ω-Conotoxin GVIA Receptors of *Discopyge* Electric Organ

CHARACTERIZATION OF ω-CONOTOXIN BINDING TO THE NICOTINIC ACETYLCHOLINE RECEPTOR*

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A peptide toxin from a *Conus* marine snail, ω-conotoxin GVIA (ω-CgTx) has been used extensively as a probe for certain types of neuronal calcium channels. It is often assumed that ω-CgTx interacts with Ca**+** channels exclusively. We have tested this assumption in a study of ω-CgTx-binding sites in the electric organ of *Discopyge ommata*. Synaptosomal membranes from this tissue contain low affinity ω-CgTx receptor sites (*Kd* = 0.6 μM) in great abundance (280 pmol/mg of protein), as first reported by Ahmad and Miljanich (Ahmad, S. N., and Miljanich, G. P. 1988) Brain Res. 453, 247–256). However, we find that a large majority of these ω-CgTx-binding sites co-purify with the nicotinic acetylcholine receptor (nAChR) and can be immunoprecipitated by monoclonal antibodies generated against the nAChR of *Torpedo*. Cross-linking experiments with radiolabeled ω-CgTx show pronounced specific labeling of the α-subunit of the nAChR but not other subunits. Specific ω-CgTx binding to the nAChR is reduced by millimolar Ca**+** but not by ω- or α-bungarotoxin, α-conotoxin, or carbamylcholine. Cross-linking experiments also reveal ω-CgTx-binding proteins of 170 and 60 kDa. The characteristics of the 170-kDa protein make it a likely candidate for the α-subunit of an N-type Ca**+** channel.

Voltage-gated Ca**+** channels couple electrical activity to Ca**+** entry into neurons and thereby control a variety of cellular responses, including transmitter release, excitability, cell metabolism, and gene expression (1–4). Several classes of Ca**+** channels (e.g. T, N, L, and P) have been distinguished by their physical and pharmacological properties and, in some cases, by their primary structures (2, 4, 5). There is considerable interest in understanding the distinctions between these multiple types of Ca**+** channels, including their ligand-binding and biochemical properties, cellular distribution, and biological roles. An important advance toward these goals was the discovery that Ca**+** channel activity can be inhibited by certain peptide toxins from the fish-hunting marine snail *Conus geographus* (6). The most extensively characterized and generally available toxin is ω-conotoxin GVIA (ω-CgTx), a 27-amino acid peptide (7). ω-CgTx is a potent inhibitor of N-type Ca**+** channels (8–10), a class of high voltage-activated Ca**+** channels that are resistant to 1,4-dihydropyridines, a group of compounds that specifically block L-type Ca**+** channels (11, 12). N-type Ca**+** channels are abundant in the nervous system and play a major role in regulating the release of certain neurotransmitters (13, 14). ω-CgTx has been synthesized (15), radiolabeled with 32P, and widely used as a probe for ligand binding and receptor purification. It has been found that ω-CgTx-binding sites are located primarily in nervous tissue, where they greatly outnumber 1,4-dihydropyridine-binding sites (16) and that ω-CgTx sites can be clustered in hot spots on the surface of individual neurons (17). Several groups have contributed to the biochemical characterization of ω-CgTx-binding proteins (18–24), but only partial purification of binding activity has been achieved to date; a major obstacle to purification has been the lack of a suitably rich source of ω-CgTx-binding sites. Most investigators have chosen avian or mammalian brain as starting material, but the density of ω-CgTx receptors in these tissues is quite low (<1 pmol/mg of protein), 50-fold lower than the density of 1,4-dihydropyridine receptors in purified skeletal muscle transverse tubules (25). Recently, however, Ahmad and Miljanich (26) have reported that synaptosomal membranes from the electric organ of the marine ray *Discopyge ommata* may be a promising source because they contain a single class of ω-CgTx-binding sites (κ ~ 1–3 μM) at a very high density (280 pmol/mg of protein). Moreover, ω-CgTx binding in this preparation closely parallels ω-CgTx block of voltage-dependent Ca**+** uptake and neurotransmitter release (26).

