Charybdotoxin Is a New Member of the K^+ Channel Toxin Family That Includes Dendrotoxin I and Mast Cell Degranulating Peptide†

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ABSTRACT: A polypeptide was identified in the venom of the scorpion *Leiurus quinquestriatus hebraeus* by its potency to inhibit the high-affinity binding of the radiolabeled snake venom toxin dendrotoxin I (^{125}I-DTX_I) to its receptor site. It has been purified, and its properties investigated by different techniques were found to be similar to those of MCD and DTX_I, two polypeptide toxins active on a voltage-dependent K^+ channel. However, its amino acid sequence was determined, and it was shown that this toxin is in fact charybdotoxin (ChTX), a toxin classically used as a specific tool to block one class of Ca^{2+}-activated K^+ channels. ChTX, DTX_I, and MCD are potent convulsants and are highly toxic when injected intracerebroventricularly in mice. Their toxicities correlate well with their affinities for their receptors in rat brain. These three structurally different toxins release [^{3}H]GABA from preloaded synaptosomes, the efficiency order being DTX_I > ChTX > MCD. Both binding and cross-linking experiments of ChTX to rat brain membranes and to the purified MCD/DTX_I binding protein have shown that the α-subunit (M_r = 76K–78K) of the MCD/DTX_I-sensitive K^+ channel protein also contains the ChTX binding sites. Binding sites for DTX_I, MCD, and ChTX are in negative allosteric interaction. Our results show that charybdotoxin belongs to the family of toxins which already includes the dendrotoxins and MCD, which are blockers of voltage-sensitive K^+ channels. ChTX is clearly not selective for Ca^{2+}-activated K^+ channel.

Mast cell degranulating peptide (MCD)[1] from bee venom and toxin I from the venom of the snake *Dendroaspis polylepis* (DTX_I) (the most potent member of the dendrotoxin family) are both convulsant and highly neurotoxic polypeptides when injected intracerebroventricularly in mouse and rat brains (Schweitz, 1984; Bidard et al., 1987a, 1989). At low concentrations MCD induces hippocampal θ rhythm (Bidard et al., 1987a) and hippocampal long-term potentiation (Cherubini et al., 1987), which is usually associated with information storage and memory. DTX_I facilitates evoked neurotransmitter release (Harvey & Karlsson, 1982; Harvey & Anderson, 1985; Weller et al., 1985). It has been recently demonstrated that both dendrotoxins and MCD block K^+ channels (Benoit & Dubois, 1986; Penner et al., 1986; Stansfeld et al., 1987; Stühmer et al., 1988). MCD and DTX_I have high-affinity receptor sites in synaptosomal membranes (Bidard et al., 1987b, 1989); these receptor sites are distinct and in negative allosteric interaction (Bidard et al., 1987b) and are located on the same protein complex (Rehm et al., 1988; Baumann et al., 1988). This protein complex has been purified (Rehm & Lazdunski, 1988). Its reconstitution leads to DTX_I-sensitive K^+ channel activity (Rehm et al., 1989). The α-subunit (M_r 76K–77K) of this protein complex contains both MCD and DTX_I binding sites (Rehm et al., 1988).

This paper describes the purification and properties of a toxin extracted from the scorpion *Leiurus quinquestriatus hebraeus* which turns out to be charybdotoxin and which has binding and physiological properties that make it a new member of the family of K^+ channel blocking neurotoxins including MCD and DTX_I.

Materials and Methods

Purification of Leiurus Toxin. Leiurus quinquestriatus hebraeus venom was obtained as a lyophilized powder from Latoxan (France). Step 1. A 150-mg sample of venom was dissolved in 20 mL of 1% acetic acid, and the undissolved mucoid material was centrifuged at 30000*g* for 20 min. Step 2. The supernatant was loaded onto a Sephadex G-50 column (3 × 38 cm) equilibrated with the same solvent. Step 3. The peptide fraction, eluting between 60% and 84% of the bed volume, was directly loaded onto a TSK SP5PW (21.5 × 150 mm) column equilibrated with buffer A (1% acetic acid adjusted at pH 3 with ammonia). A linear gradient of buffer A to buffer B (1 M ammonium acetate) was run for 100 min at 8 mL/min, and A_{280} was monitored. All the fractions were checked for their ability to prevent binding of ^{125}I-labeled toxin I from *Dendroaspis polylepis* (^{125}I-DTX_I) to rat brain synaptosomes. The active fractions eluted at about 55% B (R_T = 62 ± 5 min). Step 4. These fractions were further fractionated by reversed-phase HPLC using a 150 Å pore C8 column [Zorbax, PEP-RP1 (6.2 × 80 mm)] and solvents C [0.1% trifluoroacetic acid (TFA)] and D (0.1% TFA in 50% ethanol). The active material was loaded at 0.5 mL/min with 30% D and was eluted in the breakthrough, whereas inactive material was eluted with a gradient of ethanol. Step 5. The breakthrough was lyophilized and dissolved in 20 mL of solvent A for a second ion exchange chromatography on TSK SP5PW (7.5 × 75 mm). The material was loaded on the column at a flow rate of 0.5 mL/min; then, a steep linear gradient was applied from 0% to 40% B within 5 min at a flow rate of 1 mL/min so that the material was eluted under isocratic con-

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[1] Abbreviations: MCD, mast cell degranulating peptide; DTX_I, toxin I (from *Dendroaspis polylepis*); ChTX, charybdotoxin (from *Leiurus quinquestriatus hebraeus*).

