Characterization of High Affinity Binding Sites for Charybdotoxin in Synaptic Plasma Membranes from Rat Brain

EVIDENCE FOR A DIRECT ASSOCIATION WITH AN INACTIVATING, VOLTAGE-DEPENDENT, POTASSIUM CHANNEL

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Charybdotoxin (ChTX), a potent peptidyl inhibitor of several types of K⁺ channels, binds to sites in vascular smooth muscle sarcolemma (Vázquez, J., Feigenbaum, P., Katz, G. M., King, V. F., Reuben, J. P., Roy-Contancin, L., Slaughter, R. S., Kaczorowski, G. J., and Garcia, M. L. (1989) J. Biol. Chem. 265, 20902-20909) which are functionally associated with a high conductance Ca⁺⁺-activated K⁺ channel (PKCa). ¹²⁵I-ChTX also binds specifically and reversibly to a single class of sites in plasma membranes prepared from rat brain synaptosomes. These sites exhibit a Kₐ of 25-30 pM, as measured by either equilibrium or kinetic binding protocols and display a maximum density of about 0.3-0.5 pmol/mg of protein. Competition studies with native ChTX yield a Kᵢ of 8 pM for the noniodinated toxin. The highest density of ChTX sites exists in vesicle fractions of plasma membrane origin. Binding of ¹²⁵I-ChTX is modulated by metal ions that interact with K⁺ channels: Ba⁺⁺, Ca⁺⁺, and Cs⁺ cause inhibition of ChTX binding; Na⁺ and K⁺ stimulate binding at low concentration before producing complete inhibition as their concentration is increased. Stimulation of binding is due to an allosteric interaction that decreases Kᵢ whereas inhibition results from an ionic strength effect. Tetraethylammonium ion has no effect on binding, but tetrabutylammonium ion blocks binding with a Kᵢ of 2.5 mM. Different toxins (i.e. α-dendrotoxin, noxiustoxin) that inhibit an inactivating, voltage-dependent K⁺ channel (PKV) block ¹²⁵I-ChTX binding in brain. In marked contrast, iberiotoxin, a selective inhibitor of PKCa, has no effect on ChTX binding in this preparation. Inhibition of ChTX binding by α-dendrotoxin and noxiustoxin results from an allosteric interaction between separate binding sites for these agents and the ChTX receptor. Taken together, these results suggest that the ChTX sites present in brain are associated with PKV rather than with PKCa. Therefore, ¹²⁵I-ChTX is a useful probe for elucidating the biochemical properties of a number of different types of K⁺ channels.

Charybdotoxin (ChTX) is a 37-amino acid highly basic peptide. It has been purified to homogeneity from venom of the scorpion Leirus quinquestriatus var. hebraeus, and its primary structure has been determined (1). ChTX was first described as a potent inhibitor of the high conductance (about 250 pS) Ca⁺⁺-activated K⁺ channel (PKCa) that is present in muscle and neuroendocrine tissues (2). Subsequently, ChTX was shown to block other types of K⁺ channels including a 35-pS Ca⁺⁺-activated K⁺ channel present in Aplysia neurons (3); Ca⁺⁺-activated K⁺ channels of intermediate conductances in brain (4); a voltage-dependent K⁺ channel characterized in lymphocytes (5, 6); the Drosophila "shaker" K⁺ channel expressed in oocytes (7) but not the native channel present in fly muscle (8); a neuronal, slowly inactivating K⁺ channel that was expressed in oocytes (9); and a similar, voltage-dependent, K⁺ channel present in brain (10, 11). Despite its ability to inhibit a number of different types of K⁺ channels, ChTX does not block the apamin-sensitive, low conductance, Ca⁺⁺-activated K⁺ channel (12), a rapidly inactivating (A-type) K⁺ channel from GH₃ cells (1), a delayed rectifying K⁺ channel from brain (13), or other types of ion channels (i.e. Na⁺ channels, L- or T-type Ca⁺⁺ channels; 1, 3).

Recently, the moniodotyrosine adduct of ChTX (¹²⁵I-ChTX) was prepared (14). It blocks PKCa by a mechanism that is identical to that of the native toxin although its potency as an inhibitor is about 10-fold reduced. In this study, ¹²⁵I-ChTX was found to associate with a single class of high affinity binding sites in sarcolemmal membrane vesicles prepared from bovine aortic smooth muscle. These receptors display properties that indicate that they are functionally associated with PKCa. Recently, it has also been shown that ChTX inhibits a slowly inactivating, voltage-dependent, K⁺ channel (PKV) present in synaptosomes (10) and cultured neuronal cells (11). Although it has been suggested that ChTX interacts directly with this channel because of its ability to modulate binding of other high affinity peptidyl probes of PKV (15, 16), direct binding of ChTX to this target has either not been reported (15) or was incompletely described (16). For these reasons, it was of interest to explore the binding of ¹²⁵I-ChTX to plasma membranes prepared from brain and to determine whether the resulting data identify sites that are coupled to PKCa or PKV.
The results presented in this investigation indicate that $^{125}$I-ChTX binds to a single class of sites in rat brain synaptic plasma membrane vesicles. The characteristics of these receptors suggest that they are functionally associated with $P_{K,V}$ in this tissue. No evidence for the association of ChTX with sites coupled to $P_{K,Ca}$ or other types of $K^+$ channels has been observed. These data indicate that radiolabeled ChTX is a useful probe for elucidating the biochemical properties of other types of $K^+$ channels besides $P_{K,Ca}$ and for aiding in their purification. A preliminary report of these findings has been made in abstract form (17).