During the course of our studies on ω-CgTx binding in the *D. ommata* electric organ, we found reason to question the idea that ω-CgTx binds exclusively to Ca**+** channels, a key assumption of many efforts to count, localize and purify neuronal Ca**+** channels. In this report, we describe three proteins in electric organ synaptosomes with molecular masses of approximately 170, 60, and 42 kDa that bind ω-CgTx with micromolar affinity. Furthermore, we have determined that the 42-kDa protein, the most abundant ω-CgTx-binding protein in the preparation, is, in fact, the α-subunit of the nicotinic acetylcholine receptor.

EXPERIMENTAL PROCEDURES

Materials—125I-ω-CgTx GVIA (2200 Ci/mmol) was obtained from Du Pont-New England Nuclear, and unlabeled ω-CgTx GVIA was

*The abbreviations used are: ω-CgTx, ω-conotoxin GVIA; nAChR, nicotinic acetylcholine receptor; DMS, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody.*

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purchased from Peninsula Laboratories, Inc. (Belmont, CA). Dimethyl suberimidate (DMS) was purchased from Pierce Chemical Co. Chromatography materials were obtained from Pharmacia LKB Biotechnology Inc., and electrophoresis reagents were from Bio-Rad. Goat anti-mouse IgG-Sepharose was purchased from Cappel/Orga-non-Yenika. ω- and ε-bungarotoxin and ω-conotoxin were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Sodium deoxycholate and Nε-carboxyanhydride-ε-bungarotoxin (Nε-CbTx) were obtained from Sigma. DEAE ion exchange chromatography was performed using a 0-700 mM KH2PO4 gradient in Buffer B. Fractions were assayed for specific 125I-ω-CgTx binding activity. Fractions of interest were pooled, concentrated to 200 μl in Centricon-10 microconcentrators (Amicon), and analyzed by SDS-PAGE (3-12% gradient gels) as described above.

Immuno precipitation of the Acetylcholine Receptor—Hybridoma supernatant (mAb 05.74) or ascites fluid (mAb 88B) was incubated with 1 ml of goat anti-mouse IgG-Sepharose overnight at 4 °C. Fractions (0.6 ml) were collected from the bottom of the tube and assayed for specific 125I-ω-CgTx binding activity. Fractions of interest were pooled, concentrated to 200 μl in Centricon-10 microconcentrators (Amicon), and analyzed by SDS-PAGE (3-12% gradient gels) as described above.

RESULTS

Properties of 125I-ω-CgTx-binding Sites in Electric Organ Synaptosomes—The characteristics of toxin binding to purified electric organ synaptosomal membranes were determined by a rapid filtration assay. Specific binding was determined for a range of 125I-ω-CgTx concentrations by taking the difference between results obtained in the absence and presence of 25 μM ω-CgTx. Representative results are illustrated in Fig. 1A. The specific binding data were well fitted by a hyperbolic function appropriate for a single class of binding sites with an apparent dissociation constant (Kd) of 0.6 μM and a maximal site density (Bmax) of 280 pmol of receptor sites/mg of protein. Nonspecific binding increased linearly with increasing concentrations of ω-CgTx and accounted for 50% of the total binding at saturating concentrations. These values are representative of a total of five separate experiments and are similar to results reported by Ahmad and Miljanich (26). The results support the idea that D. ommata synaptosomes are a rich source of ω-CgTx receptors.

To determine which protein(s) bind ω-CgTx, the homobifunctional imido ester, DMS was used to cross-link the toxin to protein components of synaptosomal membranes. Three membrane proteins show specific labeling as demonstrated by competition between 125I-ω-CgTx and 25 μM unlabeled ω-CgTx (Fig. 1B). The molecular masses of these specifically labeled proteins were 170, 60, and 45 kDa as determined by SDS-PAGE. Densitometric analysis of the autoradiograms reveals that the 45-kDa protein accounts for 70% of the total binding at saturating concentrations. These values are representative of a total of five separate experiments and are similar to results reported by Ahmad and Miljanich (26).

