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Synthetic Charybdotoxin–Iberiotoxin Chimeric Peptides Define Toxin Binding Sites on Calcium-Activated and Voltage-Dependent Potassium Channels

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ABSTRACT: Charybdotoxin (ChTX) and iberiotoxin (IbTX) are highly charged peptidyl toxins which exhibit 68% sequence identity and share a similar three-dimensional structure. Despite these structural similarities, IbTX and ChTX differ in their selectivity for two types of potassium channels; large conductance calcium-activated potassium (maxi-K) channels and slowly inactivating voltage-gated ($Kv_{1.3}$) potassium channels. ChTX blocks with high affinity both maxi-K and $Kv_{1.3}$ channels, while IbTX blocks the maxi-K but not the voltage-gated channel. To identify regions of the toxins which impart this selectivity, we have constructed by solid-phase synthesis two chimeric toxins, $ChTX_{1-19}IbTX_{20-37}$ (Ch–IbTX) and $IbTX_{1-19}ChTX_{20-37}$ (Ib–ChTX), as well as a truncated peptide, $ChTX_{7-37}$. These peptides were assayed for their ability to inhibit [^{125}I]ChTX binding in sarcolemmal vesicles from smooth muscle (maxi-K binding) and [^{125}I]ChTX binding to plasma membranes from brain ($Kv_{1.3}$ binding). The ability of the peptides to block the maxi-K channel was determined from recordings of single maxi-K channels incoporated into planar lipid bilayers. Block of $Kv_{1.3}$ was determined from recordings of whole cell currents in *Xenopus* oocytes injected with mRNA encoding the cloned $Kv_{1.3}$ channel. Both chimeric toxins inhibited [^{125}I]ChTX binding to sarcolemmal membranes from smooth muscle, and they both blocked the maxi-K channel in planar lipid bilayers. In contrast, [^{125}I]ChTX binding in brain and $Kv_{1.3}$ currents expressed in oocytes were inhibited only by the chimera Ib-ChTX. Deletion of the first six amino acids from the N-terminus ($ChTX_{7-37}$) significantly weakened the interaction of ChTX with the maxi-K channel but had little effect on toxin interaction with $Kv_{1.3}$. These findings suggest that the C-terminal domain of the ChTX homologues defines the toxin–channel interaction which distinguishes between the maxi-K channel and $Kv_{1.3}$. In contrast, the N-terminal domain of ChTX imparts of high-affinity interaction with the maxi-K channel but not with $Kv_{1.3}$.

Charybdotoxin (ChTX)[1] is a highly basic peptide that was discovered in venom of the scorpion *Leiurus quinquestriatus* var. *hebraeus* and characterized as an inhibitor of a large conductance Ca^{2+}-activated K^+ (maxi-K) channel (Miller et al., 1985). Its amino acid sequence has been elucidated (Gimenez-Gallego et al., 1988; Luchesi et al., 1989; Strong et al., 1989; Schweitz et al., 1989) and confirmed by synthesis (Lambert et al., 1990; Sugg et al., 1990). The three-dimensional solution structure of ChTX has also been determined (Bontems et al., 1991). Iberiotoxin (IbTX), isolated from venom of the scorpion *Buthus tamulus*, shares 68% sequence identity (Galvez et al., 1990) and a similar three-dimensional structure (Johnson & Sugg, 1992) with ChTX. ChTX (Anderson et al., 1988; MacKinnon & Miller, 1988; Miller, 1988) and IbTX (Candia et al., 1992; Giangiacomo et al., 1992a) both block current through the maxi-K channel by binding to a site in the external "mouth" of the channel, thereby occluding passage of ions through the pore. Binding of ChTX (MacKinnon & Miller, 1988, 1989; MacKinnon et al., 1989; Vazquez et al., 1989) and IbTX (Candia et al., 1992; Giangiacomo et al., 1992a) to the maxi-K channel is dominated by electrostatic interactions between negatively charged residues near the mouth of the channel and positively charged residues on the toxins. In addition, ChTX and IbTX can completely inhibit [^{125}I]ChTX binding to maxi-K channels in smooth muscle (Vazquez et al., 1989; Galvez et al., 1990). Thus, ChTX and IbTX appear to share similar domains of interaction with the maxi-K channel.

Despite their similar structures and mechanisms of block of the maxi-K channel, ChTX and IbTX display remarkable differences in their ability to block voltage-gated potassium channels. These differences in channel selectivity are evident from electrophysiological studies and from radioligand binding studies with [^{125}I]ChTX. ChTX blocks a potassium channel clone from brain mRNA, $Kv_{1.3}$, that produces a slowly inactivating current when heterologously expressed in *Xenopus* oocytes (Swanson et al., 1990). $Kv_{1.3}$ is also expressed in T lymphocytes (Grissmer et al., 1990), and potassium currents in human T lymphocytes (Price et al., 1989; Lewis & Cahalan, 1988) and rat neurons (Schweitz, et al., 1989) that have biophysical and pharmacological properties similar to $Kv_{1.3}$ are blocked by ChTX (Sands et al., 1989). $Kv_{1.3}$ is the only voltage-gated channel shown to date to be sensitive to nanomolar concentrations of highly purified or synthetic ChTX, although other members of the *Shaker* family of potassium channels are sensitive to other peptidyl components

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[1] Abbreviations: ChTX, charybdotoxin; IbTX, iberiotoxin, maxi-K channel, large-conductance calcium-activated potassium channel; $Kv_{1.3}$, slowly inactivating voltage-gated potassium channel; Ch–IbTX, $ChTX_{1-19}IbTX_{20-37}$; Ib–ChTX, $IbTX_{1-19}ChTX_{20-37}$; [^{125}I]ChTX, monoiodotyrosine charybdotoxin; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; BSA, bovine serum albumin; TFA, trifluoroacetic acid; TEA, tetraethylammonium ion; LbTX, limbatotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; FMOC, fluorenylmethyloxycarbonyl.

