Characterization of High Affinity Binding Sites for Charybdotoxin in Sarcolemmal Membranes from Bovine Aortic Smooth Muscle

EVIDENCE FOR A DIRECT ASSOCIATION WITH THE HIGH CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNEL*

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Charybdotoxin (ChTX), a peptide inhibitor of the high conductance Ca"+-activated K" channel (PK,ca), has been radiolabeled to high specific activity with "I and resulting derivatives have been well separated. The monooiodotyrosine adduct blocks PK,ca in vascular smooth muscle with slightly reduced potency compared with the native peptide under defined experimental conditions. "IChTX, representing this derivative, binds specifically and reversibly to a single class of sites in sarcolemmal membrane vesicles prepared from bovine aortic smooth muscle. These sites display a \( K_d \) of 100 pm for the iodinated toxin, as determined by either equilibrium or kinetic binding analyses. Binding site density is about 500 pmol/mg of protein in isolated membranes. The addition of low digitonin concentrations to disrupt the vesicle permeability barrier increases the maximum receptor concentration to 1.5 pmol/mg of protein, correlating with the observations that ChTX binds only at the external pore of PK,ca and that the membrane preparation is of mixed polarity. Competition studies with ChTX yield a \( K_i \) of about 20 pm for native toxin. Binding of "IChTX is modulated by ionic strength as well as by metal ions that are known to interact with PK,ca. Moreover, tetraethylammonium ion, which blocks PK,ca with moderately high affinity when applied at the extracellular membrane surface, inhibits "IChTX binding in an apparently competitive fashion with a \( K_i \) similar to that found for channel inhibition. In marked contrast, agents that do not inhibit PK,ca in smooth muscle (e.g. tetrabutylammonium ion, other toxins homologous with ChTX, and pharmacological agents that modulate the activity of dissimilar ion channels) have no effect on "IChTX binding in this tissue. Taken together, these results suggest that the binding sites for ChTX which are present in vascular smooth muscle are directly associated with PK,ca, thus identifying "IChTX as a useful probe for elucidating the biochemical properties of these channels.

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High conductance Ca"+-activated K" channels (PK,ca) are present in the plasma membrane of both excitable and nonexcitable cells (1, 2). These channels are presumed to function in a variety of cellular activities including the control of neuroendocrine secretion, muscle contractility, and neuronal electrical activity. PK,ca may also be involved in regulation of intracellular osmolarity and cellular electrolyte concentrations. The characteristics of these channels include very high single-channel conductance, high K" selectivity, and activation by both cytoplasmic Ca"+ and membrane depolarization. Another distinctive property of PK,ca is their sensitivity to external application of tetraethylammonium ion (TEA). Recently, charybdotoxin (ChTX), a minor component of venom from the scorpion Leirurus quinquestriatus var. hebraeus, has been purified to homogeneity, and its primary structure was determined (3). This toxin, a 37-amino acid peptide, reversibly inhibits PK,ca in a number of systems, including vascular smooth muscle, when applied at the extracellular face of the channel (3-5). Mechanistic studies indicate that ChTX, which contains 8 positively charged residues, binds at the external mouth of the channel via an electrostatic interaction to inhibit K" conduction at nM potency under physiological ionic conditions (6, 7). Thus, ChTX should be useful as a probe for studying the properties of PK,ca and for defining their physiological roles.

In the present investigation, we report the characterization of high affinity binding sites for ChTX in sarcolemmal membrane vesicles derived from bovine aortic smooth muscle, a particularly rich source of PK,ca. The properties of these binding sites suggest that receptors for ChTX are directly associated with PK,ca in this tissue. Previously, two other toxins, apamin and dendrotoxin, have been used to identify and characterize binding sites that are associated with distinct types of K" channels: namely, a small conductance Ca"+-activated K" channel and a rapidly inactivating ("A-type") voltage-dependent K" channel, respectively (see, for example, Refs. 8 and 9). In analogy, the data presented herein suggest that ChTX will be useful in elucidating the biochemical and pharmacological characteristics of PK,ca and in attempting its purification. A preliminary report of these findings has been made in abstract form (10).

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* The abbreviations used are: PK,ca, high conductance Ca"+-activated K" channel(s); "IChTX, monooiodotyrosine charybdotoxin; TEA, tetraethylammonium ion; ChTX, charybdotoxin; Mops, 3-(N-morpholino)propanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; \( B_{max} \), maximum receptor concentration; HPLC, high performance liquid chromatography.
EXPERIMENTAL PROCEDURES

Materials—Lyophilized venom from the scorpion L. quinquestratus var. hebraeus was obtained from Latoxan Scorpion Farm, Rosans, France. [125I]NaI was purchased from the Amersham Corp., and the reagent IODO-GEN was bought from Pierce Chemical Co. All other reagents were purchased from commercial sources and were of the highest purity commercially available. Various drugs were obtained from the sample collection of Merck & Co. Glass fiber filters (GF/C) were supplied by Whatman.