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**FREE 125I-\(\omega\)-CgTx (\(\mu\)M)

\[ K_D = 0.6 \, \mu\text{M} \]
\[ B_{\text{max}} = 280 \, \text{pmol/mg} \]

**FIG. 1.** 125I-\(\omega\)-CgTx equilibrium binding and covalent cross-linking to *D. ommata* synaptosomal membranes. 

**A**, characterization of specific binding to synaptosomes. Membranes (1.0 mg of protein/ml) were incubated for 2 h at 4°C with 125I-\(\omega\)-CgTx in the presence and absence of 25 \(\mu\)M unlabeled \(\omega\)-CgTx to determine total and nonspecific binding, respectively (see "Experimental Procedures"). Specifically bound 125I-\(\omega\)-CgTx was determined as the difference between total and nonspecific binding and was plotted against the free 125I-\(\omega\)-CgTx concentration. Points represent the averages from five separate experiments. The continuous curve represents a saturation isotherm predicted by a single binding site model with \(K_D = 0.6 \, \mu\text{M}\) and \(B_{\text{max}} = 280 \, \text{pmol/mg}\) of protein.

**B**, covalent cross-linking of 125I-\(\omega\)-CgTx to synaptosomal proteins. Membranes (0.1 mg of protein/ml) were incubated with 50 nM 125I-\(\omega\)-CgTx for 2 h at 4°C either in the absence (lane 1) or presence (lane 2) of 25 \(\mu\)M unlabeled \(\omega\)-CgTx prior to cross-linking with 20 mM DMS for 2 h at 4°C, as described under "Experimental Procedures." The reaction was terminated by the addition of SDS reducing sample buffer, and the samples were analyzed by SDS-PAGE (3-12% gradient gel) and subsequent autoradiography. The arrows indicate those proteins (170-, 60-, and 45-kDa) specifically labeled by 125I-\(\omega\)-CgTx.

**C**, 125I-\(\omega\)-CgTx displacement by increasing concentrations of unlabeled \(\omega\)-CgTx. Cross-linking was performed as in **B**, in the presence of 0.1-10 \(\mu\)M \(\omega\)-CgTx. The 170-, 60-, and 45-kDa bands in the autoradiographs were subjected to densitometric scanning. Normalized 125I-\(\omega\)-CgTx labeling for each of the proteins is plotted against the unlabeled \(\omega\)-CgTx concentration. Points represent the averages from three separate experiments, and error bars represent the standard deviation. Curves are fitted hyperbolic isotherms.

shows displacement profiles for the 170-, 60-, and 45-kDa proteins. In each case, the data fit a single hyperbolic curve, with half-maximal displacement of 125I-\(\omega\)-CgTx occurring at a toxin concentration of 0.7-0.8 \(\mu\)M. These IC_{50} values are in good agreement with the apparent \(K_D\) determined in Fig. 1A. Thus, cross-linking experiments reveal a multiplicity of \(\omega\)-CgTx-binding proteins that contribute to the overall binding to synaptosomal membranes.

**Characterization of Solubilized 125I-\(\omega\)-CgTx-binding Sites**

To characterize further the toxin-binding proteins, synaptosomal membranes were solubilized with digitonin and 125I-\(\omega\)-CgTx-binding sites were partially purified by DEAE ion exchange and hydroxylapatite chromatography (See "Experimental Procedures"). The hydroxylapatite eluant was then subjected to sucrose density gradient (5-20%) sedimentation. The sedimentation profile (Fig. 2A) displayed two clear peaks of 125I-\(\omega\)-CgTx binding activity (I, II). Analysis of the peak fractions by SDS-PAGE and staining with Coomassie Blue is illustrated in Fig. 2B. The protein compositions of peaks I and II are basically similar; in both cases, the predominant bands show apparent molecular masses of 41, 51, 58, and 67 kDa. These are strikingly similar in pattern and apparent \(M_r\) to the subunits of the nicotinic acetylcholine receptor (nAChR) and are provisionally labeled as \(\alpha, \beta, \gamma, \) and \(\delta\). Furthermore, the sedimentation characteristics of the 125I-\(\omega\)-CgTx binding are reminiscent of the behavior of monomeric and dimeric forms of the acetylcholine receptor, as analyzed previously by sucrose density gradient sedimentation (29). Taken together, these results strongly suggest that 125I-\(\omega\)-CgTx binding activity co-sediments with the nAChR.