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derived from *Leiurus* venom (unpublished observations; J. Smith and R. MacKinnon, personal communications). Despite sequence homology with ChTX, IbTX does not block $Kv_{1.3}$ in human T lymphocytes (Deutsch et al., 1991; Leonard et al., 1992). Binding studies with [^{125}I]ChTX have revealed a single class of sites in rat brain (Vazquez et al., 1990) and human T lymphocytes (Deutsch et al., 1991) that is completely inhibitable by other peptidyl inhibitors of voltage-dependent K^+ channels such as noxiustoxin and α-dendrotoxin but is insensitive to IbTX. This pharmacological profile is consistent with that of the $Kv_{1.3}$ currents found in these tissues. This suggests that the conditions used to monitor binding of [^{125}I]-ChTX in rat brain represent binding to voltage-gated channels and not to the IbTX-sensitive maxi-K channels which are also known to exist in brain.

In order to identify regions of ChTX and IbTX which impart selectivity for the maxi-K and $Kv_{1.3}$ channels, we used solid-phase techniques previously employed with ChTX to synthesize biologically active IbTX, two chimeric toxins, Ch-$TX_{1-19}IbTX_{20-37}$ (Ch–IbTX) and $IbTX_{1-19}ChTX_{20-37}$ (Ib–ChTX), and a truncated form of ChTX, $ChTX_{7-37}$. These peptides were assayed in both binding and electrophysiological experiments. Interaction with the maxi-K channel was evaluated in protocols monitoring binding of [^{125}I]ChTX to bovine aortic smooth muscle membranes and through recordings of single maxi-K channels incorporated into planar lipid bilayers. Interaction with the $Kv_{1.3}$ channel was evaluated by [^{125}I]ChTX binding to rat brain synaptic plasma membranes and through whole cell current recordings from $Kv_{1.3}$ expressed in *Xenopus* oocytes. These studies reveal that toxin selectivity for $Kv_{1.3}$ relative to the maxi-K channel is defined by the carboxy-terminal residues 20–37. Since only six of these C-terminal amino acids differ between ChTX and IbTX, the regions of receptor selectivity must be limited to these residues. In addition, these studies reveal that the N-terminal residues 1–6 of ChTX impart high-affinity binding to the maxi-K channel but not to the $Kv_{1.3}$ channel. A preliminary report of these findings has been made in abstract form (Garcia et al., 1990; Giangiacomo et al., 1992b).

MATERIALS AND METHODS

Materials. Lyophilized venoms from the scorpions *Leiurus quinquestriatus hebraeus* and *Buthus tamulus* were obtained from Alomone Laboratories, Jerusalem, Israel, and Sigma Chemical Co., St. Louis, respectively. Native ChTX and IbTX were isolated from these respective venoms as previously described (Gimenez-Gallego et al., 1988; Galvez et al., 1990). N^{α}-FMOC-Ser(*O-tert*-butyl)-Pepsyn KA and N^{α}-FMOC-Gln-Pepsyn KA resins were purchased from Milligen, Bedford, MA. N^{α}-FMOC-amino acid pentafluorophenyl esters were obtained from Milligen and Bachem AG, Philadelphia, PA. GF/C glass fiber filters were purchased from Whatman. 1-Palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were purchased from Avanti Lipids, Inc., Birmingham, AL. Decane from Fisher Scientific, Inc., Springfield, NJ, was 99.9% mol purity. Bovine serum albumin (BSA), fraction grade V, was from Sigma Chemical Co. All other reagents were obtained from commercial sources and were of the highest purity commercially available.

Synthesis of Peptides. IbTX, the two chimeric analogs, and $ChTX_{7-37}$ were assembled by solid-phase peptide synthesis on a Milligen 9050 peptide synthesizer using N^{α}-FMOC-Ser(*O-tert*-butyl)-Pepsyn KA resin or N^{α}-FMOC-Gln-Pepsyn KA resins. N^{α}-FMOC-amino acid pentafluorophenyl esters were coupled in 4-fold or 6-fold excess in *N*-methylpyrrolidinone (Burdick and Jackson). All six cysteines were *S*-trityl derivatives. Other side chain protecting groups included for His, N^{im}-*tert*-butyloxycarbonyl; for Lys, N^{ϵ}-*tert*-butyloxycarbonyl; for Arg, N^{g}-trimethylphenylsulfonyl; for Asp or Glu, *O-tert*-butyl ester; and for Thr or Ser, *O-tert*-butyl ether. Thirty-minute coupling protocols were used except for regions 16–24 and 1–9, where extended (60-min) coupling protocols were utilized. Removal of the N^{α}-FMOC group was accomplished with 20% piperidine in *N*-methylpyrrolidinone. Syntheses were started on a 0.15-mmol (1.5 g) scale. Once position 20 was coupled, each synthesis was completed in $^1/_3$ (0.05 mmol) portions.

Peptides were cleaved from the resin and all the protecting groups were removed by treatment with 95% trifluoroacetic acid (TFA), 3% dithioethane, and 2% anisole (10 mL/g of peptide resin) for 8 h at room temperature. The resin was removed by filtration, and the TFA was evaporated in vacuo. The residue was triturated with anhydrous diethyl ether to remove organics and then dissolved in 5% aqueous acetic acid (100 mL) and lyophilized to a white solid. The crude peptide (190 mg) was dissolved in 0.1% aqueous TFA (500 mL), and the pH was adjusted to 8 with dilute ammonium hydroxide. The basic solution was allowed to stir at room temperature for 8–24 h, and cyclization of peptide was monitored by RP-HPLC. The cyclic peptides all eluted as single peaks 5–7 min earlier than the crude sulfhydryl-containing peptides. The pH was readjusted to 4 with glacial acetic acid, and the solution was lyophilized. The crude cyclic peptides were purified by gel filtration (Sephadex G-25, 2.5 cm × 100 cm) and gradient reverse-phase-HPLC (Vydac C_{18}, 1 × 25 cm, 10–20% acetonitrile, 0.5%/min, in 0.1% aqueous TFA). ChTX was synthesized using a similar methodology as previously described (Sugg et al., 1991). Composition of the peptides was confirmed either by Edman degradation techniques using a 2090E Porton microsequencer with an on-line detection system or by amino acid analysis.