**EXPERIMENTAL PROCEDURES**

**Materials**—L. quinquestratus var. hebraeus venom was obtained from Latoxan Scorpion Farm, Rosans, France; Buthus tamulus venom was purchased from Sigma. $^{125}$I was bought from Amersham Corp., and the reagent IODO-GEN was supplied by Pierce Chemical Company. $[^{3}H]PN 200-110$ was obtained from Du Pont-New England Nuclear. All other reagents were purchased from commercial sources and were of the highest purity commercially available. $\alpha$-Dendrotoxin was a gift of Dr. R. Stein, Blausen University of Medical Sciences. Monoiodotyrosine ChTX was generously provided by Drs. Ruth Nutt and Robert Stein, Merck Sharp and Dohme Research Laboratories, West Point, PA. Various drugs were obtained from the sample collection of Merck and Co. Glass fiber filters (GF/C) were bought from Whatman.

**Purification and Iodination of Toxins**—ChTX was purified to homogeneity from scorpion venom as outlined previously (1). Stock solutions of native ChTX were prepared in 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin. The concentration of ChTX in solution was calculated from the known extinction coefficients of this peptide (1). ChTX was iodinated using the IODO-GEN method (18) as described (14, 15). Briefly, $10 \mu l$ of a peptide solution containing $5 \mu g$ of ChTX in 100 mM sodium phosphate, pH 7.3, was mixed with $0.5 \mu g$ of dried IODO-GEN to which was then added $1 \mu Ci$ of Na$^{25}$I ($10 \mu l, 2000$ Ci/mmol). After 15 min, the iodination mixture was injected into a C$_{18}$ reversed-phase high performance liquid chromatography column (Vydac) equilibrated with 10 mM trifluoroacetic acid, and elution was achieved with a linear gradient (0-14%) of isopropyl alcohol/acetonitrile, 2:1, in 4 mM trifluoroacetic acid applied over a 40-min period at a flow rate of 0.5 ml/min. Using this procedure, monoisodiotyrosine ChTX is well separated from various other iodinated peptide derivatives. $^{125}$I-ChTX obtained after column chromatography was made 0.5% in bovine serum albumin, lyophilized, reconstituted with 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and stored at $-20^\circ C$ until use. Iodotritotoxin (I$^{125}$T) was purified to homogeneity from B. tamulus venom as described previously (21).

**Preparation of Synaptic Plasma Membrane Vesicles from Rat Brain**—Rats (Wistar, 150-250 g) were killed by guillotine, their brains were rapidly dissected, and this material was placed in ice-cold homogenization buffer ($250$ mM sucrose, 1 mM K$_{EDTA}$, and 10 mM Tris-HCl, pH 7.4). Synaptosomes were prepared by methods reported previously (20). The synaptosomes were lysed by osmotic shock, and high purified synaptic plasma membrane vesicles were isolated as described (21). These vesicles were resuspended in 50 mM Tris-HCl, pH 7.4, rapidly frozen in liquid $N_2$, and stored at $-70^\circ C$. The binding activity for ChTX is stable for at least 6 months.

**Binding Assays**—All ChTX binding reactions were carried out in $12 \times 75$-mm polystyrene tubes since the toxin adsorbs to glass surfaces in low ionic strength medium. The incubation medium (200 $\mu l$) consisted of 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin, unless otherwise indicated. Nonspecific binding was determined in the presence of $10 \mu M$ native ChTX, and incubations were carried out at 25°C. The binding reactions were quenched by the addition of ice-cold 100 mM NaCl, 20 mM Tris/Hepes, pH 7.4 (4 mM), followed by rapid filtration through GF/C filters that had been presoaked in 0.3% polyethyleneimine, and succeeded by two washes with quench buffer. Using this protocol, non-specific binding represents less than 20% of the total radioactivity added; and at a $K_d$ concentration of ligand, specific binding is 90% of total binding. There is no significant displaceable binding of $^{125}$I-ChTX from GF/C filters under these conditions. In each experiment, triplicate assays were routinely performed, and the data were averaged. The standard error of the mean of these results was typically less than 5%. $[^{3}H]PN 200-110$ binding studies were carried out as described previously (21).

**Analysis of Data**—The results from saturation binding experiments were subjected to a Scatchard analysis, and linear regression was performed to obtain the equilibrium dissociation constant ($K_d$) and the maximum receptor concentration ($B_{max}$). The correlation coefficients for these plots were typically greater than 0.95. Data from competition experiments were analyzed by the method of Cheng and Prusoff (22) to determine $K_i$ values. Kinetic data obtained from ligand association and dissociation experiments were subjected to the analysis of Weiland and Molinoff (23). The dissociation rate constant for ChTX ($k_d$) was determined directly from the first order plot of ligand dissociation versus time. The association rate constant ($k_a$) was determined from the equation $k_a = k_{so} [L]/([L] - [LR]_{max})$ where [L] is the concentration of ligand, [LR] is the concentration of the ligand-receptor complex at equilibrium, [LR]$_{max}$ is the maximum number of receptors present on the receptor protein which interact with the ChTX binding site at a given affinity for the ligand. The allosteric interaction is reflected by a change in $a$ on the $K_a$ of ChTX. The possibility that more than one cation interacts with the protein to produce the stimulatory effect is taken into account by introducing Hill’s approximation in the formulation. Employing these assumptions, the effect of cation concentration on $^{125}$I-ChTX binding is given by Equation 1.

