0026-895X/88/02067-04$02.00/0

MOLECULAR PHARMACOLOGY, 34:87-90

ACCELERATED COMMUNICATION

Characterization of the ω-Conotoxin-Binding Molecule in Rat Brain Synaptosomes and Cultured Neurons

BEATRICE MARQUEZE, NICOLE MARTIN-MOUTOT, CHRISTIAN LEVÊQUE, and FRANÇOIS COURAUD

Laboratoire de Biochimie, CNRS UA 1179 and INSERM U 172, Faculté de Médecine Nord, Bd Pierre Dramard, F-13326 Marseille Cedex 15, France

Received February 1, 1988; Accepted May 9, 1988

SUMMARY

ω-Conotoxin GVIA is a peptide purified from the venom of the marine snail, *Conus geographus*, that specifically blocks voltage-sensitive calcium channels in neurons. A mono-[^{125}I]iodo-ω-conotoxin was prepared and specific binding to both rat brain synaptosomal membranes and cultured neurons was detected. The interaction was irreversible and the association kinetic constant k was measured at 5–7 × 10^6 $M^{-1}s^{-1}$ in synaptosomes and at 2–4 × 10^6 $M^{-1}s^{-1}$ on intact neurons. The binding site capacities were 650 and 60 fmol/mg of protein, respectively. No competition was detected with other calcium channel blockers or with toxins acting on Na^+ or K^+ channels but the binding was lowered by the divalent cations Co^{2+} and Ca^{2+}. Photoaffinity experiments specifically labeled a single component with an apparent M_r of 222,000 ± 7,000 in brain synaptosomes and 245,000–300,000 in cultured embryonic neurons.

Voltage-sensitive calcium channels contribute to diverse neuronal functions such as spike initiation, rhythmic firing, and transmitter release. Three different channel subtypes have been shown to coexist in sensory neurons of the chick dorsal root ganglion and have been called L, N, and T (1); only the L channel is sensitive to dihydropyridine agonists or antagonists. Recently, a series of peptide neurotoxins has been purified from the venom of the marine snail, *Conus geographus* (2) and among these, ω-CgTx specifically blocks both L and N subtypes of calcium channel in chick dorsal root ganglion neurons (3). This toxin has also been shown to inhibit $^{45}Ca^{2+}$ uptake by synaptosomes (4, 5) and by cultured neurons (4), indicating that the toxin blocks voltage-sensitive calcium channels in both nerve terminals and cell bodies. Irreversible and specific binding to chick brain synaptosomes (6) (without any competition with dihydropyridine or phenylalkylamine drugs) has been detected. Very recently the molecular weight of the ω-CgTx receptor has been determined in rat brain membranes by photoaffinity labeling to be 310,000, 230,000, and 34,000 (7) and in chick brain synaptosomes by chemical cross-linking to be 135,000 (8).

In this paper, we demonstrate binding of the ω-CgTx to both synaptosomal preparations and cultured neurons from rat brain and describe use of a photoreactive toxin derivative to identify the polypeptide chains associated with the receptor.

Experimental Procedures

Materials. Synthetic ω-CgTx was obtained from the Peptide Institute (Osaka, Japan); apamin was purified from bee venom according to the method of Banks *et al.* (9). ANPAA succinimidyl ester was a gift from Dr. K. Angelides (University of Florida, Gainesville, FL) and batrachotoxin from Dr. J. W. Daly (National Institutes of Health, Bethesda, MD). Veratridine was from EGA Chemie (Steinhein, Germany), verapamil and D600 from Knoll (Ludwigshafen, Germany), tetrodotoxin from Boehringer (Mannheim, Germany), and nifedipine from Bayer AG (Wuppertal, Germany). Lactoperoxidase and protease inhibitors were from Sigma Chemical Co. (St. Louis, MO). Standard proteins were from Bio-Rad Laboratories (Richmond, CA) or Pharmacia (Uppsala, Sweden). All other products were reagent grade.

