THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 261, No. 14, Issue of May 15, pp. 6230–6233, 1986
Printed in U.S.A.

Calcium Channel Antagonists

ω-CONOTOXIN DEFINES A NEW HIGH AFFINITY SITE*

(Received for publication, November 4, 1985)

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The ω-conotoxins, a class of Ca^{2+} channel antagonists from fish-hunting marine snails, have recently been described (Olivera, B. M., McIntosh, J. M., Zeikus, R., Gray, W. R., Varga, J., Rivier, J., de Santos, V., and Cruz, L. J. (1985) *Science*, 230, 1338–1343). One of these peptide neurotoxins, ω-conotoxin GVIA, was radiolabeled with iodine, and the ^{125}I-labeled toxin was shown to bind specifically to high affinity sites on chick brain synaptosomes. The toxin-receptor complex was extremely stable; addition of an excess of unlabeled toxin did not cause significant displacement of the labeled toxin after 2 h.

Binding competition data suggest that ω-conotoxin defines a new high affinity receptor site affecting voltage-activated Ca^{2+} channels, distinct from both the verapamil and dihydropyridine target sites.

Voltage-activated calcium channels are ubiquitously found in nerve and muscle. These ion channels are believed to transduce electrical signals in excitable tissues into biochemical changes (1–4). A prominent treatise on ion channels describes the importance of Ca^{2+} channels succinctly, "without calcium channels our nervous system would have no outputs" (1).

Neuronal Ca^{2+} channels have not been extensively characterized at the molecular level, compared to acetylcholine receptors and Na^{2+} channels. To investigate the latter molecules, the use of drugs and toxins which affect the activity of the channels has been indispensable. The development of α-bungarotoxin which binds the acetylcholine receptor with high affinity was the key to a biochemical characterization of this receptor (5). The availability of guanidinium, alkaloid, and peptide toxins which bind to different sites on the Na^+ channel have greatly facilitated the dissection of this channel (6).

The agents currently in widest use for studying calcium channels are the dihydropyridines (*i.e.* nitrendipene, nifedipine, and related compounds) and anti-arrhythmic drugs such as verapamil; these agents define at least two distinct, though coupled, binding sites which affect Ca^{2+} channel activity (2–4, 7, 8). Recently, we reported the isolation and purification of a new class of presynaptic toxins, the ω-conotoxins from the venom of fish-hunting snails (9, 10). Electrophysiological work on one of these toxins, ω-conotoxin GVIA, has shown that the toxin blocks voltage-activated calcium channels at the frog neuromuscular junction, and, indeed, blocks all frog synapses tested (11). Thus, ω-conotoxins are very promising tools for studying voltage-activated calcium channels at synapses.

In this paper, we demonstrate specific high affinity binding of ω-conotoxin GVIA to synaptosomal and membrane preparations from chick brain. We also provide evidence that ω-conotoxins define a new class of target sites which affect Ca^{2+} channels, distinct from both dihydropyridine- and verapamil-binding sites.

EXPERIMENTAL PROCEDURES

Materials—ω-Conotoxin GVIA (ω-CgTx[1]) was purified as described by Olivera *et al.* (9). [5-*methyl*-^{3}H]nitrendipine was obtained from New England Nuclear. All other calcium antagonists and the protease inhibitors were purchased from Sigma. Iodogen was obtained from Pierce Chemical Co.

Membrane Preparations—Membrane fractions enriched in T tubules were prepared according to the method of Glossman *et al.* (12) from the hind leg muscle of Northern grass frogs, *Rana pipiens*.

Synaptosomes were prepared from the brain of frogs and 19–20-day-old chick embryo essentially as described by Catterall *et al.* (13) except that all sucrose solutions were buffered with 5 mM HEPES/Tris, pH 7.4, and contained the following protease inhibitors: 1 μM pepstatin A, 2 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM 1,10-phenanthroline. For chick brain, the stepwise gradient was modified such that the bottom layer contained 1.3 M instead of 1.2 M sucrose. All fractions were pelleted, resuspended in 0.32 M sucrose, 5 mM HEPES/Tris, pH 7.4, containing protease inhibitors, and stored either on ice or in the deep freezer. For some experiments, the heavy synaptosome fraction of the 1.0–1.3 M sucrose interface was lysed and the membranes fractionated as described by Catterall *et al.* (13).