Preparation of Monoiodo-ChTX—ChTX was purified from scorpion venom by methods described previously (13). Toxin concentration was calculated using the known extinction coefficients of the peptide (3). ChTX was subjected to iodination by employing the IODO-GEN method (11). Briefly, 10 μl of a solution containing 5 μg of ChTX in 100 mM sodium phosphate, pH 7.5, was added to a vial containing 0.5 pg of dried IODO-GEN. Next, 1 mCi of [125I]NaI (10 μl, 2000 Ci/mmol) was added, and the capped vial was kept at room temperature for 15 min. The iodination mixture was then injected into an HPLC C18 reversed-phase column (Vydac) equilibrated with 10 mM trifluoroacetic acid. Elution was achieved with a linear gradient (5-14%) of isopropl alcohol/acetoneitrile, 2:1, in 4 mM trifluoroacetic acid, applied over a 40-min period at a flow rate of 0.5 ml/min to separate various iodinated species. Material judged to be a monoiodo derivative based on specific activity of the radiolabeled peptide was subjected to structural determination to confirm its identity. Iodinations corresponding to this material were made 0.5% in bovine serum albumin, lyophilized, and reconstituted with 100 mM NaCl, 20 mM Tris-HCl, pH 7.4. Aliquots of [125I]ChTX were stored at -70°C. ChTX was iodinated in nonradioabeled form essentially as described above, except using a greater amount of peptide, but keeping a similar ratio of peptide to NaI to IODO-GEN as for the labeling procedure with [125I]NaI. Increasing the ratio of NaI to peptide did not improve the production of moniodoacetosine ChTX but led to the appearance of other iodinated species upon reversed-phase chromatography.

Amino Acid Sequence Determination—Purified monoiodo-ChTX was alkylated with iodoacetate and digested with endoproteinase Lys-C as described previously (3). Peptides derived from this digestion were purified using a C18 reversed-phase column. All the radioactivity was associated with one peptide that was then subjected to amino-terminal Edman degradation on Polybrene-coated filters using an Applied Biosystems 470A microsequenator. Phenylthiohydantoic acid amino acids were detected using an on-line analyzer (Applied Biosystems model 120A).

Preparation of Bovine Aortic Smooth Muscle Sarcolemmal Membrane Vesicles—Highly purified sarcolemmal membrane vesicles were prepared from bovine aortic smooth muscle by methods reported previously (12). The membrane fraction at the 8-30% interface of a sucrose density gradient has been shown to be enriched in sarcolemmal marker activities. These vesicles were resuspended in 160 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, and washed twice with ice-cold medium. Under these conditions, nonspecific binding represents less than 2% of the total radioactivity added, and at a Kd concentration of ligand, specific binding represents 75% of total binding. Displaceable binding of [125I]ChTX to glass fiber filters in the absence of membranes is negligible. Triplicate assays were routinely performed under each experimental condition, and the data were averaged. The standard error of the mean of these results was typically less than 3%. Stock solutions of CTX were prepared in 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin.

Analysis of Data—Data from saturation experiments were subjected to linear regression analysis, and linear regression was performed to yield the equilibrium dissociation constant (Kd) and maximum receptor concentration (Bmax). Correlation coefficients for these determinations were typically greater than 0.95. Data from competition experiments were analyzed by the method of Cheng and Prusoff (13) to determine Kd values. The kinetic data for ligand association and dissociation were subjected to the analysis of Weiland and Molinoff (14). The dissociation rate constant for ChTX (k-1) was determined directly for a first-order plot of ligand dissociation versus time. The rate of ligand association (k1) was determined from the equation k1 = kobs([LR]/([L] [LR]max)) where [L] is the concentration of ligand, [LR] is the concentration of the complex at equilibrium, [LR]max is the maximum number of receptors present, and kobs is the slope of the pseudo-first order plot ln([LR]/([LR] - [LR])max) versus time.

Electrophysiological Analysis—Primary bovine aortic smooth muscle cells were obtained and grown as described (15, 16). Cells were cultured for 2-4 days on 25-mm glass cover slips before use in electrophysiological experiments. P2X, activity was monitored in outside-out excised membrane patches using conventional patch-clamp methodologies employed for single-channel measurements (17). Samples of ChTX derivatives were added directly to an experimental chamber in which a microelectrode containing the excised patch was suspended.

Protein Determination—Membrane protein was determined by the amido black dye-binding method (18) using bovine serum albumin as a standard.