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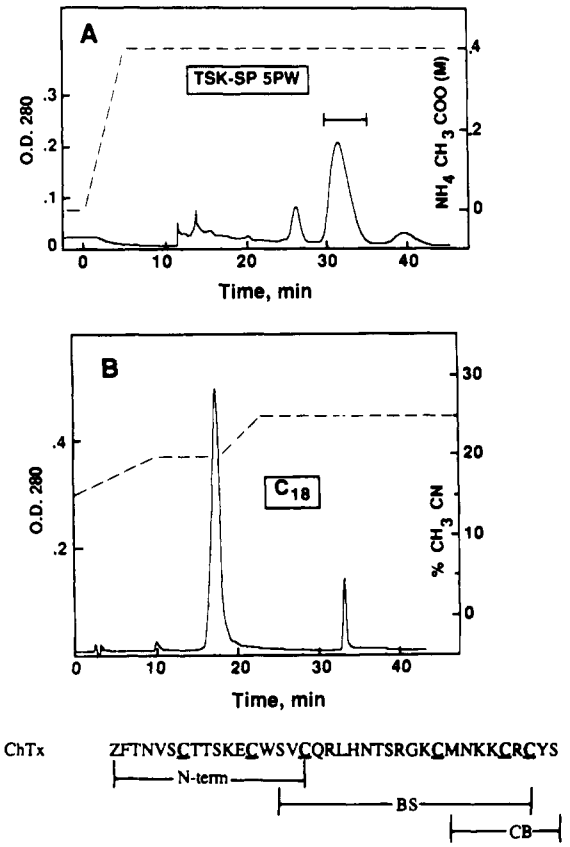

FIGURE 1: Last two steps of purification of ChTX. (A) Step 5. Ion exchange chromatography on a TSK SP5PW (7.5 × 75 mm column). Active material of step 4 (see Materials and Methods) was loaded in 1% acetic acid and eluted at 1 mL/min with 400 mM ammonium acetate. The bar indicates activity, i.e., inhibition of ^{125}I-DTX_I binding. (B) Step 6. The active fraction from step 5, above in (A), was chromatographed by reverse-phase HPLC on a Waters C18 (3.9 × 150 mm) column at 0.7 mL/min as described under Materials and Methods. All ChTX activity migrated in the major peak. (ChTX) Methods used to determine the amino acid sequence of *Leiurus* toxin. N-term: Amino-terminus sequence obtained after treatment with methanolic HCl. Inner fragments were generated by in situ chemical cleavage of S-pyridylethylated protein on glass fiber. BS: Obtained by treatment with BNPS-skatole. CB: Obtained by treatment with cyanogen bromide. No amino acids were released when the intact toxin was submitted to NH_2-terminal sequence analysis, suggesting that this position could be blocked. The peptide was unblocked by treatment with methanolic HCl. Glutamic acid and its γ-methyl ester were detected during the first degradation cycle, indicating that the amino-terminal residue is pyroglutamic acid. Further degradation allowed the determination of 16 residues. Taking advantage of the blocked NH_2-terminal residue, the S-pyridylethylated toxin was directly submitted to in situ chemical cleavages on glass fiber prior to sequence analysis. Treatments with BNPS-skatole and cyanogen bromide allowed the generation of overlapping peptides accounting for the complete 37-residue sequence.

ditions at 40% B. Most of the activity was found in the main peak (Figure 1A). Step 6. The final purification was achieved by reverse-phase HPLC on a 5-μm spherical C18 Waters column (3.9 × 150 mm) with solvents E [0.5% TFA and 0.9% triethylamine (TEA) in water] and F (the same counterions at the same concentration but in acetonitrile). The major fraction in step 5 was lyophilized, redissolved in the minimum volume of solvent E, and loaded onto the column. Elution was performed at 0.7 mL/min with the gradient shown in Figure 1B. Activity was found in the major fraction. Step 7. This fraction was lyophilized and desalted on a Sephadex G-25 column with 1 mM HCl as eluent, to give pure *Leiurus* toxin. Recovery was 0.17% of the dry venom. HPLC purifications were performed with a Waters system (pump 501, injector U6K, automated gradient controller, and spectrophotometer 4S1) equipped with a Shimadzu C-R3A integrator–recorder.

Amino Acid Sequence of Leiurus Toxin. The toxin and its fragments were sequenced on an Applied Biosystems 470A gas-phase sequenator with an on-line PTH analyzer, Model 120A. To unblock the N-terminus, 50 pmol of toxin was treated with 30 μL of 1 N methanolic HCl at 37 °C for 72 h under N_2. Reduction and alkylation of the toxin (100–300-pmol aliquots) were performed directly on a polybrene-treated glass-fiber filter with 4-vinylpyridine and tributylphosphine vapors for 2 h at 40 °C.

S-Pyridylethylated toxin adsorbed to the glass-fiber filter was then submitted to chemical cleavages. Cyanogen bromide cleavage was performed in the vapor phase for 10 h at room temperature. BNPS-skatole cleavage was performed by spotting a 100-fold excess of reagents and *N*-acetyltyrosine on the filter and reacting for 24 h at room temperature. In both cases, filters were loaded in the cartridge of the sequenator and directly sequenced.