Iodination of ω-CgTx—The toxin was iodinated according to the method of Fraker and Speck (14) using [^{125}I]NaI (0.20 mCi/nmol) as label and Iodogen as a solid phase reagent. The iodinated material was applied on a 3-ml Sephadex G-10 column and eluted with 5% acetic acid. The radioactive peak which eluted close to the void volume was pooled and purified by high pressure liquid chromatography using a VYDAC C18 column (0.46 x 25 cm; 5-μm particle, not end capped) at a flow rate of 1 ml/min. A gradient was applied using Solvent A, 0.1% trifluoroacetic acid, and Solvent B, 0.1% trifluoroacetic acid in 60% (v/v) acetonitrile (15). A representative chromatogram is shown in Fig. 2. The biological activity of the radiolabeled peaks was determined as described previously (9). The two major peaks of iodinated derivatives were biologically active. In two independent determinations/sample, iodinated conotoxin GVIA gave specific activities of 14 and 27 units/nmol for the early and 22 and 60 units/nmol for the later eluting peaks, respectively. The specific activity of unmodified toxin was 70 units/nmol. One unit of ω-CgTx produces detectable symptoms in 30 min after intracranial injection of a 16–19-day-old mouse. For the ^{125}I-labeled ω-CgTx preparation used in most of the binding assays described below, the two peaks were pooled, dried, and taken up on 0.32 M sucrose, 5 mM HEPES/Tris, pH 7.4, containing 0.2 mg/ml lysozyme. In the experiments in Fig. 3 and Table I, only

* This work was supported by Research Grant GM22737 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: ω-CgTx, ω-conotoxin GVIA; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

the larger later eluting iodinated peak was used. The ^{125}I-labeled preparation had specific activities in the range of 1–4 × 10^8 cpm/nmol.

Binding Assays—The measurement of [^{3}H]nitrendipine binding to membrane preparation from frog brain and muscle was done as described by Curtis and Catterall (16).

For measuring the binding of ^{125}I-labeled ω-CgTx to brain synaptosomes and T tubule preparations, the assay mix (200 μl) contained the following: 30–100 μg of membrane protein, ^{125}I-labeled ω-CgTx, 0.01 mg/ml lysozyme, 0.32 M sucrose, 5 mM HEPES/Tris, pH 7.4. To measure nonspecific binding, 0.25 μM cold ω-CgTx was preincubated for 30 min with the membrane preparations before ^{125}I-labeled ω-CgTx was added. The mixture was then incubated at room temperature for 30 min–1 h and diluted with 2–3 ml of ice-cold wash medium containing 160 mM choline chloride, 5 mM HEPES/Tris, pH 7.4, 1.5 mM $CaCl_2$, and 1 mg/ml bovine serum albumin. Membranes were collected on glass fiber filters (Whatman GF/C) under vacuum and washed 3 times with 2 ml of wash medium. Radioactivity of filters and sometimes of filtrates was determined.

RESULTS

ω-Conotoxin Does Not Compete with Nitrendipine for Binding to T Tubule Skeletal Muscle Sites—One of the richest sources of dihydropyridine-binding sites are T tubule preparations from skeletal muscles (12, 17); the nitrendipine-binding site has been purified from this tissue (18, 19). We have investigated ω-conotoxin competition for the high affinity dihydropyridine-binding sites of skeletal muscle.

The binding of [^{3}H]nitrendipine to the high affinity dihydropyridine sites of frog skeletal muscle is shown in Fig. 1. These data on nitrendipine binding are consistent with previous reports in the literature. These results demonstrate that while nifedipine competes effectively with the labeled nitrendipine for binding, the addition of purified ω-conotoxin does not detectably reduce the binding of [^{3}H]nitrendipine, even at 1000-fold excess. We conclude that ω-conotoxin does not compete with [^{3}H]nitrendipine for the high affinity dihydropyridine T tubule sites.