RESULTS

Chemical Modification of ChTX by Iodination—ChTX was reacted with [125I]NaI under the conditions described under “Experimental Procedures”, and resulting products were separated using HPLC techniques (Fig. 1). A peptide assumed to be the monoiodo adduct of ChTX was identified based on a specific activity corresponding to the maximum theoretical value for incorporation of 1 iodine/peptide molecule. This ChTX derivative ([125I]ChTX) elutes from reversed-phase chromatography well separated from native peptide and other iodinated species. ChTX contains single tyrosine and histidine residues, both of which are potential sites for iodination. To identify which residue is modified, a nonradioactive derivative of ChTX corresponding to the species of interest was prepared in sufficient quantities and mixed with a small amount of [125I]ChTX. This toxin mixture was carboxymethylated, digested with endoproteinase Lys-C, and applied to a reversed-phase column in order to separate peptides derived

![Figure 1](https://example.com/fig1.png)

**FIG. 1. Iodination of ChTX and HPLC separation of iodinated derivatives.** ChTX was subjected to iodination using the IODO-GEN method as described under “Experimental Procedures.” The reaction mixture was then injected into an HPLC C18 reversed-phase column equilibrated with 10 mM trifluoroacetic acid, and elution was achieved by a linear gradient (5-14%) of isopropl alcohol/acetoneitrile (2:1) in 4 mM trifluoroacetic acid (solvent B) applied over 40 min at a flow rate of 0.5 ml/min. Material eluting from the column was monitored by measuring absorbance at 210 nm and, in the case of reaction with [125I]radioactivity. Peaks A, B, C, and D correspond to unmodified ChTX, monoiodotyrosine ChTX, diiodotyrosine ChTX, and probably a monoidotyrosine species of ChTX, respectively. The small peak eluting before native ChTX does not contain iodine and most likely is a form of oxidized ChTX.
from the digestion procedure. All radioactivity was found to be associated with one peptide that was then subjected to sequence analysis by Edman degradation. The sequence of this peptide is Cys-Arg-Cys-[125]I-X-Ser. This peptide can be identified unambiguously as being derived from the C terminus of ChTX (3) where the iodinated species is in a position corresponding to a tyrosine residue.

Although under these experimental conditions, monoiodo- and diiodotyrosine adducts of ChTX are preferentially produced, sometimes a small amount of a component representing a monoiodinated species is also formed which elutes between unmodified and monoiodotyrosine ChTX. This ChTX derivative, which has not been characterized by sequence analysis, displays no biological activity (based on binding criteria; see below) and is likely to represent a monoiiodohistidine derivative of ChTX.

To test whether monoiodotyrosine ChTX retains its biological activity, P<sub>K,Ca</sub> was monitored in outside-out excised membrane patches from primary bovine aortic smooth muscle cells using patch clamp techniques. Initial experiments were carried out with symmetrical 150 mM K⁺ bathing both faces of the membrane. ChTX blocks P<sub>K,Ca</sub> under these conditions with a K<sub>i</sub> of about 2 nM; complete abolition of channel activity is achieved at 20 nM toxin (not shown). Identical data have been obtained for ChTX-block of P<sub>K,Ca</sub> in GH₃ pituitary cells (3). In contrast, monoiodotyrosine ChTX has no effect on channel activity at concentrations up to 100 nM. Since treatment of toxin with IODO-GEN in the absence of Na⁺ does not affect ChTX block of P<sub>K,Ca</sub> (not shown), it is the formation of the monoiodotyrosine adduct rather than exposure to toxin the iodination reaction conditions per se which alters the inhibitory activity of ChTX. However, if K⁺ is lowered at the external face of the membrane, iodinated ChTX does inhibit P<sub>K,Ca</sub>. In the experiment illustrated in Fig. 2, control records show the existence of a single high conductance Ca<sup>2+</sup>-activated K⁺ channel in this membrane patch. Addition of 10 nM monoiodotyrosine ChTX leads to an 80% decrease in channel activity, and inhibition is reversed upon washing away toxin (total open time is decreased from 2.8 to 0.6% in the presence of toxin and returns to 1.8% after 10 min of washout). Complete block of P<sub>K,Ca</sub> is achieved with 30–50 nM modified ChTX. It is apparent from the records of Fig. 2 that iodinated ChTX behaves mechanistically in a fashion similar to unmodified toxin: it affects neither single channel unitary conductance (expanded traces) nor channel mean open time (histogram representations). These are properties that characterize block of P<sub>K,Ca</sub> by native ChTX (6). However, it appears that the blocking activity of iodinated ChTX is much more sensitive to the ionic strength of the medium than that of ChTX. Under the ionic conditions of these experiments, monoiodotyrosine ChTX is 5- to 10-fold weaker than native ChTX at inhibiting P<sub>K,Ca</sub>. It has been noted that in addition to P<sub>K,Ca</sub>, primary bovine aortic cells possess other lower conductance K⁺ channels that are insensitive to ChTX (19). As a control, the recordings in Fig. 2 also illustrate that this iodinated derivative of ChTX has no effect on the activity of one such channel.