Purification of MCD. Bee venom was a kind gift from Charles Mraz, Beekeeper, Middlebury, VT. A total or 5.3 g of dried venom was dissolved in 100 mL of 100 mM ammonium acetate, pH 8.5, and centrifuged for 20 min at 30000*g*. The supernatant was loaded onto a series of two Sephadex G-50 columns (5 × 100 cm) and eluted with the same solvent at a flow rate of 140 mL/h. The peptide fraction eluted between the main peak (Mellitin) and the salts. It was acidified at pH 3 with acetic acid. Then, it was directly loaded in halves onto a TSK SP5PW (21.5 × 150 cm Beckman) column equilibrated with buffer A (see above). A linear gradient of buffer A to buffer B was run for 80 min at 8 mL/min, and A_{280} was monitored. MCD eluted 65 min after application of the gradient. For final purification, the MCD pool was loaded onto a RP18 column (25 × 250 mm, 7-μm particle size, Merck) and eluted at 10 mL/min with a linear gradient over 120 min of 10%–40% acetonitrile in solvent E (see above). The purified MCD peak was lyophilized and desalted on a Trisacryl GF05M column (2.5 × 20 cm) with 1% acetic acid as eluent, lyophilized, and stored desiccated at −20 °C. Recovery was 84 mg.

Purification of Toxin I from D. polylepsis (DTX_I). *D. polylepsis* venom was obtained from Latoxan, France. A 200-mg sample of the venom was dissolved in 3 mL of 2% acetic acid and chromatographed on a Sephadex G-50 column (3 × 40 cm). The peptide fraction which was eluted between 67% and 90% of the column bed volume (corresponding to a dry weight of about 120 mg) was adjusted at pH 3 with ammonia and chromatographed as in step 3 described above for the *Leiurus* toxin purification. The presence of DTX_I was checked by its toxicity after intracisternal injection in mouse brain. DTX_I was eluted at a retention time of 72 min. Recovery after lyophilization was 40 mg of extensively purified (>95%) DTX_I. Final purification was achieved by reverse-phase HPLC on a C18 column (25 × 250 mm, 7-μm particle size, Merck). The effluent of the previous chromatographic step was directly loaded onto the column and eluted at 8 mL/min over 75 min with a linear gradient of 10%–40% of acetonitrile in solvent E. Pure DTX_I was then lyophilized and desalted as for MCD.

Iodination of the Polypeptide Toxins. *Leiurus* toxin was iodinated by the iodogen method (Pierce Chemical Co.) with $Na^{125}I$ (2000 Ci/mmol) and then purified by HPLC on a TSK SP5PW column as previously described for ^{125}I-DTX_I (Bidard et al., 1989). Retention times from the start of the gradient were 26 and 32 min for 125I-labeled and unlabeled *Leiurus*

toxins, respectively. Iodination of MCD and DTX_I was previously described (Bidard et al., 1989). Biological activity of iodinated toxins (low radioactivity specific) was checked by toxicity measurements.

Toxicity Measurements. Toxicity was measured after intracisternal injection of 5 μL of toxin solution in mice slightly anaesthesized by ether as described by Schweitz (1984). The LD_{50} values were calculated according to the method of Behrens and Karber (1935) using groups of three mice. A toxin amount corresponding to 1 LD_{50} kills in about 1 h.

Bioassay. Guinea pig ileum contractions were measured as previously described (Schweitz, 1986). The strips were fixed to an UL5 microbalance screwed to an UC2 isometric force transducer which was connected to a Model 50 amplifier. Contractions induced by the toxins were recorded on a Gould 2600 graphic recorder (Gould Electronique, France). Presynaptic effects of the toxin were suppressed by a mixture of 0.2 μM tetrodotoxin, 0.2 μM atropine, 0.1 μM propranolol, 1 μM prazosine, and 1 μM yohimbine.

[^{3}H]GABA Release. Large synaptosomes were prepared from rat forebrain cortex. [^{3}H]GABA release was measured with a slightly modified version of the superfusion system described by Habermann (1983) and by Weller et al. (1985).

Briefly, large synaptosomes (0.5–1 mg of protein/mL) in Krebs–Ringer–Hepes solution containing 10 μM dipropylacetate were loaded with [^{3}H]GABA (56 Bq/pmol) for 15 min at 37 °C. Synaptosomes were centrifuged at 10000*g* for 2 min; the pellet was taken up in the original volume of the superfusion solution containing 0.5 mg/mL bovine serum albumin in the Krebs–Ringer–Hepes solution. A volume of 0.3 mL was layered on a 0.5-mL Sephadex G-10 column (40–120 μm, Pharmacia Fine Chemicals). The superfusion solution was eluted at a flow rate of 0.35 mL/min at 37 °C. After 30 min of wash-out, 2-min fractions were collected for 30 min. Two separate 2-min stimuli by the superfusion solution containing either the toxin or 50 mM K^+ (isosmolar replacement of NaCl by KCl) were started at t = 4 and 18 min, respectively. Radioactivity of each fraction was counted. At the end of the superfusion the radioactivity remaining in synaptosomes was recovered by washing the columns for 2 min with 0.2% Triton X-100. The radioactivity at zero time was the sum of total release during the collection plus the radioactivity extracted by Triton X-100 at the end of the experiment. The release during the sampling times is given in percent of the synaptosomal radioactivity at zero time. The basal efflux (control) was subtracted from the total release (in presence of toxin or 50 mM K^+) to obtain the evoked release.

Binding Assays. Synaptosomal P_3 membranes from rat brain were prepared as previously described (Taylor et al., 1984). Protein concentrations were determined according to Bradford (1976) with bovine serum albumin as a standard. ^{125}I-Labeled *Leiurus* toxin and ^{125}I-DTX_I binding to membranes was determined by a rapid centrifugation method as previously described for ^{125}I-MCD (Taylor et al., 1984) with minor modifications: the binding buffer was 50 mM Tris-HCl at pH 7.5 containing 140 mM NaCl, 1.3 mM $MgSO_4$, 5 mM KCl, and 1 mg/mL bovine serum albumin. Incubation was carried out at 4 °C for 30 min. Concentrations of ^{125}I-labeled toxin, unlabeled toxin, and membranes in the binding experiments are indicated in the legends of the figures. Membrane pellets were counted in a γ-counter. All binding data were corrected for binding to tubes measured in the absence of membranes and for nonspecific binding measured in the presence of 100 nM of the unlabeled toxin. Each data point represented the mean value of at least triplicate determinations.