$$B/Bo = \left(1 + L/K_{a0}\right) - L/K_{d} + L/K_{a0} \cdot a/K_{a0}\left(\right)$$

where $B$ is the concentration of $^{125}$I-ChTX bound, $B_o$ is the concentration of $^{125}$I-ChTX bound in the absence of metal ions, $I$. is the concentration of $^{125}$I-ChTX in solution, $K_{d0}$ is the equilibrium dissociation constant of $^{125}$I-ChTX in the absence of metal ions, $K_{a0}$ is the same dissociation constant with metal ion present, $A$ is the concentration of metal ion, $n$ is the Hill coefficient, $K_{a0}$ is the dissociation constant of one cation from its binding site, and $a$ is the factor that reflects the allosteric interaction between metal ion and ChTX binding sites.

Initially, the inhibitory effects of metal ions on $^{125}$I-ChTX binding which occur in the millimolar range were analyzed by assuming the existence of additional binding sites that increase $K_{a0}$ allosterically. In this simulation univocally demonstrated the inability of such a model to explain both metal ion activating and inhibitory effects (not shown). It was therefore assumed that inhibition of ChTX binding was due to a nonspecific effect of ionic strength on $K_{d0}$ as has been shown for the interaction of ChTX with $F_{SCG}$ (14, 24, 25). In this model, it is assumed that the charged region of fixed negative charge density near the toxin binding site, which establishes a local electrostatic potential that in turn raises the local concentration of ChTX. Increasing the salt concentration would screen the negative charge density near the toxin binding site, which establishes a local electrostatic potential that in turn raises the local concentration of ChTX. Increasing the salt concentration would screen the negative charge density near the toxin binding site, which establishes a local electrostatic potential that in turn raises the local concentration of ChTX. Increasing the salt concentration would screen the negative charge density near the toxin binding site, which establishes a local electrostatic potential that in turn raises the local concentration of ChTX.
experimental data (Fig. 4B). The numerical values of the parameters used to fit the model are shown in Table II and are discussed further under “Results.”

From Equations 1 and 2, it can be demonstrated easily that a Scatchard analysis of data obtained from equilibrium binding of 125I-ChTX in the presence of a fixed concentration of metal ion should generate a straight line. In such representations, only the $K_0$ for ChTX will be affected, with no change in $B_{max}$ values. The apparent ChTX dissociation constant ($K_{eq}$) obtained from the slope of this linear representation is given by Equation 3.

$K_{eq} = (1 + A/\alpha K_0)/(1 + a A/\alpha K_0) \cdot K_0 \cdot 10^{10}$

Protein Determination—The concentration of membrane protein was determined by the method of Lowry et al. (28) using bovine serum albumin as a standard.

RESULTS

Characterization of ChTX Binding Sites in Brain Synaptic Membranes—To determine whether binding sites exist for ChTX in rat brain synaptic plasma membrane vesicles, the interaction of 125I-ChTX with this preparation was monitored. When vesicles are incubated with increasing concentrations of radiolabeled probe until equilibrium is achieved, 125I-ChTX associates with membranes in a concentration-dependent fashion (Fig. 1A). Repetition of this experiment in the presence of 10 nM nonlabeled peptide results in a pattern of 125I-ChTX association which is linearly dependent on toxin concentration. The specific binding of ChTX, defined as the difference between total radiolabeled toxin and binding in the presence of excess native peptide, is a saturable function of 125I-ChTX concentration. A Scatchard analysis of these data (Fig. 1B) indicates the presence of a single class of binding sites for ChTX, whose $K_0$ is 29 pm and $B_{max}$ is 0.3 pmol/mg of protein. These are typical parameters for the ChTX interaction, since in five different vesicle preparations, $K_0$ values of 25–30 pm and corresponding $B_{max}$ values of 0.3–0.5 pmol/mg of protein have been measured.

Several characteristics of ChTX binding in brain have been determined. 125I-ChTX binding is linear with synaptic membrane protein up to a concentration of 80 μg of membrane protein per ml. If vesicles are first treated with trypsin (0.26 mg/ml, 30 min, at 37°C), 125I-ChTX binding is decreased by 70%, suggesting that the binding site is of a proteinaceous nature. When the temperature dependence of ChTX binding is monitored at 4, 25, and 37°C, no significant differences are found in binding parameters at either 25 or 37°C, but binding is diminished 4-fold at 4°C when measurements are made using a $K_0$ concentration of toxin. If rat brain membranes are first treated with low concentrations of detergents (e.g., CHAPS, octyl glucoside) that do not solubilize these vesicles but which disrupt their permeability barrier, binding of 125I-ChTX does not appear to be enhanced significantly. These results indicate that the majority of vesicles in this preparation are of an outside-out polarity, since ChTX associates with K⁺ channels exclusively at the external pore (cf. Ref. 14 for further evidence obtained by monitoring ChTX binding to vascular smooth muscle sarcolemmal membranes, which supports this interpretation). It is also noteworthy that the affinity measured for 125I-ChTX in brain is higher than that determined for the interaction of monoiodotyrosine ChTX with P₄₅₀ in vascular smooth muscle sarcolemmal membranes (see below; 14).