Identification of ChTX-binding Sites in Smooth Muscle

**Identification of ChTX-binding Sites in Bovine Aortic Sarcolemma**—To determine whether ChTX can be used to characterize the biochemical properties of P<sub>K,Ca</sub>, [125]I-ChTX binding was monitored in membranes derived from a tissue in which the density of these channels is very high. Purified sarcolemmal membrane vesicles isolated from bovine aortic smooth muscle represent such a system. The prevalence of P<sub>K,Ca</sub> in aorta is well established (16, 19, 20), and a procedure that yields a highly purified aortic sarcolemmal preparation with little contamination from either mitochondria or sarcoplasmic reticulum has been developed (12). When vesicles are incubated with increasing concentrations of [125]I-ChTX until equilibrium is achieved, toxin associates with membranes in a concentration-dependent fashion (Fig. 3A). In the presence of 10 nM ChTX, binding is found to be a linear function of [125]I-ChTX concentration. Specific binding, defined as the difference between total binding and binding in the presence of excess nonradiolabeled toxin, is a saturable function of [125]I-ChTX concentration. A Scatchard analysis of these data (Fig. 3B) indicates the presence of a single class of binding...
sites with a $K_d$ of 100 pm and a $B_{max}$ of 500 fmol/mg of protein. These are typical values for this binding reaction since nearly identical results have been obtained with five different vesicle preparations. If membranes are first treated with 0.1% digitonin, a concentration of detergent which does not appreciably solubilize membrane proteins but which disrupts the permeability barrier of vesicles, binding of $^{[35]S}$ChTX is increased. Enhanced binding is due to a 3-fold elevation in receptor site density ($B_{max}$) of 1.5 pmol/mg of protein with no effect on toxin $K_d$. These results are consistent with data of a previous study (12) suggesting that this aortic sarcolemmal membrane preparation consists of both right-side-out and inside-out vesicles and further indicate that ChTX binds with a defined density at the membrane surface. $^{[35]S}$ChTX binding in aortic sarcolemma is linear with protein up to a concentration of 100 µg of membrane protein/ml. When the temperature dependence of the binding reaction is investigated at 4, 22, and 37 °C, no differences are found at either 22 or 37 °C, whereas binding at 4 °C with a fixed concentration of ChTX represents 50% of that found at 22 °C (the result of a change in $K_d$).

The kinetics of ChTX binding have been measured to determine whether toxin association occurs through a simple bi-molecular reaction. The data shown in Fig. 4A indicate that when a sub-$K_a$ concentration of $^{[35]S}$ChTX is incubated with sarcolemmal membranes, there is time-dependent association of toxin which approaches equilibrium in about 10 min. The nonspecific binding component, determined in the presence of 10 nM ChTX, is time independent and has been subtracted from the experimental data. A semilogarithmic transformation of these results (Fig. 4A, inset) yields a linear dependence, as expected for a pseudo-first order reaction, and the slope of this line gives $k_{obs}$. The association rate constant, $k_a$, calculated as described under “Experimental Procedures,” is $6.8 \times 10^8$ M$^{-1}$ s$^{-1}$. Interestingly, this value is slightly greater than the diffusion control rate expected for a small peptide and suggests the possibility of an electrostatic interaction between positive charges on the toxin and negative charges at its membranous binding site. Dissociation of $^{[35]S}$ChTX from its receptor, initiated by the addition of an excess of nonradiolabeled toxin, displays a single exponential relationship with a $t_0$ of 2 min (Fig. 4B). A semilogarithmic plot of these data generates a straight line (Fig. 4B, inset), as expected for a first order reaction, and the slope of this line gives a dissociation rate constant, $k_{-1}$, of 0.0052 s$^{-1}$. The $K_d$ calculated from these values is 76 pm, a value similar to that determined under equilibrium conditions. This suggests that ChTX binding is a freely reversible process that occurs through a single step reaction. To confirm these values for the association and dissociation rates of $^{[35]S}$ChTX, rate constants have also been determined in independent experiments by measuring the kinetics of radiolabeled toxin binding at different ligand concentrations (10-100 pm). Semilogarithmic representations of these data display straight lines whose slopes yield $k_{obs}$ at each concentration of toxin. As shown in Fig. 4C, a plot of $k_{obs}$ versus ChTX concentration produces a linear representation in which $k_t$ is given by the slope, and $k_{-1}$ is obtained from the y intercept. Results of these determinations give values for $k_t$ and $k_{-1}$ of $6.3 \times 10^1$ M$^{-1}$ s$^{-1}$, and 0.0053 s$^{-1}$, respectively, which yields a corresponding $K_d$ of 84 pm.

**Fig. 3.** Binding of $^{[35]S}$ChTX to sarcolemmal membrane vesicles derived from bovine aortic smooth muscle. A, saturation binding analysis. Membrane vesicles (5.5 µg of protein) were incubated with increasing concentrations of $^{[35]S}$ChTX at 22 °C as described under “Experimental Procedures” until equilibrium was achieved. Total binding (●) and nonspecific binding determined in the presence of 10 nM ChTX (○) are represented. Specific association of toxin (●) was assessed from the difference between total and nonspecific ligand binding. B, analysis of $^{[35]S}$ChTX binding at equilibrium. Specific binding data from A were subjected to a Scatchard analysis.