Specific Binding of ω-Conotoxin to Synaptosomal Preparations—In order to examine the binding of ω-conotoxin directly, it is necessary to have radioactive biologically active toxin. The iodination of ω-conotoxin using Iodogen was carried out as described under "Experimental Procedures." A high pressure liquid chromatography analysis of the iodinated toxin is shown in Fig. 2. The biological activity of the various peaks was tested; the two major peaks (indicated by *shading*) which were radioactively labeled were biologically active (see "Experimental Procedures"). The toxin preparation used in most experiments comprised a pool of the two peaks indicated in the figure.

The radiolabeled toxin was used for binding assays as described under "Experimental Procedures." A number of tissues from frog and chick were tested in a preliminary survey. It was found that the frog skeletal muscle preparation used for the [^{3}H]nitrendipine-binding studies gave marginal levels of binding. In contrast, crude frog and chick brain preparations gave easily detectable levels of specific binding. Because of the greater accessibility of chick brain, this tissue was used in all subsequent experiments.

Chick brain synaptosomal membranes were purified (see "Experimental Procedures"). Iodinated ω-conotoxin was added to synaptosomal membranes (Figs. 3 and 4), and a saturable binding component was detected which meets all criteria for specific binding. The amount of binding at saturation is proportional to the amount of membrane preparation provided (Table I). These results demonstrate that a high affinity ω-conotoxin-binding site can be assayed by the method used. In the experiments in Figs. 3 and 4, apparent half-saturation of the receptor is achieved at subnanomolar concentrations of toxin. However, equilibrium has not been attained under these conditions since there is negligible dis-

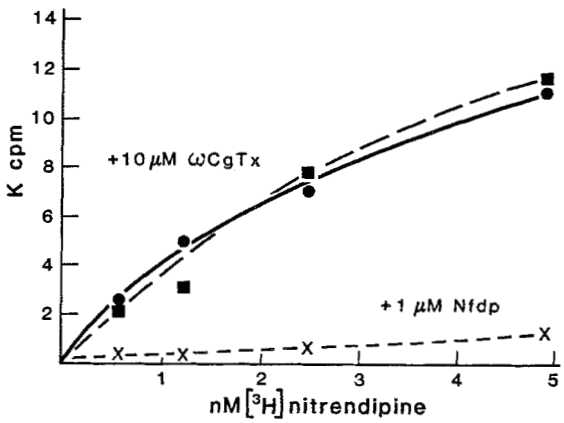

FIG. 1. **The binding of [^{3}H]nitrendipine to membrane preparation from frog skeletal muscle.** A membrane preparation enriched in T tubules was prepared, and the binding assay was done as described under "Experimental Procedures." The reaction mixture (0.2 ml) contained 1–5 nM [^{3}H]nitrendipine (81.3 μCi/nmol), 36.7 μg of membrane protein, 94 mM choline chloride, 4 mM glucose, 50 mM KCl, 1.5 mM $CaCl_2$ and 78 mM HEPES/Tris, pH 7.4. Total binding is represented by ■. Nonspecific binding was estimated by preincubating the membrane preparation with 1 μM nifedipine (*Nfdp*) for 30 min at 0 °C before adding [^{3}H]nitrendipine (×). Membrane preparations were also preincubated with 10 μM ω-CgTx (●). Incubation with [^{3}H]nitrendipine was carried out on ice for 1 h.