**Immunoprecipitation of 125I-\(\omega\)-CgTx Binding with Monoclonal Antibodies to nAChR**

As a direct test of the possibility
that $^{32}$I-$\omega$-CgTx binds to the nicotinic acetylcholine receptor, monoclonal antibodies to the nAChR were tested for interactions with solubilized $\omega$-CgTx-binding sites. Antibodies tested included mAb 35.74, an antibody directed against an antigenic determinant near the main immunogenic region of the $\alpha$-subunit of the Torpedo nAChR (30, 31), and mAb 88B, an antibody directed against an epitope common to the $\gamma$- and $\delta$-subunits (32). Immunoblot analysis (Fig. 3A, lane 1) shows that the anti-Torpedo antibody 35.74 cross-reacts with a single 42-kDa protein component of Discopyge membranes, presumably the $\alpha$-subunit of the nAChR. Next, synaptosomal membrane proteins were solubilized, immunoprecipitated with mAb 35.74, subjected to SDS-PAGE, and stained with Coomassie Blue (lane 2). The immunoprecipitate contained the 42-kDa protein and three other bands, corresponding to all four subunits of the nAChR. (Free antibody does not interfere with the SDS-PAGE analysis because mAb 35.74 was cross-linked to goat anti-mouse IgG-Sepharose beads).

To determine whether the nAChR binds $\omega$-CgTx, $^{125}$I-$\omega$-CgTx was cross-linked to membrane proteins prior to their solubilization, immunoprecipitation, and analysis by SDS-PAGE and autoradiography (Fig. 3A, lanes 3 and 4). The results show that the immunoprecipitate contains significant amounts of cross-linked toxin. Moreover, the $^{125}$I-$\omega$-CgTx specifically labels a single dominant protein with a $M_r$ of 45 kDa, corresponding to the 45-kDa protein described earlier (Fig. 1B); the apparent molecular weight is that expected for a 42-kDa nAChR $\alpha$-subunit cross-linked to a 3-kDa toxin molecule. The specificity of toxin binding is indicated by the relative lack of $^{125}$I-$\omega$-CgTx labeling in the presence of 25 $\mu$M unlabeled toxin (lane 4).

Fig. 3B demonstrates that, whereas a control antibody (mAb VF6) is ineffective, 80% of the solubilized $\omega$-CgTx-binding sites were immunoprecipitated by saturating amounts of anti-acetylcholine receptor mAb directed against either the $\alpha$-subunit (35.74) or the $\gamma$/$\delta$-subunits (88B). This is not surprising, since the 45-kDa $^{125}$I-$\omega$-CgTx-cross-linked protein accounts for the majority of the total binding in membranes (Fig. 1B) and is readily solubilized by digitonin. The 170-kDa protein is not readily solubilized under these conditions; the 60-kDa contributes a major fraction of the 20% of $^{125}$I-$\omega$-CgTx-binding sites remaining in the supernatant (results not shown).

Characterization of $^{125}$I-$\omega$-CgTx Binding to the Acetylcholine Receptor—To determine if $^{125}$I-$\omega$-CgTx interacts with previously characterized binding sites on the acetylcholine receptor, competition experiments were performed between $^{125}$I-$\omega$-CgTx and various ligands known to bind to the $\alpha$-subunit. Fig. 4A illustrates that $^{125}$I-$\omega$-CgTx binding was not significantly reduced by saturating concentrations of $\alpha$-bungarotoxin, $\kappa$-bungarotoxin, $\alpha$-conotoxin, or carbamylcholine. $^{125}$I-$\omega$-CgTx binding is, however, strongly reduced by unlabeled $\omega$-CgTx or by 1 mM CaCl$_2$. These results suggest that $\omega$-CgTx binds to a novel site on the $\alpha$-subunit that is in close association with a Ca$^{2+}$-binding site. The density of $\alpha$-subunits in the membrane preparation was estimated by measurements of the number of $\alpha$-bungarotoxin-binding sites (average $B_{max}$ = 260 pmol/mg; three experiments). The rough correspondence between estimates of $\omega$-CgTx-binding sites and $\alpha$-bungarotoxin-binding sites is consistent with a stoichiometry of one $\omega$-CgTx site/$\alpha$-subunit.