The receptor was purified up to the DTX_I–Aca 22 affinity step according to Rehm and Lazdunski (1988). Binding assays to the purified receptor were done by a rapid filtration procedure as previously described (Rehm et al., 1988).

Competition binding experiments were performed by adding an appropriate concentration of unlabeled toxin 5 min prior to adding the ^{125}I-labeled toxin.

Cross-Linking of Iodinated Toxins to Rat Brain Synaptic Membranes. ^{125}I-Labeled *Leiurus* toxin (0.2 nM, 2000 Ci/mmol) or ^{125}I-DTX_I (0.5 nM, 750 Ci/nmol) was incubated for 30 min at 4 °C with P_3 membranes (0.5 mg of protein) in the absence or in the presence of unlabeled *Leiurus* toxin or DTX_I in 0.5 mL of the binding medium described above. ^{125}I-Labeled toxins were then cross-linked to membranes by 1 mg/mL dimethyl suberimidate and analyzed by SDS–polyacrylamide (8%) gel electrophoresis and autoradiography as described previously (Rehm et al., 1988).

Results and Discussion

Amino Acid Sequence. The strategy employed for the sequence determination of *Leiurus* toxin, purified as illustrated in Figure 1A,B, is schematically summarized at the bottom of Figure 1. We were surprised to observe that this sequence is identical with that recently observed for charybdotoxin (ChTX) (Gimenez-Gallego et al., 1988), a toxin that is considered to be a specific blocker for a class of Ca^{2+}-activated K^+ channels (≈200 pS) (Cook, 1988; Miller et al., 1985; Moczydlowski et al., 1988; Smith et al., 1986). We observed indeed that our isolated toxin blocked Ca^{2+}-activated K^+ channels in smooth muscle and neuronal cells (Van Renterghem and Romey, personal communication).

Toxicity of ChTX. Symptoms of toxicity observed when ChTX was injected to mice by intracisternal injection in the brain were similar to those observed after injection of MCD and DTX_I by the same route when mice were injected with lethal doses (LD_{50}), which are 8, 7, and 4 ng/g of body weight, respectively. for ChTX, MCD, and DTX_I. At first, injection of both DTX_I and ChTX produced classical wet dog shakes (Bidard et al., 1987a); then, seizures and convulsions appeared at 3–4 min. Seizures appeared immediately with MCD (Bidard et al., 1987a, 1989). Death occurred within 30–50 min.

Effect of ChTX on Smooth Muscle Contraction. Figure 2 shows that ChTX contracts guinea pig ileum. This effect first seemed similar to that observed with DTX_I at the same concentration (50 nM) (panel A). However, there was a difference between the modes of action of the two toxins. ChTX seems to act predominantly at the postsynaptic level (panels B and C), directly on the smooth muscle, since its action was not decreased importantly in the presence of a mixture of tetrodotoxin, atropine, propanolol, and yohimbine which eliminate the presynaptic component of contraction. In contrast, DTX_I action predominantly involves a presynaptic component (panel C). MCD had no effect at all on the resting tension of guinea pig ileum (data not shown).

Effect of the Different Toxins on [^{3}H]GABA Release from Preloaded Synaptosomes. Figure 3 shows how the three toxins evoke release of [^{3}H]GABA from synaptosomes. The toxins could only release part of the loaded [^{3}H]GABA since more release could be obtained with a K^+ depolarization of synpatosomes. Moreover, K^+-evoked [^{3}H]GABA release was hardly modified by a previous pulse of ChTX or DTX_I [Figure 3A (unmodified)]. The rank order of efficiency of the three toxins at 0.1 μM concentration is DTX_I > ChTX > MCD (Figure 3B). In the same conditions, K^+ depolarization by 50 mM KCl stimulated a larger release than the one produced by the

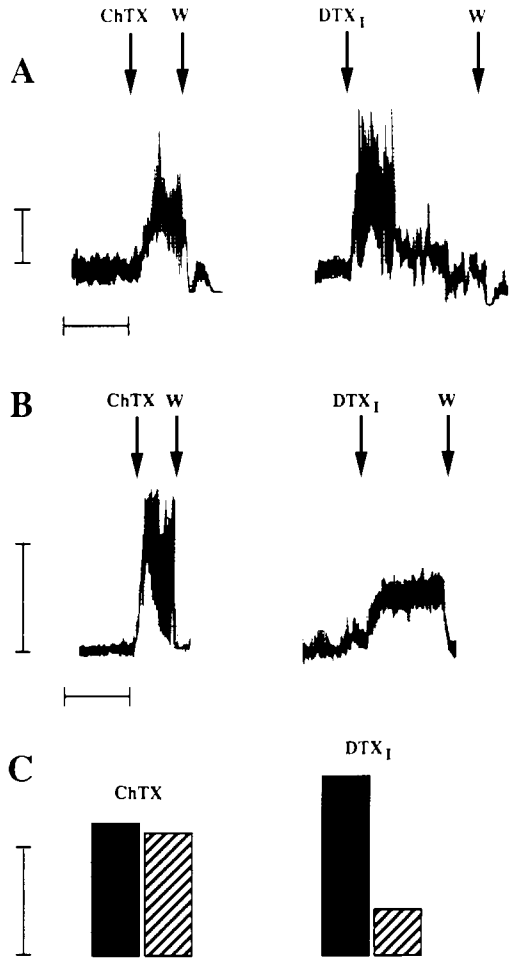

FIGURE 2: Effect of ChTX and of toxin I from *D. polylepis* (DTX_I) at a concentration of 50 nM on the tension of guinea pig ileum. (A) Direct action. (B) Presynaptic effects are suppressed by application of a cocktail of drugs, as indicated under Material and Methods, 10 min before application of toxins. W indicates washing. (C) Solid bars, direct action; shaded bars, postsynaptic action. Calibration vertical bars, 0.5 g; horizontal bars, 5 min.