The kinetics of ChTX binding have been measured to ascertain whether toxin associates with its receptor site through a simple bimolecular reaction. The data shown in Fig. 2A indicate that when vesicles are incubated with a sub-$K_0$ concentration of 125I-ChTX, toxin associates with membranes in a time-dependent fashion, and equilibrium is achieved in approximately 2 min. The nonspecific binding component of ChTX, determined in the presence of excess nonradiolabeled peptide (10 nM), is time independent and has been subtracted from the experimental data in Fig. 2A. A semilogarithmic transformation of these data (Fig. 2A, inset) yields a linear plot as expected for a pseudo-first order reaction, and the slope of this representation gives $k_{obs}$. The association rate constant for ChTX, $k_1$, calculated as described under “Experimental Procedures,” is $6 \times 10^9$ M⁻¹ s⁻¹. This value is greater than the diffusion control rate expected for a small peptide and indicates the possibility of an electrostatic interaction between the positively charged toxin and negative charges located on the ChTX receptor. Notably, this value is also 10-fold greater than $k_1$ measured for 125I-ChTX binding to P₄₅₀ channel sites in smooth muscle sarcolemmal membranes. Dissociation of prebound 125I-ChTX from synaptic plasma membranes, initiated by the addition of excess nonradiolabeled toxin, yields data that fit a single exponential relationship with a $t_1/2$ of about 30 s (Fig. 2B). A semilogarithmic plot of these data generates a linear relationship as expected for a first order reaction (Fig. 2B, inset), and the slope of this representation provides a $k_1$ value of $1.8 \times 10^7$ s⁻¹ for the dissociation rate constant. This value is 3.5-fold greater than the $k_1$ rate constant of 125I-ChTX dissociation from receptors in aortic sarcolemmal membranes. The $k_2$ calculated from the kinetic measurements made in brain is 27 pm, a value identical to that determined under equilibrium conditions. These results suggest that ChTX binding is a completely reversible process that occurs via a single step reaction. The $k_1$ and $k_2$, values for binding of ChTX in brain have also been determined independently by measuring the kinetics of 125I-ChTX binding at different ligand concentrations (5–60 pM). When these data are analyzed in semilogarithmic representations, the slopes of these plots yield $k_{obs}$ values at each toxin concentration. As illustrated in Fig. 3C, a plot of $k_{obs}$ versus ChTX concentration generates a linear representation in which $k_1$ is obtained from the slope, and $k_2$ is given by the y intercept. These results give values for $k_1$ and $k_2$, of 7.5 × 10⁹ M⁻¹ s⁻¹ and 1.95 × 10⁹ M⁻¹ s⁻¹, respectively. Such values produce a corresponding $K_0$ of 26 pm and confirm that a simple bimolecular interaction takes place with ChTX in brain plasma membranes.

To determine whether native toxin displays a similar affinity for its receptor in brain as monoiodotyrosine ChTX,
competition experiments were performed. Typical results are illustrated in Fig. 3A, and the $K_i$ value for ChTX has been determined from these data using the Cheng-Prusoff relationship (22). Native ChTX completely displaces $^{125}$I-ChTX from its receptor with a $K_i$ value of 8–10 pm in the presence of 50 mM Na$. Therefore, monosubstitution of Tyr$^3$ with iodine results in approximately a 2–3-fold loss in ligand affinity. However, this loss of activity is less than was found when $K_d$ values of native toxin are compared in brain and smooth muscle under equivalent experimental conditions, a loss of approximately 4-fold was noted (14). Interestingly, when $K_d$ values of native toxin are compared in brain and smooth muscle under equivalent experimental conditions, there is a 5-fold difference in affinity of this peptide in the two tissues, with binding being more potent in brain. These results suggest that ChTX binds to distinct sites in these two tissues.

The pH dependence of ChTX binding in brain plasma membranes has been ascertained by performing experiments in the pH range 6–9 (Fig. 3B). Binding of $^{125}$I-ChTX to synaptic membrane vesicles increases 4–5-fold from pH 6.0 to 8.0 and then plateaus before decreasing at pH 9.0. Since the $p$ of ChTX is known to be approximately 10, and binding to 2 as well as inhibition of K$^+$ channels appears to involve an electrostatic interaction between basic toxin residues and acidic functions on the channel, this pH profile probably reflects titration of negatively charged residues at the ChTX binding site. It is important to note that the magnitude of the increase in ChTX binding activity with pH in brain is significantly different from that observed for ChTX binding to $P_{K_{Ca}}$ in vascular smooth muscle.

To determine the cellular location of ChTX binding sites in brain, different fractions obtained during the purification of plasma membranes have been tested for their ability to bind $^{125}$I-ChTX. The results of these experiments are presented in Table I. To carry out these determinations, approximately the same quantity of protein from each fraction obtained during plasma membrane isolation was subjected to $P_{K_{Ca}}$ binding until equilibrium was established. In parallel experiments, binding of $[^3H]$PN 200-110, a dihydropyridine known to interact with L-type Ca$^{2+}$ channels that are localized in the plasma membrane, was also measured. ChTX binding sites co-purify with the membrane fraction enriched in PN 200-110 receptor activity (not shown). There is approximately a 4-fold purification of ChTX binding activity in the most highly purified synaptic plasma membrane fraction as compared with the starting tissue homogenate. These results indicate that ChTX receptors are present in the plasma membrane, which is consistent with the localization of K$^+$ channels within the cell.