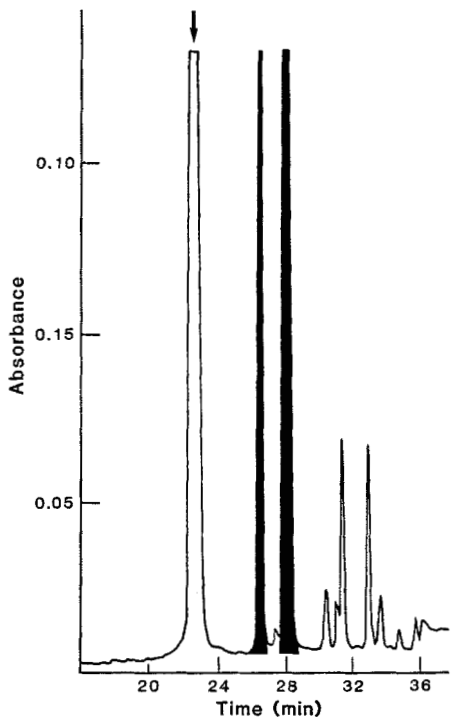

FIG. 2. **Chromatographic purification of ^{125}I-ω-conotoxin GVIA on VYDAC C18 analytical column.** Solvent A, 0.1% trifluoroacetic acid; Solvent B, 0.1% trifluoacetic acid in 60% (v/v) acetonitrile. Iodinated derivatives of GVIA were eluted at 1 ml/min with a linear gradient expressed as a percentage of Solvent B achieved at times given in parentheses (min): 10(0)/10(2)/50(42). For the chromatogram shown, 20 nmol of GVIA were iodinated using 20 nmol of [^{125}I]NaI. Unmodified ω-conotoxin GVIA is indicated by the *arrow*. The *shaded peaks* are iodinated derivatives which retained significant biological activity and which were used for the binding assays described in this report.

TABLE I

Proportionality of toxin binding to synaptosomal protein concentration

Binding assays were carried out as described under "Experimental Procedures" using the same chick brain synaptosome and ^{125}I-labeled ω-CgTx preparations as in Fig. 3. The concentrations of toxin used were saturating; the protein concentration was varied as shown. Nonspecific binding has been corrected for as described in the legend to Fig. 3.

Total protein in assay	^{125}I-labeled ω-CgTx	Toxin bound	Protein
μg	*nM*	*fmol*	*pmol/mg*
24	2.0	49	2.1
24	4.0	41	1.7
47	2.0	79	1.7
47	4.0	92	1.9
94	2.0	160	1.7
94	4.0	170	1.8

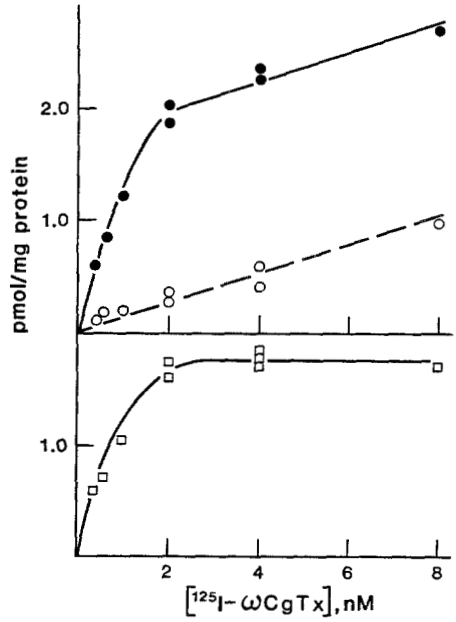

FIG. 3. **The binding of ^{125}I-labeled ω-CgTx to chick brain synaptosomes.** Synaptosomes were prepared and the binding assay done as described under "Experimental Procedures." *Upper panel*, the reaction mixture (●) contained various concentrations of ^{125}I-labeled ω-CgTx, 94.1 μg of membrane protein, 0.01 mg/ml lysozyme, 0.32 M sucrose, 5 mM HEPES/Tris, pH 7.4, in a total volume of 90.2 μl. Nonspecific binding (○) was determined in parallel reactions by preincubating synaptosomal preparations with 0.25 μM unlabeled ω-CgTx for 30 min on ice before the addition of radiolabeled toxin. Incubation with ^{125}I-labeled ω-CgTx was done for 30 min at room temperature. The ^{125}I-labeled ω-CgTx used (peak eluting at 28 min in Fig. 2) had a specific activity of 1.57×10^8 cpm/nmol. *Lower panel*, the same data as in the *upper panel* replotted after subtraction of nonspecific binding.

sociation of toxin for receptor within the assay time frame (see below).