toxins (Figure 3B). Figure 3C shows that the evoked [^{3}H]-GABA release was dependent on ChTX concentration with an EC_{50} of 10 nM. Saturation (Figure 3C) was obtained with concentrations of ChTX above 0.1 μM, and maximal release was about 22% of that obtained with 50 mM K^+.

Binding Experiments to Synaptosomes and Purified Receptor from Rat Brain. ChTX was iodinated to analyze its own binding properties (^{125}I-ChTX = ^{125}I-labeled *Leiurus* toxin). The Scatchard plot presented in Figure 4 gives a K_d value of 730 pM and a B_{max} value of 76 fmol/mg.

One cannot eliminate of course the presence of lower affinity binding sites for ^{125}I-ChTX, but their identification was prevented by the fact that the nonspecific binding component becomes as large as the specific binding component at ^{125}I-ChTX concentrations higher than 1 nM.

Inhibition of ^{125}I-ChTX binding by ChTX gives an apparent IC_{50} for ChTX action of 142 pM, corresponding to a K_d value of 140 pM (Figure 5). Figure 5 also shows that both DTX_I and MCD inhibit ChTX binding but that DTX_I is 100 times more efficient than MCD in preventing ^{125}I-ChTX binding to its binding sites; i.e., IC_{50} = 0.42 nM for DTX_I and 41 nM for MCD. Figure 6 (insets) shows that ChTX prevents with the same apparent affinity ($K_{0.5} \approx 30$ nM) both ^{125}I-DTX_I and ^{125}I-MCD binding to their respective sites. Inhibitions by ChTX are of the noncompetitive type as shown by the Scatchard plots in Figure 6 (main panels). In contrast, inhibition of ^{125}I-DTX_I binding by DTX_I ($K_{0.5}$ = 70 pM) and of ^{125}I-MCD by MCD ($K_{0.5}$ = 300 pM) was observed over 2 log units of toxin concentrations (Bidard et al., 1989). The inhibitory effect of ChTX is observed over 4 log units of toxin concentrations on both ^{125}I-DTX_I and ^{125}I-MCD binding, suggesting the existence of two types of ChTX receptors of higher and lower affinity, both of them related to high-affinity receptors for both DTX_I and MCD.

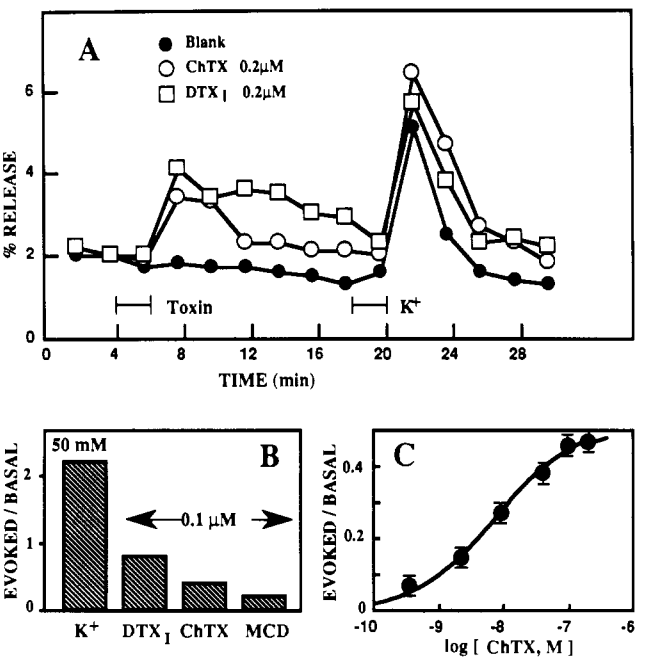

FIGURE 3: [^{3}H]GABA release from preloaded synaptosomes in superfusion experiments. (A) Illustration of the [^{3}H]GABA release evoked successively by 0.2 μM toxin (4–6 min) and 50 mM K^+ (18–20 min). The release during the sampling time is given as a percent of the synaptosomal radioactivity at zero time. Protein content was 0.16 mg/sample. Radioactivity before stimulation was 3500 Bq/sample (63 pmol). All data represent the means of two values. Standard deviations are less than 8% of the mean value. (B) Comparison of the [^{3}H]GABA release evoked by 0.1 μM toxins or by 50 mM K^+. (C) Dose-dependent ChTX-evoked [^{3}H]GABA release. In (B) and (C), toxin or K^+ pulses were between 4 and 6 min. The basal efflux (blank) was subtracted from the total release to obtain the evoked release. Data are given as the ratio of evoked to basal release.