**Fig. 4.** Association and dissociation kinetics of $^{[125]I}$ChTX in aortic sarcolemmal membrane vesicles. A, association kinetics. Sarcolemmal membrane vesicles were incubated with 36 pm $^{[125]I}$ChTX at 22 °C for different periods of time. Nonspecific binding measured in the presence of 10 nM ChTX is time invariant and has been subtracted from the data. Inset, a semi-logarithmic representation of the pseudo-first order association reaction, where $B_p$ and $B_T$ represent ligand bound at equilibrium and time $t$, respectively. $B$, dissociation kinetics. After incubating vesicles with 36 pm $^{[125]I}$ChTX at 22 °C for 60 min, toxin dissociation was initiated by the addition of 10 nM ChTX. Samples were taken after different periods of time, and toxin that remained associated with vesicles was measured. Inset, a semi-logarithmic representation of the first order dissociation reaction. C, relationship between $k_{obs}$ and ChTX concentration. Sarcolemmal membrane vesicles were incubated at 22 °C with the indicated concentrations of $^{[125]I}$ChTX for different periods of time. The slopes ($k_{obs}$) of each semi-logarithmic representation of the various pseudo-first order association reactions that were obtained are plotted as a function of toxin concentration.
To ascertain whether unmodified toxin displays a similar affinity for the receptor as [\textsuperscript{125}I]ChTX, competition experiments were performed. These data are shown in Fig. 5A and have been subject to the Cheng-Prusoff analysis (13) to determine a K, value. Noniodinated ChTX completely displaces [\textsuperscript{125}I]ChTX from its binding site with a K, typically of 10–20 pm in the presence of 20 mM Na`. Therefore, substitution of the tyrosine with an iodine results in about a 5- to 10-fold loss of binding affinity. Perhaps, modification of this residue disrupts the conformation of the peptide to some extent. Notably, neither the diiodotyrosine species of ChTX nor the derivative thought to be a moniodohistidine adduct is able to displace [\textsuperscript{125}I]ChTX (not shown), indicating that both of these products have lost the ability to bind to the receptor. As found in electrophysiological experiments (6), increasing the salt concentration of the medium decreases the binding affinity of ChTX: at 50 mM Na`, the K, of ChTX is 50 pm (Fig. 5A).

The pH dependence of CaTX binding has been studied by performing experiments in the pH range 6–9 (Fig. 5B). Binding of [\textsuperscript{125}I]ChTX to aortic sarcolemmal membranes increases 1.7-fold from pH 6.0 to 8.0 and then plateaus. Since the pI of ChTX is known to be slightly greater than 10.0, and both binding as well as blockade of \( P_{K_{Ca}} \) (6, 7) appear to involve electrostatic interactions between positively charged residues on the toxin and negative charges on the receptor, this pH profile probably reflects titration of functional groups at the ChTX-binding site. Alternatively, this relationship could represent block of ChTX binding by protons via a mechanism analogous to that observed with other ions (see below).

If ChTX-binding sites are associated with \( P_{K_{Ca}} \), then they should be localized at the sarcolemma. To confirm this, different membrane fractions obtained during purification of bovine aortic sarcolemmal vesicles have been tested for their ability to bind [\textsuperscript{125}I]ChTX. Results of these experiments are presented in Table I. To obtain these data, equivalent quantities of protein from each membrane fraction were subjected to binding data in each case are presented relative to toxin association at 22 °C until equilibrium was achieved. Specific binding data in each case are presented relative to toxin association in the presence of 20 mM Tris-HCl, pH 7.4.

![Fig. 5. Effect of salt concentration, peptidyl toxins, and pH on [\textsuperscript{125}I]ChTX binding to aortic sarcolemmal membrane vesicles.](image)

### Table I

**Localization of [\textsuperscript{125}I]ChTX-binding sites in bovine aortic smooth muscle membranes**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[\textsuperscript{125}I]ChTX bound pmol/mg protein</th>
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<tbody>
<tr>
<td>Homogenate</td>
<td>0.003</td>
</tr>
<tr>
<td>Applied sample</td>
<td>0.011</td>
</tr>
<tr>
<td>8/30% interface-FI</td>
<td>0.107</td>
</tr>
<tr>
<td>30/40% interface-FII</td>
<td>0.047</td>
</tr>
<tr>
<td>Pellet</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Membranes were prepared from bovine aortic smooth muscle and fractionated on a discontinuous sucrose density gradient as described (12). Marker activities associated with the sarcolemmal membrane are enriched in fraction FI. Aliquots of each fraction containing equivalent protein concentrations were incubated with 28 pm [\textsuperscript{125}I]ChTX at 22 °C until equilibrium was achieved. Specific binding data are presented in each case.

![Fig. 6. Effect of ions on [\textsuperscript{125}I]ChTX binding to aortic sarcolemmal membrane vesicles.](image)

36-fold purification as compared with the starting tissue homogenate and a recovery of about 30% of the binding sites in the most highly purified sarcolemmal fraction.