Competition of Nifedipine, Verapamil, and ω-Conotoxin for Binding to Brain Synaptosomes—Two classes of pharmacologically active agents have been used to study voltage-activated Ca^{2+} channels, the anti-arrhythmic drugs (typified by verapamil) and the dihydropyridines. We have examined whether verapamil will compete with ω-conotoxin binding using brain synaptosomal preparations (see Fig. 4). The presence of an excess of verapamil (1 mM) does not affect ^{125}I-labeled ω-conotoxin binding to the high affinity site. We conclude that verapamil-binding sites and ω-conotoxin-binding sites are distinct from each other in chick brain synaptosomes. A number of other voltage-activated Ca^{2+} channel antagonists are known, some of which are believed to bind at the same site as verapamil and some (such as diltiazem) which have clearly different structures and pharmacological effects (4) and have been suggested to bind at different sites (20). These antagonists were tested for their ability to inhibit binding by ^{125}I-labeled ω-conotoxin. As shown in Table II, none of these agents inhibit ω-conotoxin binding to the high affinity chick brain site.

The dihydropyridines, such as nifedipine, bind to a site which has been well defined by numerous studies. We tested whether nifedipine would influence ^{125}I-labeled ω-conotoxin binding; the presence of nifedipine does not detectably affect ^{125}I-labeled ω-conotoxin binding to chick brain synaptosomes (Table II). These data as well as the results in Fig. 1 lead to the conclusion that dihydropyridine-binding sites and the high affinity ω-conotoxin target are distinct both in frog

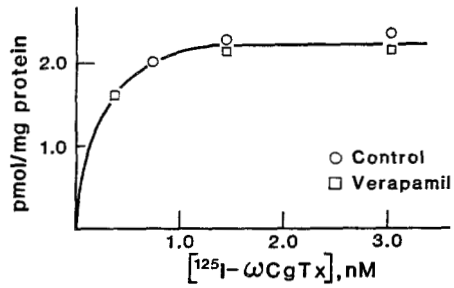

FIG. 4. **Effect of verapamil on ^{125}I-labeled ω-CgTx binding.** The synaptic membrane preparation from chick brain and the assay procedure are as described under Fig. 3. For each point, the nonspecific binding measured in the presence of 0.25 μM unlabeled ω-CgTx was subtracted from total binding by ^{125}I-labeled ω-CgTx. The concentration of (±)verapamil used was 0.5 μM.

TABLE II

Effect of Ca^{2+} channel antagonists on ^{125}I-labeled ω-conotoxin binding

The reaction mixture contained 100 μg of protein of chick brain synaptosomal preparation, various calcium antagonists as indicated below, and other components as stated under "Experimental Procedures." In Experiment 2, all tubes contained 0.92 nM ^{125}I-labeled ω-CgTx. Incubation with labeled toxin was done at room temperature for 1 h.

Experiment 1

Concentration of ^{125}I-labeled ω-CgTx added	Counts/min on filter		
	Control	+ 0.25 μM unlabeled ω-CgTx	+ 10 μM nifedipine
nM			
0.18	1,227	545	1,394
0.46	3,253	1,278	3,325
0.92	6,164	2,336	6,195
3.7	17,339	10,013	17,551

Experiment 2

Inhibitors added	% of control
Control	100
+ Nifedipine, 10 μM	101
+ Prenylamine lactate, 1 μM	101
+ Prenylamine lactate, 20 μM	99
+ Diltiazem, 1 μM	111
+ Diltiazem, 20 μM	106
+ Flunarizine, 1 μM	109
+ Flunarizine, 20 μM	100
+ Cinnarizine, 1 μM	102
+ Cinnarizine, 20 μM	98

TABLE III

Effect of various salts on toxin-complex formation and stability

In Experiment 1 the reaction mixture contained 0.5 μg/μl protein of chick brain synaptosome, 0.86 nM ^{125}I-labeled ω-CgTx, the salts given below, and other components as stated under "Experimental Procedures." Incubation with labeled toxin was done at room temperature for 30 min. In Experiment 2, a 1.6-ml reaction mixture containing 2.5 μg/μl protein of chick brain synaptosome, 1.3 nM ^{125}I-labeled ω-CgTx and the other components of a regular binding medium was incubated for 30 min. This was centrifuged at 16,000 rpm (Sorvall SS34) for 20 min, and the pellet was resuspended in 2 ml of wash medium. After centrifugation, the washed pellet was resuspended in 750 μl of 0.1 M sodium phosphate, pH 7.4, containing 1 μM pepstatin A, 2 μM leupeptin, 1 mM 1,10-phenanthroline, and 1 mM phenylmethylsulfonyl fluoride. For measurement of complex stability, 50-μl aliquots were used. The final reaction mixture (200 μl) contained 1.33 μg/μl protein, 75 mM sodium phosphate, pH 7.4, and the salts indicated below. The mixtures were filtered and washed after incubation at room temperature for 2 h. Standard deviations are shown for 3 or more independent measurements.