**FIG. 3. Effect of native toxin and pH on $^{125}$I-ChTX binding to brain synaptic plasma membrane vesicles.** A, membrane vesicles were incubated with 18 pm $^{125}$I-ChTX in the absence or presence of increasing concentrations of native ChTX at 22°C until equilibrium was achieved. Inhibition of binding was assessed relative to toxin association in untreated vesicles. B, membrane vesicles were incubated with 23 pm $^{125}$I-ChTX at 22°C in media of different pH until equilibrium was achieved. Specific binding data are presented versus pH.

**FIG. 2. Association and dissociation kinetics of $^{125}$I-ChTX in brain synaptic plasma membrane vesicles.** A, association kinetics. Membrane vesicles were incubated with 8.5 pm $^{125}$I-ChTX at 22°C for different periods of time. Non-specific binding determined in the presence of 10 nm ChTX is time invariant and has been subtracted from the data. Inset, a semilogarithmic representation of the pseudo-first order association reaction, where $B_a$ and $B_t$ represent ligand bound at equilibrium and time $t$, respectively. B, dissociation kinetics. After incubating vesicles with 8.5 pm $^{125}$I-ChTX at 22°C for 30 min, toxin dissociation was initiated by addition of 10 nm ChTX and incubating at 22°C for different periods of time. Inset, a semilogarithmic representation of the first order dissociation reaction. C, relationship between $k_{off}$ and $^{125}$I-ChTX concentration. Membrane vesicles were incubated at 22°C with the indicated concentrations of $^{125}$I-ChTX for different periods of time. The slopes ($k_{off}$) of each semilogarithmic representation of the various pseudo-first order association reactions are plotted as a function of toxin concentration.

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$^{125}$I-ChTX Bound pmol/mg protein</th>
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<tbody>
<tr>
<td>Homogenate</td>
<td>0.036</td>
</tr>
<tr>
<td>Lysed synaptosomes</td>
<td>0.077</td>
</tr>
<tr>
<td>0.6/1.0 M interface-fraction I</td>
<td>0.15</td>
</tr>
<tr>
<td>1.0/1.2 M interface-fraction II</td>
<td>0.078</td>
</tr>
<tr>
<td>Pellet</td>
<td>0.039</td>
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</tbody>
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Localization of $^{125}$I-ChTX binding sites in brain

Membrane vesicles were prepared from rat brain and fractionated on a discontinuous sucrose density gradient as described (21). The homogenate refers to a crude synaptosomal preparation, derived after tissue homogenization and centrifugation to remove debris; the applied sample consists of lysed synaptosomes isolated after high speed centrifugation. Aliquots of each fraction containing 0.6 mg of protein were incubated with 27 pm $^{125}$I-ChTX in 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin at room temperature until equilibrium was achieved. Specific binding data are presented in each case.
varying degrees of potency (27–31). For example, Cs⁺ and Ba²⁺ block voltage-dependent K⁺ channels whereas TEA is a relatively potent inhibitor of Pₓₐₓ. Certain monovalent cations, besides the substrate K⁺, also interact with K⁺ channels, although some of these metal ions display limited permeability (e.g. Na⁺, Ca²⁺). Therefore, it was of interest to determine the effects of different ions on the ChTX receptor in brain membranes.

When vesicles are incubated with ¹²⁵I-ChTX in the presence of increasing concentrations of either Ba²⁺, Cs⁺, Ca²⁺, TBA, or TRA, a variety of effects on ChTX binding is noted (Fig. 4A). The first four ions produce concentration-dependent inhibition of toxin binding activity with Ki values of 25 μM, 50 μM, 500 μM, and 2.5 mM, respectively. In contrast, TBA has no effect whatever on the interaction of ChTX with brain membranes. This is in marked contrast to observations made with TEA in vascular smooth muscle, where this agent is a potent inhibitor of ChTX binding (14). However, TBA, which is a known inhibitor of Pₓₐₓ, does block the interaction of ChTX in brain membranes.

Interestingly, when ChTX binding is monitored in the presence of either K⁺ or Na⁺, a unique pattern of receptor modulation is observed. At low concentrations (below 20 mM), these ions stimulate ChTX binding approximately 2.5- and 1.5-fold, respectively, before producing complete inhibition of binding as their concentrations are increased (Fig. 4B). Li⁺, on the other hand, causes only inhibition of ChTX binding (Fig. 4B), as does choline (not shown). The concentration dependence of each of these ions as inhibitors of ChTX binding is very steep. This pattern likely reflects the effects of ionic strength on toxin binding because any electrostatic interaction between peptide and receptor would be diminished by increasing the ionic concentration of the medium. In contrast, the marked enhancement of ChTX binding caused by low concentrations of K⁺ and Na⁺ probably reflects a direct interaction of these ions with the ChTX receptor. The data shown in Fig. 4B have been described by a mathematical model that accounts for the activating and inhibitory behavior of the various ions (see “Experimental Procedures”). The numerical values of the parameters used to fit this model to the experimental data are listed in Table II. They suggest that two K⁺ bind to the ChTX receptor moiety whereas only a single Na⁺ interacts at this locus. In addition, the dissociation constants obtained for K⁺ and Na⁺ (about 0.1 and 2.0 mM, respectively) indicate that the affinity of K⁺ is 10-fold greater than that of Na⁺. Furthermore, the analysis predicts that upon binding of K⁺ or Na⁺, the Kₐ of ChTX is allosterically decreased, either 4- or 3-fold, respectively (κ-value). The small variation in β from one ion to another, upon inhibition of binding, argues in favor of a nonspecific interaction for K⁺, Na⁺, and Li⁺ in the high millimolar concentration range, the behavior expected for an ionic strength effect.