**Effect of \( P_{K_{Ca}} \) Modulators on [\textsuperscript{125}I]ChTX Binding**—Several metal ions such as Cs`, Ba`, and Ca` as well as the organic cation, TEA, are known to block \( P_{K_{Ca}} \) by different mechanisms with relatively good potency (21–26). High affinity binding sites for Cs` and Ba` have been postulated to exist along the conduction pathway of this channel. TEA, on the other hand, blocks \( P_{K_{Ca}} \) by binding to a site of moderately high affinity located on the outside face of the channel. Recently, it has also been shown that K` binds with high affinity to a site that is accessible from the external medium and in so doing can trap Ba` within the channel. Given these observations, the effects of different ions on ChTX binding were ascertained. When vesicles are incubated with [\textsuperscript{125}I]CHTX in the presence of increasing concentrations of either K`, Cs`, Ba`, or TEA, there is concentration-dependent inhibition of toxin-binding activity (Fig. 6A). Inhibition is complete for Ba`, Ca`, and TEA, but only partial for K` and Cs`. K` values calculated by the Cheng-Prusoff relationship for Ba`, K`, Ca`, Cs`, and TEA are 12, 17, 80, 100, and 140 μM, respectively. These data are in general agreement...
with $K_d$ values measured for the interaction of the various ions with $P_{K,PKC}$. In contrast, when ChTX binding is monitored in the presence of either Na$^+$ or Li$,^+$ both ions weakly inhibit toxin binding and do so in an equivalent fashion with a profile that displays a very steep concentration dependence (Fig. 6B). Noteworthy is the finding that $K^+$ and Ca$^{2+}$, which cause partial inhibition of binding at mM concentrations (see above), produce complete inhibition of binding over this same mM concentration range. Thus, these latter two ions display biphasic inhibition curves (not shown). This pattern of inhibition by mM concentrations of ions likely reflects the effects of ionic strength on ChTX binding such that the electrostatic interaction between toxin and receptor is weakened by increasing the ionic composition of the medium. These results are expected given the mechanism that has been postulated for blockade of $P_{K,PKC}$ by toxin (6, 7). Although it is technically difficult to perform binding experiments above 50 mM salt, the effect of increasing the NaCl concentration from 20 to 50 mM appears to reflect a change in toxin affinity (cf. Fig. 5A). The change in $K_d$ is not due to alteration of ChTX dissociation rates (not shown) and therefore probably involves an effect on $k_1$, consistent with an electrostatic interaction being involved in the binding reaction.

To study the mechanisms by which TEA, Ca$^{2+}$, and $K^+$ block ChTX binding to aortic sarcolemmal vesicles, saturation experiments were carried out with increasing concentrations of $[\text{H}]$ChTX in the absence or presence of each inhibitor. Scatchard analyses of the resulting data are shown in Fig. 7. Increasing concentrations of TEA inhibit ChTX binding by decreasing toxin affinity with no effect on receptor site density. Ca$^{2+}$ inhibits toxin binding in a similar manner. However, $K^+$ decreases the maximum number of high affinity ChTX receptor sites available with no marked effect on toxin $K_d$. To determine whether any of these ions alters ligand dissociation kinetics, $k_1$, of $[\text{H}]$ChTX was measured in the absence or presence of an excess of each ion. Interestingly, no significant effects on toxin dissociation kinetics were observed in any case (not shown). On the other hand, $k_i$ of toxin binding was decreased 50% by a $K_i$ concentration of TEA and Ca$^{2+}$, whereas $K^+$ had no significant effect on this parameter. Taken together, these findings suggest that the first two agents may be acting competitively to block binding, whereas $K^+$ exerts its effect through a strictly allosteric mechanism.

A variety of agents with differing pharmacological activities have been tested for effects on ChTX binding in aortic sarcolemmal membranes. These agents include L-type Ca$^{2+}$ channel blockers (i.e. nifedipine, dilazepam, D-600, fluspirilen, cinnarizine, flunarizine); N-type Ca$^{2+}$ channel blockers (i.e. omega-conotoxin, Plectreurys tritrix spider venom (27)); low conductance Ca$^{2+}$-activated $K^+$ channel modulators (i.e. spamin, leuurotin I (28)); other $K^+$ channel modulators, some of which are active in smooth muscle (i.e. tetraethyl-ammonium ion, BRL 34915, 4-aminoopyridine, glyburide); various antiarrhythmic agents (i.e. bepridil, quinidine, bertylum, AQA-39, cloflium, amiiodarone), and Na$^+$ channel modulators (i.e. (S$^-$)DPI 201-106, R$^+$)DPI 201-106, lidocaine). None of these compounds, even when tested at high concentrations, has any significant effect on $[\text{H}]$ChTX binding, suggesting that the ChTX receptor possesses a unique pharmacology. Interestingly, two other scorpion toxins that display high sequence homology with ChTX, noxiustoxin isolated from Centruroides noxius (29) and leuurotin II isolated from L. quinquestratus, have no significant effect on $[\text{H}]$ChTX binding when assayed at concentrations up to 500-fold greater than the $K_i$ of ChTX (Fig. 5A). This finding is in accord with the observation that neither of these toxins affects $P_{K,PKC}$ electrical activity in bovine aortic smooth muscle cells at concentrations of 1 mM (not shown). These data, then, support the postulate that ChTX-binding sites in vascular smooth muscle are directly associated with $P_{K,PKC}$.