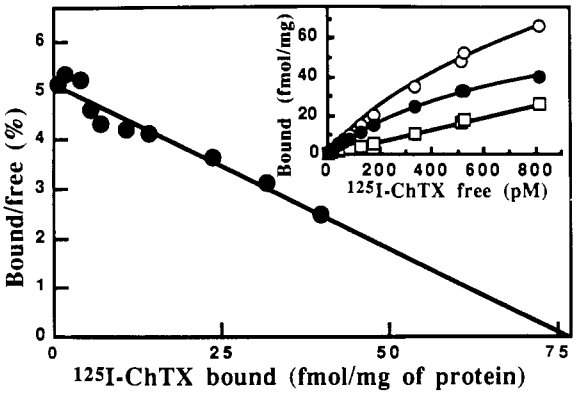

FIGURE 4: Binding of ^{125}I-ChTX to synaptosomal membrane from rat brain. Synaptic membranes (500 μg of protein/mL) were incubated with various concentrations of ^{125}I-ChTX (9–850 pM). Inset shows a saturation curve for ^{125}I-ChTX-specific binding to membranes (●) calculated after subtraction of nonspecific ^{125}I-ChTX binding (□), determined by including 100 nM ChTX in the incubation medium, from total ^{125}I-ChTX binding (○), determined in the absence of unlabeled ChTX. Binding to the tubes themselves, measured in incubation mixtures containing no membranes, was subtracted. Main panel shows a Scatchard analysis of the free and bound ^{125}I-ChTX concentrations calculated from the data in the inset (●).

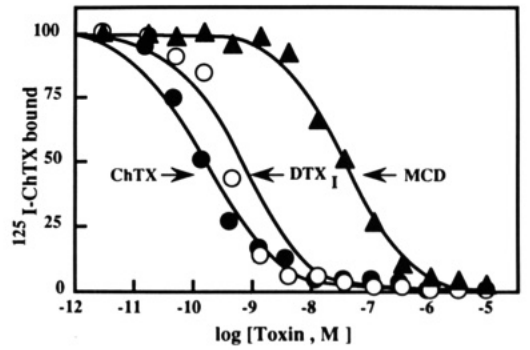

FIGURE 5: Determination of the dissociation constant for ChTX binding to synaptosomal membranes from rat brain. The ability of ChTX, DTX_I, and MCD to displace ^{125}I-ChTX specifically bound to the brain membrane was tested by equilibrating mixtures of the unlabeled toxins at various concentrations with ^{125}I-ChTX (8 pM) and membranes (500 μg of protein/mL). The nonspecific binding component, determined by including 100 nM ChTX in the incubation medium, was subtracted from the total binding.

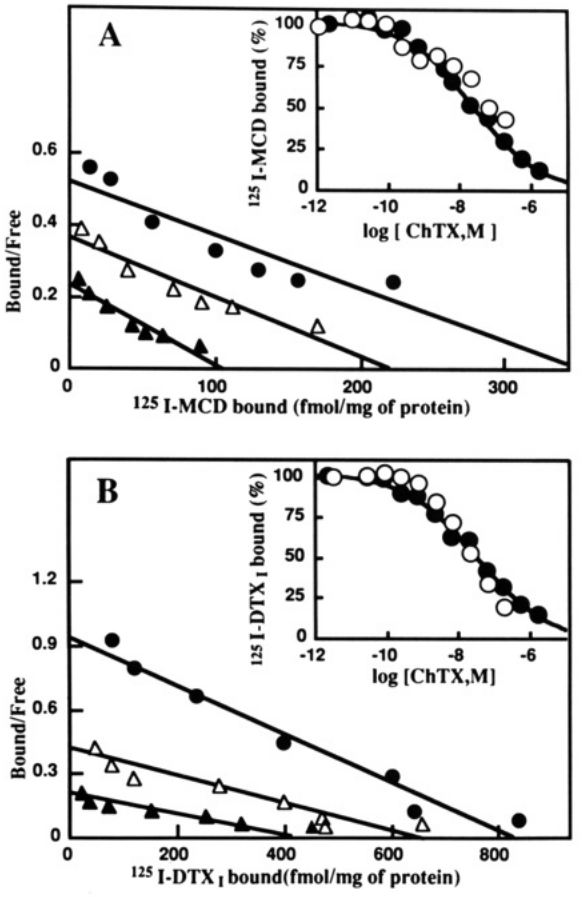

FIGURE 6: Effect of ChTX on ^{125}I-MCD (A) and ^{125}I-DTX_I (B) binding to synaptosomal receptors from rat brain. Main panels show Scatchard analysis of the free and bound ^{125}I-labeled toxin concentrations. The incubation mixtures contain no ChTX (●) or ChTX at concentrations of 3 (△) and 30 nM (▲). (A) Synaptic membranes (210 μg of protein/mL) were incubated with various concentrations of ^{125}I-MCD (3–340 pM). (B) Synaptic membranes (35 μg of protein/mL) were incubated with various concentrations of ^{125}I-DTX_I (6–400 pM). Insets show inhibition by ChTX of specific ^{125}I-labeled toxin binding to synaptic membranes (●) or to purified receptor (○). 10–20 pM ^{125}I-MCD (A) and ^{125}I-DTX_I (B) was incubated in the presence of various concentrations of ChTX either with synaptic membranes at 150 and 50 μg of protein/mL, respectively, or with purified receptor at 0.08 μg of protein/mL.