Binding Studies. The interaction of [^{125}I]ChTX with either bovine aortic sarcolemmal membrane vesicles or rat brain synaptic membrane vesicles was monitored as previously described (Vazquez et al., 1989, 1990). Binding assays were performed in triplicate, and the standard error of the mean was less than 3%. Data from competition experiments were analyzed by the method of Cheng and Prusoff (1973) to determine K_i values. Data from saturation experiments were subjected to a Scatchard analysis and linear regression was employed to calculate toxin affinity (K_d) and maximum receptor density (B_{max}). Correlation coefficients were typically greater than 0.96.

Recordings of Maxi-K Channels in Planar Lipid Bilayers. Planar lipid bilayers were formed by painting a mixture of POPE and POPC in a 7:3 molar ratio dissolved in decane (50 mg/mL) across a 250-μm hole in a partition separating two aqueous compartments. Plasma membrane vesicles from bovine aortic smooth muscle that contain maxi-K channels were fused with the bilayer under an osmotic gradient. Single channel currents were measured with a Dagan 3900 voltage clamp in the mixed RC mode that was connected to the bilayer chamber with silver/silver chloride wires and agar bridges filled with 0.2 M KCl. The polarity of channel insertion in the bilayer was determined from the voltage and calcium sensitivity of each channel. The voltage and current are expressed in the normal electrophysiological convention with the extracellular side of the channel defined as 0 mV and with

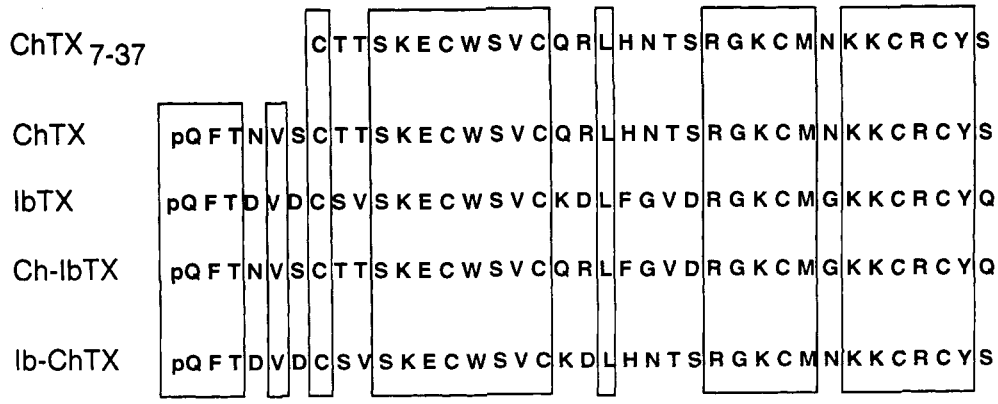

FIGURE 1: Amino acid sequences of ChTX-like toxins. The amino acids which are conserved with ChTX are enclosed.

current flowing from the intracellular side to the extracellular side plotted as positive current.

Single channel currents were recorded on video cassette tape with a VR 10 (Instrutech Corp., Elmont, NY) digital data encoder and played back on a Gould chart recorder (2200S) or into a DEC 11/73 computer (Digital Equipment Corp., Marlboro, MA) for analysis of toxin-blocked and -unblocked durations. Toxin-blocked durations were distinguished from closed times due to normal channel gating by defining a minimum cutoff time for toxin-blocking events. This cutoff time was determined during the control period of each experiment and was assigned a value that was longer than the longest closed times seen in control. Channel closings observed in the presence of toxin that were longer than the cutoff time were deemed toxin blocking events. With the exception of $ChTX_{7-37}$, the cutoff time was typically about 1 s in most experiments. The cutoff time for $ChTX_{7-37}$ was 100 ms.

We used the average duration of toxin blocked and unblocked times to calculate the toxin dissociation rate constant (k_{off}) and the pseudo-first-order association rate constant (k_{on}[toxin]) where

$$k_{off} = 1/(\tau_{block}) \quad (1)$$

$$k_{on}[\text{toxin}] = 1/(\tau_{unblock}) \quad (2)$$

where τ_{block} is the mean blocked time and $\tau_{unblock}$ is the mean unblocked time observed in the presence of toxin. The equilibrium dissociation constant (K_d) values were calculated from the association and dissociation rate constants where

$$K_d = k_{off}/k_{on} \quad (3)$$

Recordings of Rat $Kv_{1.3}$ Channels Expressed in Oocytes. RNA was transcribed in vitro from a cDNA encoding the rat $Kv_{1.3}$ potassium channel (Swanson et al., 1990), kindly provided by R. Swanson, Merck Research Labs. *Xenopus laevis* were obtained from commercial suppliers, and oocytes were isolated by collagenase treatment of ovarian lobes removed by surgical excision under general anesthesia. Isolated, defolliculated oocytes were injected with ~5 ng of $Kv_{1.3}$ RNA. Voltage clamp recordings were obtained after 24–28 h of incubation in oocyte Ringer's (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Na–HEPES, pH 7.2) supplemented with 50 mg/mL gentamycin sulfate. Oocytes were voltage clamped using a conventional two-electrode circuit (DAGAN 8500). The voltage-measuring electrode was filled with 3 M KCl, and the current-passing electrode was filled with 4 M KAc. A Ag/AgCl pellet was used as a reference electrode. Recordings were made in oocyte Ringer's supplemented with 0.05% BSA, and peptides were diluted from concentrated stocks to their final concentrations in the BSA-containing Ringer's. Addition of BSA appears to inhibit binding of the peptides to plastic in the perfusion system and results in reproducible dose–response determinations. Oocytes were continuously superfused during recordings at a rate of 2 mL/min. Test pulses were delivered from a holding potential of –70 mV.