To confirm the predicted mechanisms by which various ions modulate ChTX binding in brain, their effects have been studied by monitoring the ChTX interaction under saturation conditions (Fig. 5). A Scatchard analysis of these results indicates that stimulation of toxin binding by low concentrations of either Na⁺ or K⁺ is due to an increase in ligand affinity, consistent with the manifestation of an allosteric interaction and the assumptions made to develop the mathematical model. In the case of K⁺, the Kₛ of ChTX is allosterically decreased, either 4- or 3-fold, respectively (κ-value). The small variation in β from one ion to another, upon inhibition of binding, argues in favor of a nonspecific interaction for K⁺, Na⁺, and Li⁺ in the high millimolar concentration range, the behavior expected for an ionic strength effect.

The data from Fig. 4B were fitted to the model described under “Experimental Procedures” by a nonlinear, least squares, Newton-Gauss iterative method. Parameters are expressed as the optimum value ± S.D.

![Figure 4: Effect of ions on ¹²⁵I-ChTX binding to brain synaptic plasma membrane vesicles.](http://www.jbc.org/)
effect whatsoever on 125I-ChTX binding in brain. These re-
ibertiotoxin is a selective inhibitor of PK,~,* it appears that
ulate ChTX binding in either a competitive or allosteric
33) and NxTX3 are known to block PK,V in brain whereas
significant effect on ligand affinity. This pattern suggests that
modulation of ChTX binding by these two toxins occurs via
an allosteric interaction with a distinct binding site(s) for LY-
DaTX and NxTX.

in Fig. 6B, both c-DaTX and NxTX inhibit ChTX binding
increasing concentrations of either NxTX (A), c-DaTX (i), or IbTX
representation.

To elucidate further the pharmacological properties of the
ChTX site in brain, various compounds that are known to
interact with different types of ion channels were tested for
their ability to modulate the binding reaction. The first group
of agents studied is K+ channel toxins. As shown by the data
in Fig. 6A, both a-dendrotoxin (a-DaTX) and noxiustoxin
(NxTX) are able to inhibit completely the binding of ChTX
to brain plasma membranes in a concentration-dependent
fashion. The K values for these two toxins are 20 and 8 pM,
respectively. The Hill coefficient for NxTX is unity whereas
a-DaTX displays a nH greater than 1. In marked contrast,
iberiotoxin has no effect on ChTX binding in brain at con-
centrations up to 100 nM (Fig. 6A). Since a-DaTX (16, 32,
33) and NxTX are known to block Pk,V in brain whereas
iberiotoxin is a selective inhibitor of Pk,~,* it appears that
the ChTX binding site under present investigation is func-
tionally associated with an inactivating, voltage-dependent
K channel. To ascertain whether a-DaTX and NxTX mod-
ulate ChTX binding in either a competitive or allosteric
fashion, the action of these toxins was studied under satura-
tion binding conditions. As shown by the Scatchard analyses
in Fig. 6B, both a-DaTX and NxTX inhibit ChTX binding
by noncompetitively decreasing receptor site density, with no
significant effect on ligand affinity. This pattern suggests that
modulation of ChTX binding by these two toxins occurs via
an allosteric interaction with a distinct binding site(s) for a-
DaTX and NxTX.

To elucidate further the pharmacological properties of the
ChTX site in brain, the actions of a variety of different ion
channel modulators were monitored for their ability to inter-
fere with this binding reaction. These agents include L-type
Ca2+ channel blockers (i.e. nitrendipine, diltiazem, D-600,
fluspirilene, cinnarizine), N-type Ca2+ channel blockers (i.e.,
~ω-conotoxin, Plectreurys tristes spider venom (21)), low
conductance Ca2+−activated K+ channel modulators (i.e. apamin,
leurotoxin 1 (34)), other K+ channel mediators (BRL 34915,
4-aminopyridine, glyburide), different classes of antiar-
rythmic agents (i.e. bepridil, quinidine, beryllium, AQA-39,
clofibrate, amiodarone), and Na+ channel modulators (i.e., S-
(−)DP1 201-106, R(+)DP1 201-106, lidocaine). None of these
agents, even when tested at high concentrations, has any
effect whatsoever on 125I-ChTX binding in brain. These re-

results suggest that the ChTX receptor in synaptic plasma
membrane vesicles possesses a unique pharmacology and fur-
ther support the hypothesis that this binding site is directly
associated with Pk,V since none of these compounds is an
effective modulator of that ion channel's activity.

