay system) can be interpreted in the context of a simple model. The inhibitory activity of OI and BPTI may be explained by assuming that their proteinase contact regions are also responsible for K_{Ca} channel interactions. This is consistent with the competitive interaction of OI and BPTI, the fact that both inhibitors mediate the production of discrete apparent substates, and the finding that the BPTI−trypsin complex is inactive when applied to the channels. Taken together, these results are most economically explained by the hypothesis that the α-subunit of maxi K_{Ca} channels contains a serine proteinase-like domain that recognizes and binds the inhibitory loops of various naturally occurring proteinase inhibitors.

DISCUSSION

Serine Proteinase-like Pharmacology of Cloned Maxi K_{Ca} Channels. This work represents the first step in an effort to identify the molecular location of the intracellular-facing binding site that mediates substate-inducing activity of certain proteinase inhibitors on maxi K_{Ca} channels. Such activity was first described for the effect of a homolog of the dendrotoxin family of mamba snake neurotoxins known as toxin I (Lucchesi & Moczydlowski, 1990). Dendrotoxins are small protein neurotoxins that block various K_V channels by binding to the extracellular P-region (Dufton, 1985; Hurst et al., 1991). They are also structurally homologous to the Kunitz trypsin-inhibitor domain exemplified by BPTI (Skarzynski, 1992). Recently, a family of sea anemone proteins homologous to dendrotoxins and Kunitz inhibitors called kalicludines have been described that also display inhibitory activity against K_V channels and trypsin (Schweitz et al., 1995). Thus, Kunitz-type proteins have acquired dual functional activities, targeting K^+ channels and/or SerP's. These seemingly incongruous observations converge in the phenomenon investigated here, where maxi K_{Ca} channels exhibit intimations of SerP-like pharmacology at an intracellular site associated with the channel-forming subunit.

The results in this paper support the conclusion that the site mediating the substate effect of BPTI and related inhibitors is located directly on the K_{Ca} channel α-subunit. This is inferred by the fact that the cloned α-subunit of mammalian Bslo or *Drosophila* Dslo K_{Ca} channels expressed in HEK293 cells exhibit similar sensitivity to BPTI as observed for K_{Ca} channels from native tissues incorporated in planar bilayers or from intact cultured smooth muscle cells (Moss & Moczydlowski, 1996). It might still be argued that an accessory subunit fortuitously present in HEK293 cells mediates the BPTI effect, but this is unlikely since the kinetics of the BPTI−channel interaction specifically differs for the mammalian vs insect α-subunit (in both native cells and after bilayer reconstitution). A small 191-residue

β-subunit has been identified that greatly enhances the sensitivity of mammalian K_{Ca} channels to activation by Ca^{2+} (Knaus, 1994; McManus et al., 1995; Wallner et al., 1995). This β-subunit has also been found to be required for activation of mammalian maxi K_{Ca} channels by dehydrosoyasaponin I, a triterpenoid glycoside natural product (McManus et al., 1993, 1995). However, this mammalian β-subunit is incapable of modulating the gating of the *Drosophila* K_{Ca} channel (Meera et al., 1996). Also, BPTI readily induces substates when applied to native channels from skeletal muscle, a tissue that does not appear to express high levels of the β-subunit (Folander & Swanson, 1995). Thus, sensitivity to BPTI and the kinetics of the BPTI−channel interaction correlate with the species of α-subunit rather than with the expression of the modulatory β-subunit, implying that the former protein most likely contains the proteinase inhibitor binding site.

The finding that BPTI and OI act on both mammalian and *Drosophila* K_{Ca} channels indicates that this site has been conserved through evolution. Since Dslo shares only ~60% sequence identity with Bslo, this result is not necessarily expected. In contrast, other studies have shown that the external binding site that mediates K_{Ca} channel block by the scorpion toxin, charybdotoxin, is not conserved across all species (Adelman et al., 1992). Charybdotoxin is a potent and rather specific blocker of mammalian maxi K_{Ca} channels and certain K_V channels (Miller et al., 1985; Miller, 1995). However, 1 μM charybdotoxin was found to have little effect on Dslo currents expressed in *Xenopus* oocytes (Adelman et al., 1992), while this concentration is known to completely block most mammalian maxi K_{Ca} channels. These findings imply that there is stronger evolutionary pressure to maintain the integrity of the internal Kunitz inhibitor binding site than the external binding site for charybdotoxin on maxi K_{Ca} channels. If a more extensive species survey confirms that a serine proteinase-like pharmacology is in fact a broadly conserved feature of this particular class of ion channels, this would suggest that the binding site mediating this effect has an important functional role.