RESULTS

Synthesis of Peptides. The synthesis of ChTX using an FMOC-pentafluorophenyl ester strategy followed by oxidation of the resulting hexasulfhydryl peptide into biologically active material has recently been accomplished (Sugg et al., 1990). Employing similar synthetic and peptide folding techniques, IbTX, two chimeric analogs of ChTX and IbTX, Ch-$TX_{1-19}IbTX_{20-37}$ (Ch–IbTX) and $IbTX_{1-19}ChTX_{20-37}$ (Ib–ChTX), and a truncated form of ChTX, $ChTX_{7-37}$, have now been produced. The respective sequences of these peptides are shown in Figure 1. The sequences were chosen such that each chimera contains 6 of the 12 amino acids that differ between ChTX and IbTX and such that in the Ib–ChTX molecule, three of four acidic residues present in IbTX are included. Truncated ChTX lacks the first six N-terminal amino acid residues of the native toxin. Solid-phase syntheses of these peptides can be completed in less than two days. After oxidation, the peptides were purified by gel filtration, followed by reverse-phase HPLC. Synthetic IbTX eluted with native toxin during HPLC, giving a preliminary indication that it had folded properly (not shown). The chimeric analogs and $ChTX_{7-37}$ cyclized readily to provide single products upon HPLC which elute approximately in the same region as ChTX. All four synthetic peptides are analytically pure by reverse-phase HPLC. Under these conditions, ChTX and the two chimeric peptides elute at ca. 10% organic solvent (2-propanol/acetonitrile 2:1), while IbTX and $ChTX_{7-37}$ elute at 18% and 8% organic solvent, respectively. The composition of the peptides was confirmed by either sequence analysis using Edman degradation techniques or amino acid analyses.

Effects of Peptides on Maxi-K Channels. The interaction of the toxin analogs with maxi-K channels was investigated using electrophysiological and ligand binding techniques. Block of maxi-K channels was monitored electrophysiologically after plasma membrane vesicles from bovine aortic smooth muscle were fused with planar lipid bilayers. The same membrane preparation was used in binding experiments that measured the ability of toxin analogs to affect [^{125}I]ChTX binding to maxi-K channels. All of the peptides blocked the maxi-K channel in electrophysiological experiments, and all of the toxins inhibited [^{125}I]ChTX binding.

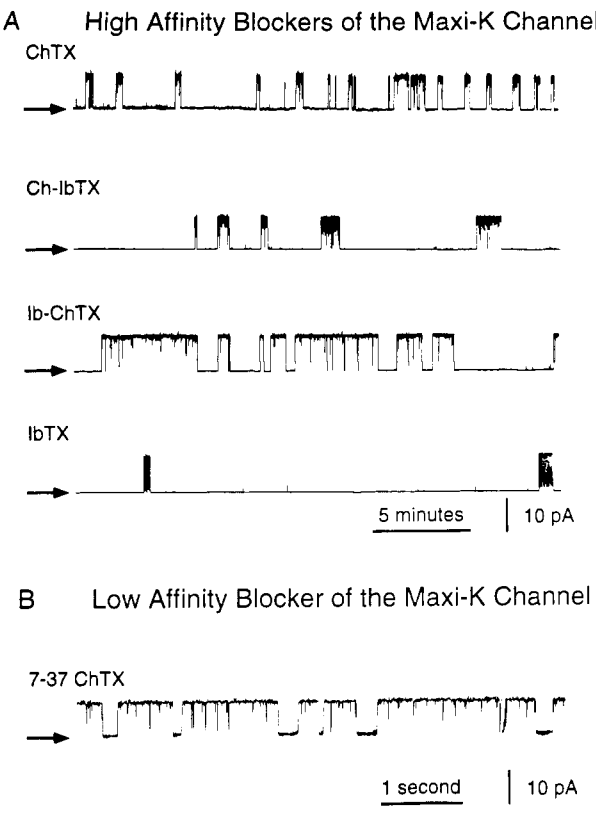

FIGURE 2: ChTX-like toxins inhibit the maxi-K channel incorporated into planar lipid bilayers. Currents through single maxi-K channels are shown in the presence of 10 nM each ChTX, Ch-IbTX, Ib-ChTX, and IbTX in part A and 1 μM $ChTX_{7-37}$ in part B. The closed-channel current level is indicated by the arrows to the left of the traces. Conditions: 150 mM KCl inside and outside; 20–50 μM $CaCl_2$ inside; 30 μg/mL BSA outside; +40 mV.

Charybdotoxin blocks the maxi-K channel by binding to a site in the external mouth of the channel with a 1:1 stoichiometry and occluding the conduction pathway for potassium (Anderson et al., 1988; MacKinnon & Miller, 1988; Miller, 1988). The quaternary ammonium ion, tetraethylammonium (TEA), a maxi-K inhibitor also interacts in this region (Yellen, 1984; Villarroel et al., 1988). As predicted from the electrophysiological data, [^{125}I]ChTX binds to smooth muscle sarcolemmal membranes in a bimolecular reaction (Vazquez et al., 1989). Iberiotoxin shares the same blocking mechanism as ChTX in bilayer experiments (Candia et al., 1992; Giangiacomo et al., 1992a). The two chimeric toxins, Ch–IbTX and Ib–ChTX, and $ChTX_{7-37}$ all blocked maxi-K channels in the same bimolecular fashion as the parent toxins and were competitive with TEA.

Figure 2 shows current recordings of maxi-K channels from bovine aortic sarcolemmal membranes vesicles incorporated into planar lipid bilayers in the presence of 10 nM ChTX, Ch–IbTX, Ib–ChTX, IbTX, or 1 μM $ChTX_{7-37}$. All of the peptide toxins blocked the maxi-K channel by causing silent periods interspersed with periods of normal channel activity. ChTX produced blocked times with a mean duration of ~65 s (see Table I) in symmetric 150 mM KCl and at +40 mV. The truncated form of ChTX, $ChTX_{7-37}$, gave the briefest blocked times, with a mean blocked time of 300 ms. Thus, removing the first six residues of ChTX caused approximately a 200-fold reduction in the mean toxin-blocked time of the channel. IbTX caused the longest blocked times (~500 s), while the two chimeric toxins gave similar blocked times of intermediate duration. The mean blocked times were 125 s for Ch–IbTX and 133 s for Ib–ChTX.