It has been reported that the receptor for a-DaTX in brain
is either a 65- or 78-kDa protein (Refs. 35 and 16, respec-
tively). The receptor was identified using bifunctional cross-
linking reagents to incorporate α-DaTX into its binding site
covalently. Moreover, the same 78-kDa protein was presum-
ably identified by the cross-linking of radiolabeled ChTX in
this tissue (16). In an effort to perform similar experiments
with 125I-ChTX, brain synaptic plasma membranes were
incubated with toxin until equilibrium was established, and
then this preparation was treated with a variety of different
cross-linking reagents. These agents include disuccinimidyl-
suberate, dimethylsuberimidate, dimethyladipimidate, and
bio(sulfosuccinimidyl)suberate. After washing membranes
and subjecting them to sodium dodecyl sulfate-polyacrylamide
gel electrophoresis analysis, there was no clear indication of
125I-ChTX covalent incorporation into any membrane protein
(not shown). In some instances, using extensive exposure
for autoradiography (5 days) and disuccinimidylsuber-
itate as a reagent, a radiolabeled 100-kDa protein could be
visualized which is reduced in intensity if the cross-linking
reaction is carried out in the presence of excess ChTX. These
results are reminiscent of studies with vascular smooth muscle
sarcolemmal membranes in which 125I-ChTX specifically labels
a 95-kDa protein that is thought to be a subunit of Pκ,ca
(36). These results indicate that Pκ,ca is present at very low
density in brain synaptic plasma membranes. They suggest
further that although the receptor site for ChTX in brain is
probably Pκ,~, this is not easily confirmed using cross-linking
paradigms.

**DISCUSSION**

The results presented in this study demonstrate unequivoc-
ally for the first time that a single class of high affinity
receptors exists for ChTX in brain. Binding of toxin to these
sites occurs through a simple bimolecular reaction and dis-
plays a Kd in the low pM range. These sites appear to be
functionally associated with a specific K+ channel. Data char-
acterizing the pharmacological properties of the ChTX site
support this conclusion. As might be predicted, a number of
metal ions that interact with K+ channels affect ChTX bind-
ing, and several have been found to modify toxin binding
through an allosteric mechanism. In addition, certain organic
cation inhibitors of K+ channels, such as TBA, also block
ChTX binding. Perhaps the most compelling piece of evi-
dence, however, is that several selective K+ channel toxins
modulate ChTX binding in brain. Specifically, both a-DaTX
and NxTX, which have been shown by electrophysiological
(32, 33) and ion flux (37) techniques to inhibit an inactivating,
voltage-dependent K+ channel, modify the binding of ChTX
allosterically. These data, taken together with the observa-
tion that ChTX also blocks this channel (9−11), strongly suggest
that the ChTX binding site is coupled to Pκ,~ in brain.

Although NxTX is also known to block a delayed rectifying
K+ channel in neural tissue (38, 39), this interaction is weak
compared with its effects on Pk,~; and ChTX does not inhibit
the former channel type (13). These findings provide further
support for the claim that the site of ChTX binding in brain
is Pk,~. Therefore, although ChTX clearly recognizes more
than one channel type, it can still be used as a selective probe
to study the biochemical and pharmacological properties of
specific K⁺ channels if the toxin is employed in selected tissues.  

¹²⁵I-ChTX has been shown previously to bind to Pk,cₐ in vascular (14) and airway (40) smooth muscle sarcolemma as well as to Pk,cₐ in t-tubular membranes derived from skeletal muscle. Since ChTX-sensitive Pk,cₐ are also present in neural tissue (3, 4), it is important to review the evidence demonstrating that the ChTX receptor under current investigation is not related to Pk,cₐ and to determine why this site is, in fact, not detected. The data indicating that the ChTX binding site observed in brain is not associated with Pk,cₐ are compelling. For example, the basic properties of toxin binding in brain and smooth muscle membranes (14) are markedly different. The KD for ¹²⁵I-ChTX in brain is 20-fold higher in affinity than that in smooth muscle, under identical ionic conditions. As anticipated, the h₁ and h₋₁ rate constants for radiolabeled toxin binding are also much faster in brain. The affinity for native ChTX is approximately 5-fold higher in brain at equivalent ionic strengths (i.e. 50 mM NaCl). Consequently, there is less loss of binding affinity in brain than in smooth muscle due to the formation of the moniodotyrosine adduct during radiolabeling of the toxin. The pH dependence for the interaction of ChTX in brain does not mirror the profile observed in smooth muscle. The modulation of ChTX binding by metal ions in the two tissues is also completely different, not only in terms of the absolute potencies detected, but also in the profile of effects observed. For example, neither K⁺ nor Na⁺ stimulates ChTX binding in smooth (14) or skeletal muscle. Furthermore, ChTX binding in brain is not affected by either TEA or IbTX. The former organic cation is a moderately potent inhibitor of Pk,cₐ which competes at the ChTX receptor in smooth muscle (14); the latter peptide is a selective high affinity probe for Pk,cₐ which modulates binding at the ChTX site allosterically. Moreover, although it is possible to identify the ChTX receptor in smooth muscle by cross-linking studies with ¹²⁵I-ChTX (36), similar paradigms have failed to label this protein in brain. When taken together, these data provide an inexorable argument that the ChTX site in brain is not associated with Pk,cₐ.