Inhibition of ^{125}I-MCD and ^{125}I-DTX_I binding by ChTX has also been observed with the purified MCD/DTX_I binding protein (Figure 6, insets). The inhibition curve is quite similar

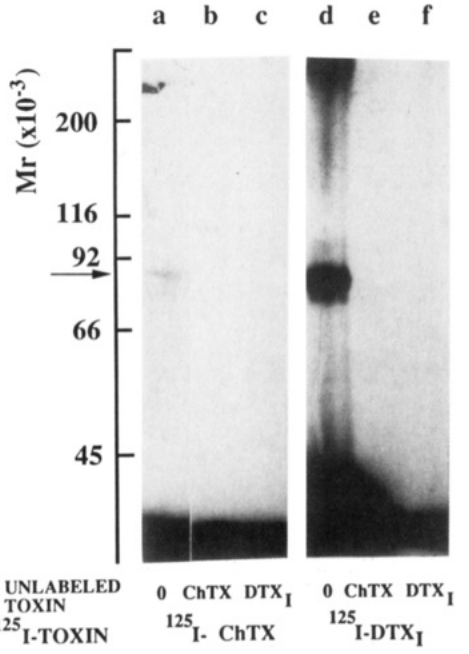

FIGURE 7: Cross-linking of ^{125}I-ChTX and ^{125}I-DTX_I to rat brain membranes. The indicated ^{125}I-labeled toxin was incubated in the absence (0) and in the presence of 200 nM indicated unlabeled toxin. Radiolabeled bands were detected by autoradiography of the Coomassie-stained dried gel after 21 days. The specifically labeled bands are indicated by an arrow.

to that observed with brain membranes (Figure 6, insets). This result clearly shows that the α-subunit of the MCD/DTX_I-sensitive K^+ channel also contains ChTX binding sites.

High-affinity binding sites in neuronal membranes are in different stoichiometries for ^{125}I-DTX_I (847 fmol/mg of protein) (Bidard et al., 1989), ^{125}I-MCD (163 fmol/mg of protein) (Bidard et al., 1989), and ^{125}I-ChTX (76 fmol/mg of protein). Nevertheless, both MCD and ChTX inhibit completely ^{125}I-DTX_I binding at its high-affinity sites. For MCD, this property is due to the fact that, depending on the brain region (Bidard et al., 1989), high-affinity ^{125}I-DTX_I binding sites are associated not only with high-affinity ^{125}I-MCD binding sites but also with a family of lower affinity MCD binding sites. The same situation holds for charybdotoxin. High-affinity DTX_I binding sites are clearly associated not only with high-affinity ChTX binding sites (Figure 5) but also with lower affinity ChTX binding sites (Figure 6). Similarly, if high-affinity ChTX binding sites seem to be associated with relatively low-affinity MCD binding sites (Figure 5), high-affinity MCD binding sites are clearly associated with low-affinity ChTX binding sites (Figure 6A). Low-affinity ChTX binding sites ($K_{0.5} \approx 10$ nM), probably because they are more prevalent as compared to high-affinity ($K_{0.5} = 142$ pM) binding sites, seem to have a major contribution in controling neurotransmitter release from total synaptosomes (Figure 3).

Labeling of ^{125}I-ChTX and ^{125}I-DTX_I Binding Sites by Cross-Linking Experiments. Cross-linking experiments with dimethyl suberimidate have been very useful in identifying the polypeptide chain (M_r = 76K–77K), which contains binding sites for DTX_I and MCD (Rehm et al., 1988). Cross-linking experiments with ^{125}I-ChTX identified a polypeptide component of M_r = 83K ± 2K (M_r = 79K ± 2K after correction for the M_r of the toxin) (Figure 7). Protection by homologous unlabeled ChTX and by DTX_I has demonstrated that the affinity labeling is specific. Figure 7 also shows that cross-linking of ^{125}I-DTX_I to its target, which reveals a very intense

band at M_r = 83K ± 2K (M_r = 76K ± 2K after correction of the M_r of the toxin), is protected not only by unlabeled DTX_I but also by ChTX.

The difference in efficacy of cross-linking experiments with ^{125}I-ChTX and ^{125}I-DTX_I is most probably due to the difference in the number of high-affinity binding sites for the two toxins.

Conclusion. ChTX has been previously described as a specific blocker of high-conductance Ca^{2+}-activated K^+ channels (Miller et al., 1985; Smith et al., 1986) whereas both dendrotoxins and MCD have been described as blockers of a class of voltage-sensitive K^+ channels with a conductance of 5–10 pS which are insensitive to concentrations of intracellular Ca^{2+} (Penner et al., 1986; Stansfeld & Feltz, 1988; Harvey & Anderson, 1985; Stansfeld et al., 1987).

The purification procedure described in this work used the property of one of the toxins of the venom of the scorpion *L. quinquestriatus hebraeus* to inhibit ^{125}I-DTX_I and ^{125}I-MCD binding to their respective sites. The purified toxin not only inhibited ^{125}I-DTX_I and ^{125}I-MCD associations to their receptors but also produced neurotoxic symptoms and neurotransmitter release from synaptosomes, which are also similar to those of DTX_I and MCD although these three toxins have quite different sequences. Therefore, it came as a surprise to us to finally find out that the *Leiurus* toxin was in fact charybdotoxin, a toxin classically used as a specific tool to block one class of Ca^{2+}-activated K^+ channels (Cook, 1988; Miller et al., 1985; Moczydlowski et al., 1988; Smith et al., 1986). Charybdotoxin is clearly not selective for the Ca^{2+}-activated K^+ channel. Properties of its brain membrane receptor sites indicate that it belongs to the family of toxins which already includes DTX_I and MCD (Penner et al., 1986; Stansfeld & Feltz, 1988; Harvey & Anderson, 1985; Stansfeld et al., 1987), two toxins which block a class of voltage-sensitive K^+ channels. In addition, recent patch–clamp experiments have shown (Schweitz et al., 1989) that charybdotoxin does indeed block DTX_I-sensitive K^+ channels. It may be that charybdotoxin predominantly blocks the voltage-sensitive K^+ channel in the brain whereas it is mainly active on Ca^{2+}-activated K^+ channels in other cell types such as intestinal smooth muscle cells (Figure 2). All results presented in this work suggest that ^{125}I-ChTX only identifies voltage-dependent K^+ channels in brain membranes: (i) ^{125}I-ChTX binding is completely inhibited by DTX_I and MCD, two toxins which are inactive on Ca^{2+}-activated K^+ channels; (ii) affinity labeling experiments (Figure 7) only identify one polypeptide, which corresponds to the DTX_I/MCD binding protein known to be a voltage-dependent K^+ channel. There is no indication from this work that charybdotoxin could serve as a probe for the identification of the Ca^{2+}-activated K^+ channel in brain membranes. A possible interpretation is that the number of charybdotoxin-sensitive Ca^{2+}-activated channels is very small as compared to the number of charybdotoxin-sensitive voltage-dependent K^+ channels [maybe because of the high conductance (≈200 pS) of the Ca^{2+}-activated K^+ channels].