The significance of BPTI binding to the maxi K_{Ca} channel might be questioned because of the low affinity of this interaction relative to trypsin. The K_D of BPTI measured here for the Bslo K_{Ca} channel (4.4−7.1 μM, +40 mV) is about 8 orders of magnitude higher than that of BPTI binding to trypsin but is comparable to that reported for BPTI binding to other catalytically inactive SerP homologs such as trypsinogen and human heparin-binding protein/azurocidin (Vincent & Lazdunski, 1976; Petersen et al., 1993). Weak BPTI binding to trypsinogen, the zymogen precursor of trypsin, induces an allosteric change resulting in an active conformation of the zymogen-activation pocket (Bode, 1979). This is interesting in the present context because BPTI binding to maxi K_{Ca} channels appears to exert its substate-inducing effect via relatively weak binding and an allosteric change that alters the equilbrium of fast fluctuations of the channel pore (Moss & Moczydlowski, 1996). Thus, known aspects of BPTI interactions with inactive SerP homologs leave open the possibility that the BPTI-binding site on the K_{Ca} channel may be structurally and functionally analogous to interactions with this class of proteins.

Such considerations have led us to hypothesize that maxi K_{Ca} channels contain a SerP-like domain (Moss et al., 1996). This hypothesis predicts that other members of the numerous families of naturally occurring proteinase inhibitors (Laskowski & Kato, 1980) may also recognize a putative SerP domain on the channel. This was tested in the present work by screening examples of several different classes of these inhibitors, which led to the finding of the inhibitory activity of chicken OI on Bslo and Dslo. The structure−activity relationships of proteinase inhibitors are complex and depend on numerous specific molecular interactions between the proteinase and the contact region of the inhibitor. For example, homologous ovomucoid inhibitor proteins from different species of birds exhibit a wide spectrum of activity against various members of the SerP family of enzymes. Some of these inhibitors exhibit affinities on the order of 1 nM, while others have affinities too weak to measure for a given proteinase (Empie & Laskowski, 1982; Laskowski et al., 1987). Thus, the observation that some examples of SerP inhibitors are inactive (chicken ovomucoid inhibitor, soybean Bowman−Birk inhibitor, soybean Kunitz inhibitor) and some are active on K_{Ca} channels (BPTI, OI) may be construed as a pattern reminiscent of SerP pharmacology. The fact that some of these inhibitors are inactive reinforces the notion that this particular channel−ligand interaction is chemically specific, since not all types of proteinase inhibitors are capable of inducing the characteristic "subconductance" behavior.

Numerous crystallographic studies have shown that protein−inhibitor interactions with SerP enzymes share the unifying principle of structural complementarity between the contact surface of the inhibitor and the proteinase (Read & James, 1986; Bode & Huber, 1992). OI is a seven-headed member of the Kazal inhibitor family. The seven homologous Kazal domains of OI exhibit different specificities for serine proteases (Tomimatsu et al., 1966; Scott et al., 1987). Although the crystal structure of OI has not been determined, structures of other related Kazal domain proteins reveal a striking similarity in their inhibitory site region with the corresponding region of Kunitz inhibitors such as BPTI (e.g., Figure 8). Both classes of inhibitors contain a protruding loop of ~6 residues that fits into the substrate recognition cleft of SerP's. Although residues in the inhibitory loop differ considerably from inhibitor to inhibitor (Reed & James, 1986; Laskowski et al., 1987), the peptide backbone of this region has a common canonical conformation (Bode & Huber, 1992). This is illustrated in Figure 8 by structural superposition of the inhibitory loop of BPTI with the Kazal domain of turkey ovomucoid third domain as first demonstrated by Reed and James (1986). In Figure 8, the peptide backbone of six residues (usually denoted P3-P2-P1-P1′-P2′-P3′) surrounding the P1−P1′ scissile peptide bond of both proteins is superimposed, showing the similar fold in this inhibitory site region contrasted with lack of similarity in the rest of the protein "scaffold". This key feature of structural similarity between Kunitz and Kazal inhibitor domains provides a natural explanation for the results in this paper, that BPTI and OI inhibit K_{Ca} channels by the same mechanism with competitive binding.