Table I: Toxin-Blocking Kinetics from Single-Channel Recordings of the Maxi-K Channel in Planar Lipid Bilayers[a]

toxin	K_D (nM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
$ChTX_{7-37}$	2900 ± 300	(1.1 ± 0.1) × 10^6	3.3 ± 0.6
ChTX	3 ± 1	(5 ± 0.5) × 10^6	0.015 ± 0.003
Ch–IbTX	6 ± 1	(1.4 ± 0.2) × 10^6	0.008 ● 0.001
Ib–ChTX	19 ± 2	(0.43 ± 0.07) × 10^6	0.0075 ± 0.0001
IbTX	1.7 ± 0.5	(1.2 ± 0.1) × 10^6	0.002 ± 0.0006

[a] The second-order association rate constant, k_{on}, values for toxin block and the toxin dissociation rate constant, k_{off}, values were determined from toxin-unblocked and -blocked times, respectively, as described in Materials and Methods. The equilibrium dissociation constant, K_d, values were calculated from the k_{off} and k_{on} values as described in Materials and Methods. Each value shown was determined from two to nine separate experiments; the errors represent the standard errors of the mean. Conditions are the same as described in Figure 2 except that the membrane potential ranged from +30 to +40 mV.

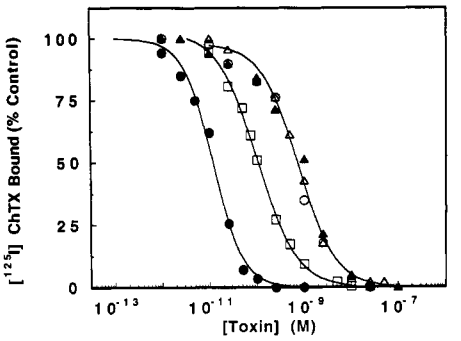

FIGURE 3: Effect of toxins on [^{125}I]ChTX binding to aortic sarcolemmal membrane vesicles. Sarcolemmal membrane vesicles were incubated with 30 pM [^{125}I]ChTX in 20 mM NaCl, 20 mM Tris–HCl, pH 7.4, 0.1% bovine serum albumin, and 0.1% digitonin in the absence or presence of increasing concentrations of ChTX (●), $ChTX_{7-37}$ (□), IbTX (△), Ch-IbTX (○), or Ib-ChTX (▲). Incubations were carried out at room temperature until equilibrium was achieved. Specific binding data in each case are presented relative to an untreated control.

The average durations of unblocked periods in the presence of the toxins are equal to the inverse of the pseudo-first-order association rate constant. The briefest unblocked times in Figure 2 were seen with ChTX, indicating that ChTX has the fastest rate of association under these conditions. The association rate measured for $ChTX_{7-37}$ was about 5-fold slower than for ChTX. The unblocked times seen with IbTX and Ch–IbTX were similar and were about 5-fold longer than with ChTX. The Ib–ChTX chimera exhibited the longest unblocked times which were ca. 10-fold longer than those observed with ChTX.

Table I summarizes the effects of the five peptides shown in Figure 2. The structural differences between the toxins lead to large differences in mean channel-blocked times, with nearly a 2000-fold difference between the mean blocked times for $ChTX_{7-37}$ and IbTX. In contrast, with the exception of Ib–ChTX, the association rates of the different peptides differ by less than 10-fold. $ChTX_{7-37}$ was the weakest inhibitor of the maxi-K channel under these experimental conditions, with a K_d of 2.9 μM. This was a consequence of a very fast dissociation rate, since the association rate was only 5-fold slower than that of ChTX. Thus, removal of the six amino acids from the N-terminus of ChTX caused a very significant reduction in the toxin's ability to block the maxi-K channel at physiological potassium concentrations.

Figure 3 demononstrates the ability of the toxin analogs to inhibit binding of [^{125}I]ChTX to bovine aortic sarcolemmal membrane vesicles. Under the low ionic strength conditions employed in these experiments, synthetic ChTX inhibited [^{125}I]-

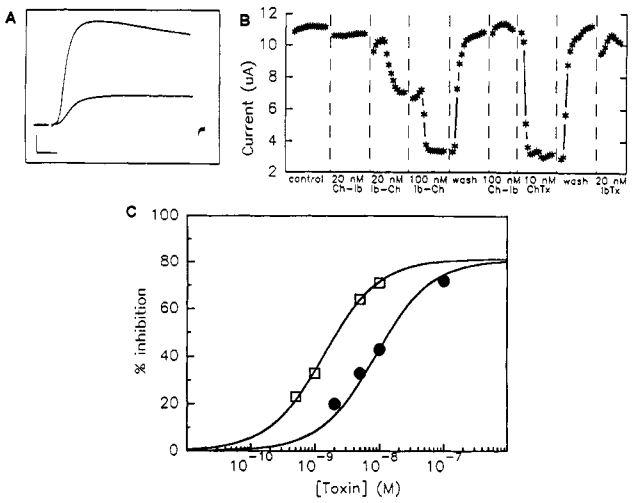

FIGURE 4: Effects of hybrid toxins on $Kv_{1.3}$ currents expressed in *Xenopus* oocytes. Oocytes were injected with approximately 5 ng of mRNA transcribed in vitro from a cDNA encoding the rate $Kv_{1.3}$ potassium channel. Two days after injection of RNA, oocytes were assayed for expression of potassium channels using a two-electrode voltage clamp. Panel A shows the response to a depolarizing test pulse to +30 mV from a holding potential of –80 mV before (top trace) and after (bottom trace) addition of 5 nM ChTX to the extracellular solution. No leakage subtraction or series resistance compensation was employed. The calibration bar is 2 μA vertical, 20 ms horizontal. Panel B shows the relative potencies of the various ChTX/IbTX hybrids at inhibiting the current elicited by a test pulse such as that shown in panel A. Data are from a single oocyte. Test pulses (to +30 mV from –70 mV for 70 ms) were delivered every 15 s, during constant superfusion of the cell with saline containing either no peptide (control, wash) or ChTX, IbTX, or a hybrid toxin as indicated along the abscissa. The graph plots the magnitude of the current measured at the end of each test pulse. Symbols connected by a line indicate responses at 15-s intervals. Breaks in the line indicate cessation of test pulses for periods between 30 s to 2 min. Panel C compares the potencies of native ChTX (□) vs $ChTX_{7-37}$ (●) on $Kv_{1.3}$ currents from two separate oocytes that expressed nearly identical levels of current. The data were fit to a standard logistic dose–response curve, with the slope constrained to unity. In all experiments, the extracellular saline contained 96 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, and 0.05% BSA. The pH was 7.2. All recordings were obtained at room temperature (22–24 °C). Peptides were diluted into saline from a concentrated aqueous stock.