	Experiment 1, ^{125}I-labeled toxin complex formation	Experiment 2, preformed complex stability after 2 h
Control	100	100
+ $CaCl_2$ (30 mM)	34	92 ± 14
+ $MgCl_2$ (30 mM)	5	99
+ $CoCl_2$ (10 mM)	3	118
+ NaCl (1 M)	18	99 ± 5
+ Cold toxin	0	96 ± 16

skeletal muscle and in chick brain synaptosomes.

These experiments suggest that ω-conotoxin does not compete with any of the Ca^{2+} channel antagonists tested and, therefore, defines a new site which affects voltage-activated Ca^{2+} channel activity.

Tests for Reversibility of Binding—We have tested for the reversibility of binding by ω-conotoxin. No significant dissociation of ω-conotoxin from receptor complex was found after 2 h, even in the presence of $CoCl_2$, $CaCl_2$, or high NaCl, even though these salts inhibited formation of the complex (see Table III). When an excess of unlabeled ω-conotoxin was added after the complex was formed, little decrease in ^{125}I-labeled ω-CgTx binding was detected. These results suggest that once the toxin binds its target, dissociation of the toxin is very slow under the conditions examined. This functional irreversibility makes ω-conotoxin potentially more useful for many applications than Ca^{2+} channel antagonists which dissociate rapidly from their targets.

DISCUSSION

In this report, we have demonstrated specific high affinity binding of ω-conotoxin to synaptosomal membrane fractions from chick brain. The level of saturation suggests that in chick brain synaptosomes, the concentration of channels is of the order of 1.5 pmol/mg protein (Fig. 3). In the microsomal fraction, it is ≈0.5 pmol/mg protein.

The results suggest that the specificity of ω-conotoxin is nonoverlapping with verapamil, diltiazem, and the dihydropyridines. Dihydropyridines and ω-conotoxins do not compete for sites in a frog muscle preparation and in a chick brain synaptosomal preparation. Since both dihydropyridines and ω-conotoxins are believed to block voltage-activated calcium channels, these results have at least two classes of explanations. It is possible that these calcium channel antagonists bind to different sites on the same channel, and by binding at different sites, are able to effect a channel block. An alternative explanation is that ω-conotoxin and the other Ca^{2+} channel antagonists bind different types of voltage-activated calcium channels. A combination of these hypotheses is of course possible. We are presently carrying out a number of experiments to distinguish between these alternatives.

The significance of nitrendipine-binding sites has recently been a subject of considerable discussion in the literature (see Refs. 21–23). Although it is clear that dihydropyridines can demonstrably block certain types of voltage-activated calcium channels, there is no concensus as to whether calcium channels in neuronal tissue are blocked by these agents. In frog, ω-conotoxin GVIA has been found to block every type of synapse so far tested including the neuromuscular junction, a ganglionic synapse, and a spinal cord synapse (11).[2] Thus, differences in specificity between ω-conotoxin and the dihydropyridines defined by electrophysiological studies are corroborated by the binding evidence presented here.

The data in this report demonstrate that the ω-conotoxin GVIA may define a new site which affects Ca^{2+} channel activity and will be a unique probe to explore the structure, function, and diversity of voltage-activated calcium channels. Because these peptides block synaptic transmission, ω-conotoxins should be particularly useful for analyzing neuronal voltage-activated Ca^{2+} channels.

Acknowledgments—We are grateful to Drs. D. Yoshikami and W. R. Gray for stimulating discussion. David Johnson, Aryan Azimi, Garth LeCheminant, and David Griffin made significant contributions to the experiments described in this report.

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[2] D. Yoshikami, unpublished results.