Iodination of toxins. ω-CgTx was iodinated by the lactoperoxidase method as follows: 1.5 nmol of toxin was reacted for 2 min with 0.2 mCi of carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) in a 50 mM phosphate buffer at pH 7.2. The molar ratio of iodide to peptide was low, 1:15, in order to minimize the appearance of diiododerivatives. Monoiodo toxins were separated as described by Cruz and Olivera (6). A Beckman C-18 column (0.46 × 25 cm; 5 μm particles; not end-capped) was used at a flow rate of 1 ml/min and a linear gradient was applied with 0.1% trifluoroacetic acid as solvent A and 0.1% trifluoroacetic acid in 60% (v/v) acetonitrile as solvent B. As shown in Fig. 1a, native toxin was eluted after 14 min and three radioactive peaks (I, II, and III) were detected at 23, 24.5, and 26 min, in good agreement with results from Cruz and Olivera (6), considering that the slope of the gradient was lower in our experiments. These three peaks probably correspond to three different monoiodo-ω-CgTx derivatives inasmuch as the toxin contains three tyrosine residues. All peaks were active and specifically bound to rat brain synaptosomes; only peak III was used in the present study. When a higher iodide to toxin ratio (10:1) was chosen, other radioactive peaks appeared after

ABBREVIATIONS: ω-CgTx, ω-conotoxin GVIA; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ANPAA, 4-azido-2-nitrophenylamino acetyl; SDS, sodium dodecyl sulfate.

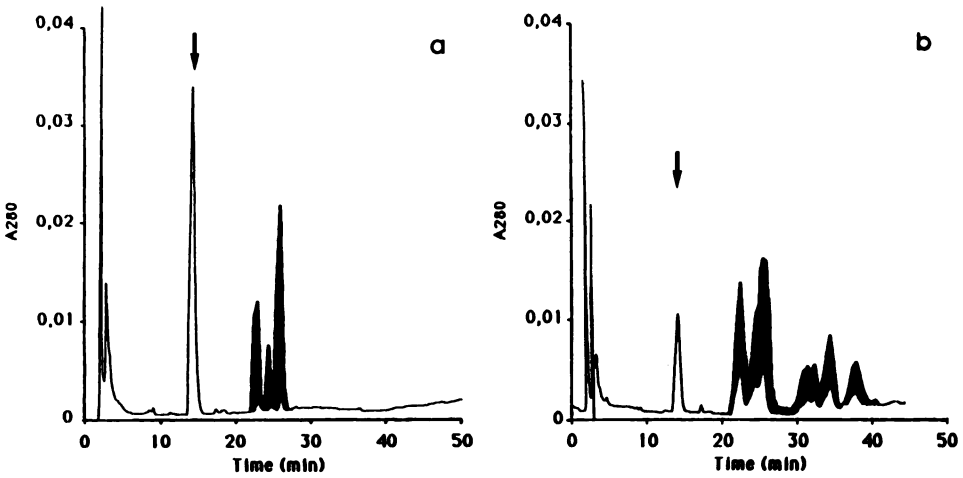

Fig. 1. Purification of ^{125}I-ω-CgTx derivatives by reverse phase high performance liquid chromatography. A total of 1.5 nmole of ω-CgTx was incubated as described in Experimental Procedures with either 0.1 nmol of carrier-free Na^{125}I (a) or 15 nmol of KI plus 0.05 nmol of Na^{125}I (b). Derivatives were separated using a linear gradient expressed as a percentage of solvent B achieved at times given in parenthesis (min): 30 (0), 50 (40). Native toxin was eluted at the position of the *arrow* and the *shaded peaks* corresponding to iodinated derivatives were superimposed on the absorbance profile.

30 min elution (Fig. 1b). These are probably di- or multi-iodinated toxins.

Biological material. Lysed synaptosomal membranes were prepared from rat brain as described (10). Some preparations were done in the presence of a mixture of protease inhibitors, 0.1 mM phenylemethylsulfonyl fluoride, 1 mM iodoacetamide, 1 mM phenanthroline, 1 μM pepstatin, and 1 mM EDTA. Protein was assayed by a modified Lowry method (11).

Embryonic rat brain neurons were cultured as previously described (12) except that half of the 5% serum-containing medium was replaced by a serum-free medium (13) every fourth day in culture. Cells were cultured in 24-mm multiwell plates (Flow Laboratories, Dublin, VA) for binding experiments and 60-mm dishes (Corning, Corning, NY) for photoaffinity labeling. The plating density was 3×10^5 cells/cm^2 with 2% of glial fibrillary acidic protein-positive cells (astrocytes) at day 14 *in vitro*. The amount of protein per dish was measured after washing the cell culture twice with 2 ml of bovine serum albumin-free medium.