To characterize further the properties of $\omega$-CgTx binding to the nAChR, various cations were tested for their effects on $^{125}$I-$\omega$-CgTx binding. First, ionic strength effects were assessed by measuring specific $^{125}$I-$\omega$-CgTx binding in the presence of increasing concentrations of NaCl. Fig. 4B illustrates that high concentrations of Na$^+$ (IC$_{50}$ = 100 mM) were required to displace specific $^{125}$I-$\omega$-CgTx binding from the nAChR. This result demonstrates that the binding of the positively charged toxin to the receptor is not simply the result of weak, nonspecific charge interactions. It raises the possibility, however, that $\omega$-CgTx binding to the nAChR may be partially inhibited...
been reported to have potent inhibitory effects on the binding either Ca$^{2+}$ or La$^{3+}$. Both divalent and trivalent cations have experiments were performed with increasing concentrations of inhibition of w-CgTx binding to the nAChR by these cations at physiological salt concentrations. Next, displacement experiments were performed with increasing concentrations of either Ca$^{2+}$ or La$^{3+}$. Both divalent and trivalent cations have been reported to have potent inhibitory effects on the binding of w-CgTx to putative Ca$^{2+}$ channels in rat brain (16). Fig. 4B shows that both Ca$^{2+}$ and La$^{3+}$ inhibit w-CgTx binding at concentrations much lower than that required by Na$^+$. Half-maximal displacement of $^{125}$I-w-CgTx binding occurs at 1.5 mM for Ca$^{2+}$ and 0.35 mM for La$^{3+}$. The order of potency for inhibition of w-CgTx binding to the nAChR by these cations (La$^{3+} > $ Ca$^{2+} > $ Na$^+$) is similar to that reported for inhibition of w-CgTx binding in brain (12). Moreover, an IC$_{50}$ of 1.5 mM for Ca$^{2+}$ is in excellent agreement with the results of a previous study in which a Ca$^{2+}$-binding site ($K_d = 1$ mM) was identified on the $\alpha$-subunit of the nAChR (33).

### DISCUSSION

This report contains a number of unexpected findings regarding receptors for $\omega$-conotoxin GVIA. One of the main discoveries is that not all w-CgTx binding sites are voltage-dependent Ca$^{2+}$ channels. Indeed, we obtained several pieces of evidence indicating that w-CgTx binds to the nicotinic acetylcholine receptor: (a) w-CgTx binding activity co-purifies with the nAChR, (b) w-CgTx binding activity can be immunoprecipitated by mAbs directed against either the $\alpha$- or $\gamma$- and $\delta$-subunits of the nAChR, and (c) the immunoprecipitate contains an $^{125}$I-w-CgTx-protein complex that displays the molecular weight expected for a toxin molecule cross-linked to a nAChR $\alpha$-subunit. Under the same conditions, cross-linking of radiolabeled toxin to other subunits of the nAChR is not detectable.

The effect of w-CgTx on nAChR function remains to be determined in D. ommata. $\omega$-CgTx has been reported to have no effect on postsynaptic cholinergic responses in both frog neuromuscular (34) and Narke japonica electric organ preparations (35). Similarly, w-CgTx does not appear to block responses of sympathetic neurons to acetylcholine. These experiments were performed, however, at NaCl and CaCl$_2$ concentrations that, based on our results, may have inhibited toxin binding. Whether or not cholinergic function is affected, the mere binding of w-CgTx to nAChRs is an important result with respect to the interpretation of radioligand binding and cell biological studies. Our results suggest caution in the use of w-CgTx to count or localize Ca$^{2+}$ channels. The extent of toxin binding to nAChRs will vary with the number and affinity of nAChRs in individual systems. Problems of interpretation might be most serious in cells in which nAChRs are plentiful and highly clustered.