There are at least two explanations for the inability of ¹²⁵I-ChTX to identify Pk,cₐ sites in brain. First, the density of these channels in rat brain synaptic plasma membrane vesicles may be very low. Given the specific activity of the radiolabeled toxin used in these experiments, it should be possible to detect binding sites with reasonable affinity at a minimum density of 10–30 fmol/mg of protein. Of course, the presence of a second high affinity site with a significant KD is apparent that iodination of toxin alters its structure in such fashion that there is a 10-fold loss in binding affinity for Pk,cₐ in smooth muscle. This has been confirmed in electrophysiological experiments in which the functional activity of moniodotyrosine ChTX was monitored (14). Thus, if Pk,cₐ in brain is more sensitive to conformational differences between iodinated and native ChTX than the smooth muscle channel, it may not be possible to use ¹²⁵I-ChTX to study Pk,cₐ in brain. Since both the diiodotyrosine and monoiodohistidine adducts of ChTX have been found to be biologically inactive (14), study of the brain channel may require a different peptide labeling strategy. In any event, this hypothesis could be tested by determining the functional activity of moniodotyrosine ChTX as an inhibitor of Pk,cₐ from brain synaptic plasma membranes reconstituted into artificial lipid bilayers (4).

The use of radiolabeled ChTX as a means of studying K⁺ channels is controversial. Some authors have suggested that iodination of this peptide results in a 1,000- to 10,000-fold loss in potency as a blocker of Pk,cₐ in muscle (19). This is clearly not the case since high affinity sites associated with this channel have been discovered in vascular (14) and airway (40) smooth muscle as well as in t-tubules isolated from skeletal muscle. Two other groups have attempted to use ¹²⁵I-ChTX to probe the interaction of this toxin with membranes derived from rat brain (15, 16). In the first of these reports (15), the authors were unable to prepare iodinated toxin in a biologically active form that would bind to brain membranes although ChTX inhibited the interaction of α₁₂⁵I DaTX with its own receptor in the same preparation. In the second report (16), ¹²⁵I-ChTX binding was detected, but the properties observed are markedly different from those noted in the present investigation. For example, the KD reported for iodinated toxin is 30-fold lower in affinity, and the site density is 5-fold less in magnitude than the same parameters measured in the current study. Under the experimental conditions used in the previous study, the nonspecific binding component was also very large (16). Such different KD and Bₘₐₓ values may result from the improper separation of moniodotyrosine ChTX from other iodinated species after radiolabeling and from the use of partially purified plasma membrane fractions for the binding experiments. Strangely, the KD of native toxin was also 10–20-fold reduced, and the KD of α-DaTX as an inhibitor of ChTX binding was about 20-fold lower in value than these same parameters measured in our investigation. Moreover, there is a 10-fold difference in the potencies reported for ChTX as an inhibitor of α₁₂⁵I-DaTX binding in the two studies cited above (15, 16). Perhaps the basis for these differences is related to the dissimilar ionic conditions used in the various studies (i.e. low ionic strength buffers in the work from this laboratory versus media with significant levels of Na⁺ present in the other reports) as well as to the techniques employed to separate bound from free ligand (i.e. filtration in the present study versus centrifugation in the other two investigations). In addition, we were unable to identify the ChTX binding site in brain as the α-DaTX receptor by cross-linking with ¹²⁵I-ChTX as has been reported previously (16). This was the case even though the membranes used in our study have a 5-fold higher density of ChTX sites than in the previous investigation (thus, being comparable to the density of α-DaTX sites noted in that study), and similar cross-linking protocols had been used. The reason for this discrepancy is not clear. Nonetheless, we are in agreement with the conclusion of the other studies cited (15, 16) that the ChTX sites in brain are associated with Pk,cₐ.

A number of distinct sites are coupled allosterically to the ChTX receptor in brain. Binding of metal ions increases the

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¹ M. L. García, S. Fleischer, and G. J. Kaczorowski, unpublished observations.
affinity of this site for ChTX through a positive heterotropic interaction. Apparently, either two K⁺ or a single Na⁺ can promote this effect (Table II). Whether the binding sites for these metal ions overlap or are distinct entities remains to be established. Both α-DaTX and NtxTX block ChTX binding noncompetitively through a negative heterotropic interaction. No studies have addressed whether these two toxins share a common receptor on the channel protein. In addition, mast cell degranulating peptide blocks ChTX binding through a low affinity interaction (16). It is possible that additional toxin binding sites will also be discovered on Pk,v. For example, other peptide toxins have been identified in L. quinquestratius var. hebraeus venom which inhibit ¹³¹I-ChTX binding in brain, and these have been purified to homogeneity.³ Determination of their primary structures indicates that the resulting peptide sequence are unique. Furthermore, another peptide has been purified to homogeneity from B. taenulus venom, the source from which IbTX was isolated, and this also blocks the interaction of ChTX in brain.³ This peptide is more basic than ChTX and is currently undergoing sequence analysis. In order to ascertain the number of unique binding sites that exist on Pk,v for different toxins, each peptide must be radiolabeled and its binding properties determined. It is expected that these toxins will aid further in the purification of Pk,v as well as be useful in pioneering the molecular pharmacology of this and other types of K⁺ channels.

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