ChTX binding completely with a Hill coefficient of ca. 1 and a K_i value of 10 pM. These values are identical to those previously determined in smooth muscle binding experiments with native ChTX (Vazquez et al., 1989). Synthetic IbTX also produced complete inhibition of [^{125}I]ChTX binding in digitonin-treated aortic sarcolemmal membranes and displayed a K_i of 540 pM; a profile which is identical to that of native IbTX (Galvez et al., 1990). The two chimeric peptides also caused total inhibition of [^{125}I]ChTX binding with identical K_i values of ca. 500 pM. In addition, $ChTX_{7-37}$ caused complete inhibition of toxin binding with a K_i of 100 pM. Clearly, the affinity of the peptides for the maxi-K channel is enhanced under conditions of low ionic strength.

It has previously been demonstrated that synthetic ChTX is functionally identical to native toxin as an inhibitor of the maxi-K channel (Sugg et al., 1990). Similarly, synthetic IbTX and native toxin isolated from *Buthus tamulus* venom displayed identical properties in the experiments presented here. These data provide a strong argument that each synthetic peptide has folded properly.

Several other peptides were synthesized to investigate the structure–activity relationship for ChTX block of the maxi-K channel. ChTX analogs in which the first or the first and second disulfide bridges were deleted by replacing Cys residues with Ala were completely inactive in binding. These findings support the view that the compact folded structure of ChTX determines its ability to block the maxi-K channel. Iodination of the Tyr residue penultimate from the C-terminus of ChTX results in a significant loss in the ability of ChTX to inhibit the maxi-K channel (Vazquez et al., 1989; Luchesi et al., 1989). Replacement of Phe_2 with a Tyr and of Tyr_{36} with a Phe in the analog [Tyr_2, Phe_{36}]ChTX abolished the activity of this molecule as an inhibitor of the maxi-K channel, while [Tyr_2, Tyr_{36}]ChTX was completely functional in both ligand binding and electrophysiological experiments. Part of the loss of activity for the [Tyr_2, Phe_{36}]ChTX variant may have been due to peptide folding problems, since several forms were observed upon reverse-phase HPLC.

Effects of Peptides on Voltage-Gated K^+ Channels. The interaction of the toxin analogs with voltage-gated channels was investigated using electrophysiological and ligand binding techniques. Block of $Kv_{1.3}$ channels was tested electrophysiologically by voltage clamp of oocytes expressing the cloned channel from rat brain. [^{125}I]ChTX binding inhibition experiments were carried out using rat brain synaptic plasma membranes. In contrast to the results obtained with maxi-K channels, only peptides containing the C-terminal half of ChTX were potent inhibitors of current through the $Kv_{1.3}$ channel and of [^{125}I]ChTX binding to brain membranes.

Figure 4 shows the relative potencies of ChTX, IbTX, and the two chimeras for inhibition of $Kv_{1.3}$ current in the small cell. Application of brief (80 ms) depolarizing voltage steps to +30 mV from a holding potential of –70 mV elicited an outward K^+ current that inactivated only slightly during the course of the pulse (Figure 4A). As shown in Figure 4B, repetitive pulses given 15 s, apart in control Ringer's yielded a consistent response. Successive presentation of the various peptides revealed a very different pattern of sensitivity from that of the maxi-K channel. Addition of 10 nM ChTX caused ca. 70% reduction of the current. In contrast, the current was insensitive to both IbTX and Ch–IbTX when tested at concentrations up to 100 nM. Ib–ChTX, however, was an effective inhibitor, although with an approximately 10-fold lower potency than ChTX. Interestingly, $ChTX_{7-37}$, which was 1,000-fold less potent than ChTX as an inhibitor of the maxi-K channel in lipid bilayers, blocked $Kv_{1.3}$ channels with nearly the same potency as ChTX (Figure 4C). Note that the dose–inhibition curve for the peptides in Figure 4C displays an apparent maximum inhibition of 80%. The residual current arises from $Kv_{1.3}$ channels, since addition of 1 mM 4-aminopyridine resulted in its complete inhibition. The failure of the peptides to block all of the current probably indicates inaccessibility of the peptides to channels located within the deepest invaginations of the oocyte membrane.

Binding studies were performed using rat brain synaptic plasma membrane vesicles (Figure 5). It was previously shown with native toxins that ChTX inhibits [^{125}I]ChTX binding in these membranes with a K_i of ca. 10 pM, while IbTX is without effect up to 4 orders of magnitude higher in concentration (Vazquez et al., 1990). Similar findings are presented here with synthetic ChTX and IbTX. The K_i value of ChTX was 15 pM, while IbTX had no effect until the concentration reached 100 nM. The two chimeric peptides also displayed markedly different activities. Ib–ChTX was a complete inhibitor of [^{125}I]ChTX binding and displayed a K_i value of 750 pM, which was about 50-fold reduced in potency from that of ChTX. However, Ch–IbTX behaved like IbTX, with little inhibitory activity until concentrations of 100 nM were