**DISCUSSION**

The results presented in this report describe for the first time the characterization of high affinity binding sites for ChTX in purified sarcolemmal vesicles prepared from bovine aortic smooth muscle. Although others have been unable to detect high affinity binding of ChTX in membranes (30), the present studies have been made possible by the synthesis and purification of an iodinated peptide of defined structure which retains biological activity. This probe binds to sites in smooth muscle sarcolemma which appear directly linked to $P_{K,PKC}$. Several lines of evidence are consistent with this conclusion including: 1) localization of ChTX-binding sites to the sarcolemmal membrane; 2) sensitivity of the binding reaction to TEA and metal ions that bind along the conduction pathway of $P_{K,PKC}$; 3) similarity between binding and electrophysiological investigations with respect to data concerning the mechanism of block by toxin; 4) confirmation that an electrostatic interaction exists between ChTX and its receptor; and 5) the unique pharmacological characteristics associated with the ChTX-binding site. Taken together, these findings provide a convincing argument that the ChTX receptor and $P_{K,PKC}$ are the same entity in vascular smooth muscle. Nevertheless, proof of this will ultimately require purification of the receptor to homogeneity followed by reconstitution and demonstration of $P_{K,PKC}$ activity in the resulting preparation.

ChTX has been shown to inhibit $P_{K,PKC}$ reversibly in primary cultures of bovine aortic smooth muscle (3, 19) and in sarcolemmal membranes prepared from that source which have been reconstituted into planar lipid bilayers. Indeed, the actions of ChTX in these systems are similar to those observed previously during studies of $P_{K,PKC}$ inhibition in skeletal muscle t-tubules (6, 7). Single channel recordings in all cases reveal that toxin blocks the channel by binding with high affinity exclusively at the extracellular pore. Therefore, localization of the ChTX receptor to the sarcolemma of smooth muscle and demonstration that binding occurs with a defined

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2. C. Dunnwiddie, E. Simpson, and J. Jacobs, unpublished observations.

polarity at the membrane surface are both consistent with the idea that ChTX-binding sites comprise part of the channel protein.

A unique characteristic of $P_{K,ca}$ from different sources is its sensitivity to block by externally applied TEA (26). This agent inhibits channel activity from the outside in the high $\mu$M concentration range, whereas it is much less effective when added at the inner membrane surface. The observation that ChTX binding is inhibited by TEA with a $K_i$ nearly identical to that reported for inhibition of $P_{K,ca}$ in electrophysiological experiments coupled with the finding that this agent appears to affect toxin binding in a competitive fashion suggest that ChTX and TEA may share part of a common receptor site at the external pore of the channel. A similar conclusion has been reached in an independent electrophysiological investigation (31). In addition to TEA, several metal ions that block $P_{K,ca}$ by binding to high affinity sites within the channel will also inhibit ChTX binding. Inhibition of channel activity by Ba$^{2+}$ and Cs$^+$ has been well characterized in a number of systems (21–24). Moreover, although Ca$^{2+}$ at low concentration activates the channel, higher concentrations of this ion will inhibit channel activity (25). All of these metal ions cause concentration-dependent inhibition of ChTX binding with a rank order of potency which resembles that for block of $P_{K,ca}$, suggesting that the various ion-binding sites either comprise part of the ChTX receptor or are coupled allosterically to that site. It is interesting that Ca$^{2+}$ displays only a blocking effect on ChTX binding. It might be expected that since low Ca$^{2+}$ concentrations promote channel opening, they might similarly stimulate ChTX binding, if toxin associates only with the open form of the channel as originally hypothesized. However, no Ca$^{2+}$ stimulation of ChTX binding has been observed. Recently, it was reported that ChTX binds in a nearly equivalent fashion to both open and closed $P_{K,ca}$ (6), thus making it difficult to discriminate these particular conformational states through binding studies with ChTX.

One surprising finding of the present investigation is the fact that ChTX binding in vascular smooth muscle is very sensitive to the presence of a Ca$^{2+}$ channel in the medium ($K_i$ of 17 $\mu$M).