Since charybdotoxin can block both Ca^{2+}-sensitive and voltage-sensitive K^+ channels, these two classes of channels probably have very significant structural homologies.

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Registry No. ChTX, 95751-30-7; GABA, 56-12-2; DTX, 107950-33-4; MCD, 83856-13-7.

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Calcium-Binding ATPase Inhibitor Protein of Bovine Heart Mitochondria. Role in ATP Synthesis and Effect of Ca^{2+} †

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ABSTRACT: Submitochondrial particles (A particles) and phosphorylating electron-transport particles (ETP_H) were prepared from bovine heart mitochondria. The A particles either were supplemented with or were depleted of the mitochondrial calcium-binding ATPase inhibitor protein (CaBI). The CaBI-depleted A particles still retained the Pullman–Monroy ATPase inhibitor protein (PMI), and the other particles all contained both CaBI and PMI. ATP synthase and ATPase activities of the particles were measured in similar reaction mixtures by luminescence of firefly luciferin–luciferase. Succinate was the respiratory substrate, and the adenylate kinase inhibitor P^1,P^5-di(adenosine-5′) pentaphosphate was obligatory. The ATP synthase activity of CaBI-depleted A particles was 30–40% of that of the A and ETP_H particles, and its ATPase activity was 7–8 times greater. Reconstitution of the CaBI-depleted A particles with CaBI restored the original ATP synthase and ATPase activities. ATP synthase activity rose about 1.7-fold when A particles were supplemented with additional CaBI and ATPase activity dropped to 9% of the original. Varying Ca^{2+} levels had little or no effect on the ATP synthase and ATPase activities of the CaBI-depleted A particles. In contrast, ATP synthase activity of the other particles was decreased by as much as 70% at the optimal Ca^{2+} concentration of 1 μM, and the ATPase activity of the A and ETP_H particles rose concomitantly by 7–8-fold. The ATP synthase and ATPase activities of all the particles in 1 μM Ca^{2+} became like those of the CaBI-depleted A particles. These changes were reversible; normal activities were restored as Ca^{2+} concentrations were raised above 1 μM. Thus, CaBI influence both ATP synthesis and hydrolysis, and Ca^{2+} modulates both through CaBI.

The calcium-binding ATPase inhibitor protein (CaBI)[1] of mitochondria of rat skeletal muscle and bovine heart (Yamada et al., 1980) was discovered during the course of studies to determine why the energy-dependent uptake of Ca^{2+} by mitochondria takes preference over oxidative phosphorylation (Rossi & Lehninger, 1964; Vercesi et al., 1978) and results in the stimulation of mitochondrial ATP hydrolysis (Hunter et al., 1976; Bygrave, 1977). It was found that Ca^{2+}, in micromolar concentrations, caused the release of CaBI from submitochondrial particles with a concurrent stimulation of from 5-fold to just over 8-fold in the ATPase activity of the particles (Yamada et al., 1980, 1981). The CaBI-depleted particles (Yamada & Huzel, 1988) still retained some amounts of the ATPase inhibitor protein (PMI) first isolated by Pullman and Monroy (1963) from bovine heart mitochondria. The dimeric form of CaBI was the active inhibitor (Yamada et al., 1981). The molecular weights of the monomeric and dimeric forms and the cleavage of the CaBI dimer by thiol compounds were reminiscent of ATPase inhibitor proteins isolated earlier which were believed to be forms of PMI (Knowles & Penefsky, 1972). Paradoxically, however, PMI isolated by the conventional method of alkaline extraction (Pullman & Monroy, 1963) was not cleaved by thiol compounds (Knowles & Penefsky, 1972). Moreover, unlike CaBI, PMI does not contain cysteine residues (Frangione et al., 1981). Later, PMI and CaBI were both isolated from bovine heart mitochondria and shown to be distinct proteins (Yamada & Huzel, 1988).

Pullman and Monroy (1963) showed that PMI inhibited the hydrolytic activity of the ATP synthase·ATPase complex of submitochondrial particles concomitantly with recoupling of the particles and an increase in P:O ratios. Whether PMI simply conserved ATP or had a direct effect on ATP synthesis was the subject of later research by others [see Schwerzman and Pedersen (1986) and Lippe et al., (1988a,b)]. It has been proposed that PMI must be released or translocated to a new site in order that ATP synthesis can proceed. Yet another proposal was that PMI exists in active and inactive forms (Panchenko & Vinogradov, 1985). Recently, the entrapment

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[1] Abbreviations: PMI, Pullman and Monroy mitochondrial ATPase inhibitor protein; CaBI, Ca^{2+}-binding mitochondrial ATPase inhibitor protein; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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