Another important finding is that the electric organ contains a 170-kDa w-CgTx-binding protein, distinct from the nAChR, that appears as a clear, sharp band in cross-linking experiments. This protein is thus a candidate for identification as the $\alpha_1$-subunit of the N-type Ca$^{2+}$ channel. This

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2 K. R. Bley, personal communication.
the results are expressed as the percentage of binding in the absence of control. The experimental Procedures, and the results are expressed as a percentage of protein aggregates.

The possible significance of ω-CgTx interactions with both nAChRs and Ca^{2+} channels is not clear. It is conceivable that the toxin binding reflects some hitherto unrecognized common structural feature of both these channels. A potential structural similarity is reflected in the ability of Ca^{2+} and La^{3+} to inhibit the binding of ω-CgTx to both the Ca^{2+} channel and the nAChR. A Ca^{2+}-binding site (K_D = 1 mM) on the α-subunit of the nAChR has been well characterized (33); however, little is known about the structural aspects of Ca^{2+} binding to voltage-gated Ca^{2+} channels. Our results raise the possibility that a Ca^{2+}-binding site common to both classes of ion channels has been highly conserved during the evolution of ligand-gated and voltage-gated channels from a common ancestor (38). ω-CgTx may serve as a useful probe for studying the properties of Ca^{2+} binding to both classes of ion channels.

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References


FIG. 4. Characterization of [125I]-ω-CgTx Binding to the Acetylcholine Receptor. A, effects of acetylcholine receptor ligands on [125I]-ω-CgTx binding. Membranes were incubated with 50 nM [125I]-ω-CgTx alone as a control (Ctrl) or with 50 nM [125I]-ω-CgTx in the presence of 10 μM ω-CgTx (ω-CgTx), 0.1 μM α-bungarotoxin (α-BTX), 10 μM ω-bungarotoxin (ω-BTX), 1 μM carboxylmethyl (Carb), or 1 mM CaCl_2 (Ca^{2+}). Specific [125I]-ω-CgTx binding was determined for each treatment as described under “Experimental Procedures,” and the results are expressed as a percentage of control. The data bars represent the means of three experiments done in duplicate, and the error bars represent the standard deviation. B, effects of tri-, di-, and monovalent cations on [125I]-ω-CgTx binding. Membranes (1.0 mg of protein/ml) were incubated with either 0.1-10 mM LaCl_3 (Λ), 0.1-44 mM CaCl_2 (Ca^{2+}), or 4.4-1000 mM NaCl (Na^+). Specific binding of 50 nM [125I]-ω-CgTx was determined at each cation concentration, as described under “Experimental Procedures,” and the results are expressed as the percentage of binding in the absence of added cations. Points represent the averages from four separate determinations, and error bars represent the standard deviation.

The hypothesis is supported by a number of observations: (a) the characteristics of [125I]-ω-CgTx binding to the 170-kDa subunit are consistent with the dose-dependent block of depolarization-induced Ca^{2+} influx and transmitter release (26), (b) the estimated molecular weight of 170 kDa is consistent with α-subunits of Ca^{2+} channels in other systems and remained unchanged under nonreducing or reducing conditions, as expected for an α-subunit (36, 37), and (c) ω-CgTx binding was not displaced by other Ca^{2+} channel antagonists, such as nifedipine or verapamil. It is not clear whether the 170-kDa ω-CgTx-binding protein in the electric organ is related to ω-CgTx-binding proteins found in other preparations. Earlier results show cross-linking to 300-kDa (20, 21), 220-kDa (23, 24), and 140-kDa (19, 23) proteins. Possible causes of these differences are (a) a diversity of toxin receptors in different animals, (b) different degrees of posttranslational processing, and (c) cross-linking of multiple subunits to form larger protein aggregates.

Our results suggest that the electric organ may yet be useful as a starting material for purification of N-type calcium channels. Although the affinity of ω-CgTx-binding sites in D. ommata synaptosomal membranes is considerably lower than in many other tissues, the system offers the great advantage of an abundance of sites. Even if the 170-kDa protein represents only 20% of the total ω-CgTx-binding sites (60 pmol/mg), this is roughly 2 orders of magnitude more abundant than the ω-CgTx receptors in mammalian brain.


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