A high affinity site for K$^+$ might not be expected to be part of a channel that displays high K$^+$ conductance (>200 picosiemens). Nevertheless, recent experiments describing $P_{K,ca}$ block by Ba$^{2+}$ have indicated the presence of a high affinity K$^+$-binding site near the extracellular face which "locks" Ba$^{2+}$ in the channel (22). This binding site and that at which K$^+$ interacts to alter ChTX binding have similar affinities and may be identical. Another K$^+$ site has been described along the ion conduction pathway at which K$^+$ entering from the inner membrane surface will bind and thereby facilitate ChTX dissociation (7). If this site is involved in inhibition of ChTX binding, then it might be expected that K$^+$ would alter the dissociation kinetics of toxin. Since K$^+$ blocks ChTX binding without affecting $I_{K,ca}$, it is unlikely that this site for K$^+$ is the same as that detected in the latter electrophysiological experiments. The findings that K$^+$ produces partial inhibition of binding at low concentration and also decreases the density of ChTX sites, probably by conversion of receptors to a low affinity state, imply that the K$^+$ site is not part of the ChTX receptor. Rather, they suggest that this ion acts allosterically to modulate ChTX binding. An interesting and puzzling observation is that even though K$^+$ inhibits toxin binding in the low $\mu$M range, ChTX inhibition of $P_{K,ca}$ can still be demonstrated in the presence of high mM concentrations of this ion albeit much higher concentrations of toxin are required to block the channel than are required for the binding experiments due to ionic strength considerations. The following argument may reconcile this apparent discrepancy. The high affinity site for K$^+$ present near the outer face of the channel has been identified under conditions in which the channel is blocked by Ba$^{2+}$ (22). Thus, in this situation, the site is isolated and is in thermodynamic equilibrium only with the extracellular medium. However, under normal K$^+$ flux conditions, other sites along the ion conduction pathway are accessible to K$^+$, and occupancy of these sites causes a marked decrease in the inherent affinity of the high affinity site due to ion repulsion (22, 23). Therefore, inhibition of $P_{K,ca}$ by ChTX can take place in the presence of mM K$^+$ because the high affinity K$^+$ site is in a much lower affinity state.

Recent electrophysiological studies on the mechanism by which ChTX blocks $P_{K,ca}$ in skeletal muscle have illustrated that this highly positively charged toxin binds through an electrostatic interaction with fixed negative charges at the mouth of the channel, perhaps to occlude the pore and thereby inhibit K$^+$ conduction (6). The association rate of ChTX measured in those studies is very rapid, and increasing ionic strength lowers toxin affinity by decreasing $k_f$. Furthermore, after modification of carboxyl groups on the channel protein by exposure to trimethylxonium, the affinity of ChTX is greatly reduced, and toxin-blocking activity is not highly dependent on ionic strength (32). Therefore, it might be predicted that ChTX binding would be sensitive to the ionic composition of the medium. The experimental findings of the present investigation support this expectation; increasing ionic strength with either Na$^+$ or Li$^+$ produces a very steep decrease in toxin-binding activity. Noteworthy is the finding that high affinity ionic modulators of ChTX binding, which are partial inhibitors at low concentration (e.g. Ca$^{2+}$ and K$^+$), also elicit complete block of binding as concentration is elevated into the range at which Na$^+$ and Li$^+$ function. Moreover, the kinetics of ChTX association appear slightly faster than the diffusion control rate expected for a small peptide, agree with kinetic measurements from independent electrophysiological studies conducted under similar experimental conditions, and are altered by the ionic strength of the medium. All of these properties confirm observations from electrophysiological investigations that an electrostatic interaction exists between toxin and the channel protein.

In characterizing the molecular pharmacology of the ChTX receptor in vascular smooth muscle, it was noted that many different agents that are known to affect other ion channels do not influence the binding of ChTX. Even other peptidyl toxins displaying high sequence homology with ChTX have no effect on toxin interactions with sarcolemmal membranes. Importantly, none of these agents modulates $P_{K,ca}$ in smooth muscle, as predicted. Therefore, this channel is likely to have a unique pharmacology that will be developed by discovering novel agents that do affect ChTX binding.

The specificity of ChTX action has been investigated in several systems. Whereas ChTX has no effect on many different ion channels (e.g. Na$^+$ channels, L- and T-type Ca$^{2+}$ channels, various types of K$^+$ channels including an apamin-sensitive low conductance Ca$^{2+}$-activated K$^+$ channel, and an A-type K$^+$ channel in GH3 cells), it will block other K$^+$ channels besides $P_{K,ca}$ (e.g. a 55-pS Ca$^{2+}$-activated K$^+$ channel in aplysia neurones (33), a voltage-dependent K$^+$ channel in lymphocytes (34), the drosophila "shaker" K$^+$ channel expressed in oocytes, but not the native channel present in fly muscle (35), and a neuronal A-type K$^+$ channel expressed in oocytes (36)). These findings suggest that some K$^+$ channels may share common structural domains. This may not be surprising given the fact that the primary structures of the
Na$^+$ channel, the L-type Ca$^{2+}$ channel, and the K$^+$ channel responsible for the shaker phenotype in drosophila all display extensive homology (37). Such observations raise the question as to what determines the specificity and function of a particular channel protein. High affinity channel probes like ChTX will undoubtedly play a significant role in answering this question as well as being important for elucidating the biochemical properties and physiological function of various target ion channels.

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