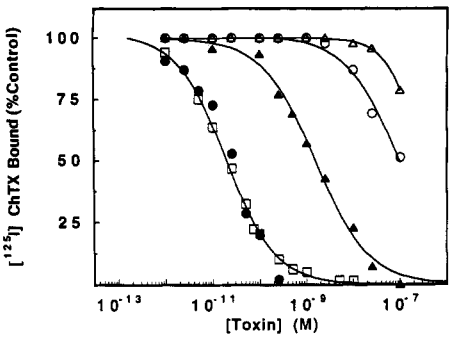

FIGURE 5: Effect of toxins on [^{125}I]ChTX binding to brain synaptic plasma membrane vesicles. Membrane vesicles were incubated with 15 pM [^{125}I]ChTX in the absence or presence of increasing concentrations of ChTX (●), $ChTX_{7-37}$ (□), Ib-ChTX (▲), Ch-IbTX (○), or IbTX (△). The incubation medium consisted of 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin and incubations were carried out at room temperature. Inhibition of binding was assessed relative to toxin association in untreated vesicles.

tested. In addition, $ChTX_{7-37}$ caused complete inhibition of ChTX binding with a potency close to that of ChTX. The results presented in Figures 4 and 5 indicate that, for the voltage-gated K^+ channel, Ib–ChTX behaves as a ChTX-like peptide, while Ch–IbTX resembles IbTX.

Saturation experiments (not shown) with [^{125}I]ChTX in synaptic membranes indicated that the toxin bound to a single class of sites at a K_d value of 25 pM and B_{max} of 0.30 pmol/mg of protein. In the presence of increasing concentrations of the Ib–ChTX chimera, there was a gradual increase in K_d of the iodinated toxin, with no effect on receptor site density. Thus, Ib–ChTX showed the expected activity of a competitive inhibitor of [^{125}I]ChTX binding in brain. The truncated analog, $ChTX_{7-37}$, produced a similar profile.

As was found for the maxi-K channel, ChTX analogs with the first or the first and second disulfide bridges deleted, were completely inactive in binding experiments. [Tyr_2, Phe_{36}]-ChTX was inactive against the maxi-K channel in binding experiments but was only 10-fold reduced in potency as an inhibitor of [^{125}I]ChTX binding in brain, consistent with other binding data indicating that modification of Tyr_{36} is less critical for the interaction of ChTX with the $Kv_{1.3}$ channel (Deutsch et al., 1991).

DISCUSSION

The data presented in this study highlight a number of interesting features regarding the interaction of peptide toxins with two ChTX-sensitive K^+ channels: the maxi-K channel from vascular smooth muscle and the $Kv_{1.3}$ channel from neuronal tissue. It has previously been demonstrated that the maxi-K channel of aortic smooth muscle is potently inhibited by both ChTX and IbTX (Giangiacomo et al., 1992a) but that $Kv_{1.3}$ is blocked with high affinity only by ChTX (Deutsch et al., 1991; Leonard et al., 1992). Since ChTX and IbTX exhibit 68% sequence identity (Galvez et al., 1990) and a nearly identical peptide backbone structure (Bontems et al., 1991; Johnson & Sugg 1992), these differences in selectivity suggest that specific amino acid residues of the ChTX-like toxins account for the specificity of their interaction with the maxi-K and $Kv_{1.3}$ potassium channels. In order to identify regions of the toxins which impart selectivity for the two channels, IbTX, ChTX, the chimeras Ib–ChTX and Ch–IbTX, and a truncated form of ChTX, $ChTX_{7-37}$, were synthesized by solid-phase methodology. The functional activity of each of these peptides was examined by both binding and electrophysiological protocols.

Using these synthetic toxins, the regions of the ChTX-like molecules which impart selectivity for the maxi-K and $Kv_{1.3}$ channels have been identified. The chimera Ib–ChTX blocked with high affinity both the maxi-K and $Kv_{1.3}$ channel, while the Ch–IbTX chimera blocked only the maxi-K channel with high affinity. Thus, residues 20–37 in the C-terminal region of these peptides appear to confer much of the observed specificity. Given that the three-dimensional structures of IbTX and ChTX are very similar, it is reasonable to assume that the hybrid structures may also be similar. Consequently, the differences in selectivity are likely to arise predominantly from one or all of the six residues which differ between ChTX and IbTX in this C-terminal region (i.e., residues 21–24, 30, and 37). Additional insight about selectivity can be gained by comparing the sequences of ChTX and IbTX with other peptidyl scorpion toxins which exhibit selectivity for the maxi-K channel. For example, limbatotoxin (LbTX), an IbTX-like peptide isolated from the new world scorpion *Centruroides limbatus* (Novick et al., 1991), blocked the maxi-K channel with high affinity and displayed similar kinetic behavior to IbTX ($k_{off} = 2.6 \times 10^{-3}$), but it did not block the $Kv_{1.3}$ channel (unpublished observations). Comparison of the primary amino acid sequences of IbTX and LbTX reveals that both toxins contain four common residues which are not found in ChTX; these are Asp_4, Gly_{22}, Asp_{24}, and Gly_{30}. Ch–IbTX contains Gly_{22}, Asp_{24}, and Gly_{30}, while Ib–ChTX contains Asp_4. If determinants of selectivity for the maxi-K channel are limited to the residues which are conserved between LbTX, IbTX, and the hybrid Ch–IbTX, then it is likely that any or all of the three residues, Gly_{22}, Asp_{24}, and Gly_{30}, impart this specificity.