As previously discussed for the rat muscle K_{Ca} channel (Moss et al., 1996), the experiments with Dslo channels incorporated into planar bilayers demonstrate that BPTI−channel interactions are prevented by complexation of BPTI by trypsin (Figure 7). Since most of the surface of BPTI is exposed in the BPTI−trypsin complex (Janin & Chothia,

FIGURE 8: Structural similarity of the inhibitory loop regions of two different serine proteinase inhibitors. Crystal structures of the Kunitz domain of BPTI in red (Wlodawer et al., 1987) and the Kazal domain of turkey ovomucoid third domain in green (Fujunaga et al., 1987) taken from the Protein Data Bank (5pti.pdb, 1cho.pdb) have been superimposed in the region of their known proteinase contact loops shown with side chains in stick representation (Pro13 to Ile18 of BPTI; Cys16 to Arg1 of the ovomucoid), while the rest of each protein, the scaffold, is shown in ribbon representation. Insight II software from Biosym was used for superpositioning and molecular graphics.

1990), this result also implicates the inhibitory loop of BPTI as the site of the interaction with the K_{Ca} channel. Thus on the basis of several independent lines of experimental evidence presented here, we infer that the maxi K_{Ca} channel α-subunit is likely to contain an intracellular domain that structurally resembles a SerP and that this domain mediates interactions with SerP inhibitors that functionally result in subconductance behavior. In Figure 1 we have boxed a region of ∼250 residues in Bslo and Dslo that we believe to be the most likely candidate region for this domain as based on a comprehensive sequence analysis that is described in detail in another paper (Moss et al., 1996).

Implications. Regarding the possible function of a SerP-like domain in maxi K_{Ca} channels, it is interesting to consider known examples of calcium regulation in the SerP family. Many members of the trypsin−chymotrypsin family contain a calcium binding site which is important in maintaining the active enzyme conformation and regulating protein−protein interactions (Bode & Schwager, 1975; Chiancone et al., 1985). For example, binding of Ca^{2+} to a site on the SerP domain of coagulation factor VIIa has been found to change the K_D for the accessory ligand, tissue factor, by ∼150 000-fold (Sabharwal et al., 1995; Banner et al., 1996). One might imagine that this allosteric effect of Ca^{2+} on the activity of factor VIIa could be analogous to the mechanism by which Ca^{2+} activates the K_{Ca} channel. Since our sequence analysis suggests that Bslo and Dslo channels contain a Ca^{2+}-binding loop related to that found in SerP's (Moss et al., 1996), it is intriguing to speculate that there may be a mechanistic correspondence between Ca^{2+} regulation in SerP's and K_{Ca} channels.

In recent years numerous protein inhibitors have been isolated from various natural sources which have been useful probes of the structure of K^+ channels (Miller, 1995; Swartz & MacKinnon, 1995). In view of the difficulty in obtaining crystallographic information on membrane proteins, a dual mutagenesis approach using toxin proteins and cloned K^+ channels has been widely exploited to obtain indirect information on the structure of K^+ channel domains such as the extracellular pore region. In this paper we have taken the first steps to define the structural determinants underlying the BPTI−maxi K_{Ca} channel interaction. Assuming that our inference regarding a SerP-like domain is correct, a large portion of the K_{Ca} channel protein (∼200−250 residues) would be expected to adopt the characteristic tertiary fold of SerP enzymes. Given the wealth of crystallographic information on SerP's and SerP−inhibitor complexes, definitive identification of this region may be expected to provide a significant advance in understanding the structure and function of K_{Ca} channels.

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