In addition to identifying those regions of ChTX-like toxins which govern selectivity for the maxi-K channel, we found that the determinants which impart high-affinity toxin–channel interactions differ for the maxi-K and $Kv_{1.3}$ channels. $ChTX_{7-37}$ interacted with the $Kv_{1.3}$ channel in a similar fashion to ChTX. [^{125}I]ChTX binding in rat brain membranes revealed that the IC_{50} values for ChTX and $ChTX_{7-37}$ were identical at ca. 20 pM. In addition, the K^+ current associated with $Kv_{1.3}$ expressed in oocytes was inhibited to similar extents by 1 nM ChTX and 5 nM $ChTX_{7-37}$. In contrast, the interaction of $ChTX_{7-37}$ with the maxi-K channel was significantly weakened relative to ChTX. The effect was most dramatic in single-channel recordings of the maxi-K channel in planar lipid bilayers where the affinity for $ChTX_{7-37}$ was weakened 1000-fold relative to ChTX. Remarkably, the IC_{50} value determined for $ChTX_{7-37}$ inhibition of [^{125}I]ChTX binding to the maxi-K channel was only weakened 10-fold relative to unlabeled ChTX. The possibility cannot be ruled out that deleting the first six residues from ChTX caused structural changes which influence toxin binding to the maxi-K channel. However, the fact that $ChTX_{7-37}$ and ChTX exhibited similar affinities for the $Kv_{1.3}$ channel supports the view that the N-terminus of ChTX contributes to its interaction with the maxi-K channel and that this interaction is weak or does not exist with the $Kv_{1.3}$ channel. Similar findings have been obtained after removal of the two N-terminal residues of ChTX with chymotrypsin; the resulting peptide is an effective blocker of $Kv_{1.3}$ in human T lymphocytes (Price et al., 1989) but is much less potent than ChTX as a blocker of the maxi-K channel in skeletal muscle (Price et al., 1989) or aortic smooth muscle (unpublished observations).

Despite the well-established model for simple bimolecular block of maxi-K channels by ChTX (Anderson et al., 1988; MacKinnon et al., 1989) and IbTX (Giangiacomo et al., 1992a), it is difficult to compare data from electrophysiological and binding studies directly. Qualitatively, these measurements yield similar results, but quantitatively they do not agree. For both channel types, all of the peptides were more potent as inhibitors of [^{125}I]ChTX binding than they were as channel blockers in electrophysiological experiments. This is not unexpected, since the ionic strength conditions for binding and electrophysiology differ significantly. Binding studies were done in low salt while electrophysiological experiments were performed under higher ionic strength conditions. The potencies of both ChTX and IbTX have been shown to depend upon external ionic strength, reflecting the electrostatic component which dominates the association of toxin with the maxi-K channel pore. Furthermore, K^+ was not included in the incubation medium under any binding condition. Intracellular K^+ is known to promote the rate of ChTX and IbTX dissociation from the maxi-K channel. For $Kv_{1.3}$, the rank order of potencies for binding and block were parallel in the sense that molecules with a ChTX-like C-terminus were potent in both binding and electrophysiological testing, while those with the C-terminus of IbTX did not exhibit a high-affinity interaction in either assay. For the maxi-K channel, the picture was less straightforward. All of the peptides were functional in binding studies, and all but $ChTX_{7-37}$ were potent blockers in the bilayer experiments. However, the rank orders of potencies in the two assays were not the same (i,e., ChTX > $ChTX_{7-37}$ > Ch–IbTX, Ib–ChTX, and IbTX in binding versus IbTX > ChTX > Ch–IbTX > Ib–ChTX >>> $ChTX_{7-37}$ in electrophysiological assays), suggesting that perhaps interaction of each of the peptides with the maxi-K channel is influenced to a different degree by ionic strength.

Another explanation for the discrepancies observed between toxin binding and channel block is that binding determinants for monoiodinated ChTX might differ from those of unmodified ChTX and the other ChTX-like peptides. Several observations lend support to this hypothesis. Binding of [^{125}I]-ChTX to maxi-K channels in aortic sarcolemma exhibits a K_d value 10-fold higher than that of native toxin as determined from competition experiments (Vazquez et al., 1989). There also appear to be some differences in the effects of ionic strength and K^+ on the interaction of [I]ChTX and ChTX with the maxi-K channel, since [I]ChTX is a much weaker channel inhibitor than native peptide in single-channel experiments performed under equivalent salt conditions (Vazquez et al., 1989). Finally, binding and electrophysiological data obtained with the maxi-K channel differ with respect to the nature of the ChTX and IbTX binding sites. Inhibition of [^{125}I]ChTX binding to bovine aortic smooth muscle by IbTX exhibits apparently noncompetitive behavior (Galvez et al., 1990). This phenomenon has also been observed in the present study with the Ch–IbTX chimera, but not with the Ib–ChTX hybrid which, as predicted for a ChTX-like peptide, displayed competitive behavior in a saturation analysis (data not shown). These findings imply that [I]ChTX and IbTX do not occupy the same site on the maxi-K channel. In contrast, kinetic measurements of toxin block of single channels in planar lipid bilayers suggest that ChTX (Miller, 1988) and IbTX (Giangiacomo et al., 1992a) compete with the small organic cation TEA for the same site in the mouth of the maxi-K channel. Thus, functional experiments suggest that IbTX and ChTX must occupy overlapping sites on the maxi-K channel, while binding experiments suggest that the ChTX and IbTX receptor domains do not strictly overlap. Again, one explanation which would account for these discrepancies is that monoiodination of ChTX changes the structure of the peptide so that its binding determinants are modified relative to native toxin.

The three-dimensional structures of both ChTX and IbTX have been determined by solution ^{1}H NMR techniques, and the backbones of these molecules are oriented similarly (Bontems et al., 1991; Johnson & Sugg, 1992). These studies indicate that most of the positively charged amino acid residues which are conserved between the two toxins are located on one face of the peptide. This surface is comprised of three antiparallel β strands which form a β-sheet. In addition, the residues that are presumably important for selectivity, Gly_{22}, Asp_{24}, and Gly_{30}, are contained in the β-sheet face of IbTX, as are the four N-terminal residues of ChTX which appear to impart a high-affinity interaction with the maxi-K channel. The tyrosine penultimate to the C-terminus of ChTX and IbTX is also located on the β-sheet. This β-sheet face may be important for toxin interaction with both the maxi-K and $Kv_{1.3}$ channels.

Although the structure of the maxi-K channel remains to be determined, the primary structure of $Kv_{1.3}$ is known. Recent studies (Oliva et al., 1991; Golstein et al., 1992) demonstrate that a single phenylalanine to glycine substitution in the presumed mouth of the ion channel pore of $Kv_{1.3}$ generates the sensitivity to ChTX that distinguishes this member of the *Shaker* family of potassium channels from its close relatives. Continuing comutagenesis studies with both the $Kv_{1.3}$ channel and selected toxins should further elucidate which residues in each molecule are involved in the binding reaction.

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