Binding experiments. Binding and wash buffers consisted of 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 10 mM glucose, 20 mM HEPES, and 0.5% bovine serum albumin adjusted to pH 7.2 with Tris base. Binding studies with synaptic membranes were carried out in 0.3 ml of buffer. The bound ligand was separated by rapid filtration over polyethyleneimine-pretreated (14) glass fiber filters (GFC-Whatman). After washing the filters four times with 2 ml of ice cold buffer, bound ligand was measured by gamma counting with 60% efficiency.

In competition experiments, membranes were preincubated 30 min at 37° with divalent cations or saturating concentrations of drugs. Then, ^{125}I-ω-CgTx was added, and toxin binding was measured after a further 40 min incubation at 37°.

Cultures were incubated in 0.4 ml of buffer. Binding was terminated by aspiration of the medium and the cell layer was washed three times, each time for 15 sec, with 1 ml of ice cold buffer. Cells were then collected in 1 ml of 0.1 M NaOH for gamma counting. Experimental points are the mean of duplicate determinations.

Photoaffinity labeling procedure. ANPAA succinimidyl ester was incubated for 2 hr at room temperature in the dark with mono-[^{125}I]iodo-ω-CgTx in a 0.2 M Tris·HCl buffer, pH 10, containing bovine serum albumin. The ratio of reagent to reactive amino groups (of the iodotoxin plus serum albumin) was 0.8. Synaptosomal membranes or cultured cells were incubated for 60 min at 37° in the dark with 0.1 mM ^{125}I-ANPAA-ω-CgTx in the presence or absence of 0.1 μM native toxin and irradiated on ice for 5 min at about 5 cm from a ventilated 125 watt Philips mercury vapor lamp (λ_{max} = 356 nm).

Synaptosomal membrane samples were washed twice with 2 ml of ice-cold wash buffer and twice with 2 ml of 25 mM Tris·HCl, 10 mM KCl buffer, pH 7.5. Irradiated cells were washed three times with 5 ml of ice-cold wash buffer, the last wash in the absence of bovine serum albumin, then harvested and homogenized in 5 ml of 0.32 M sucrose, 1 mM Tris·HCl buffer, pH 7.4. Nuclei and unbroken cells were eliminated by centrifugation for 10 min at 600 × *g*, and mitochondria for 30 min at 10,000 × *g*. Membranes were then pelleted at 105,000 × *g* for 30 min.

Synaptosomal and cell membranes were solubilized, denatured, and analyzed by SDS polyacrylamide gel electrophoresis and autoradiography as previously described (15).

Results and Discussion

^{125}I-ω-CgTx binding to synaptosomal membranes. Results of typical experiments demonstrating saturable binding of ω-CgTx to synaptosomal membranes are shown in Fig. 2. The binding was irreversible as shown in Fig. 2b; no release of bound toxin was observed after addition of an excess of native toxin and incubation for 24 hr at 4°. Only 20% of the binding was lost after 5 hr at 37°. A control experiment showed that this small decrease was due to a partial denaturation of the membrane receptor.

We have thus used an irreversible model to describe the interaction:

$$R + L \xrightarrow{k} RL$$

where R, L, and RL are the concentrations of unoccupied receptor, free ligand, and bound ligand, respectively, and k the association kinetic constant.

In all binding experiments, we have chosen concentrations of membranes and ^{125}I-ω-CgTx so that the ratio $RL/(RL + L)$ never exceeded 0.15. Under these conditions, one can calculate (16):

$$RL = (R_T)(1 - e^{-k(L)t}) \quad (1)$$

where R_T is the total concentration of binding sites. Eq. 1 can be linearized:

$$\log ((R_T - RL)/R_T) = -k(L)t \quad (2)$$

If time is kept constant, these expressions provide a concentration dependency relation (Fig. 2a) whereas if concentration is maintained constant, a kinetic equation is obtained (Fig. 2c). After linearization using Eq. 2, k was calculated to be 5.5×10^6 (Fig. 2a) and 6.9×10^6 M^{-1}s^{-1} (Fig. 2c). The binding site capacity of ^{125}I-ω-CgTx was 650 fmol/mg of protein on rat brain synaptosomal membranes, in good agreement with previously published data on chick synaptosomes (1.5 pmol/mg of protein) (6). No competition was obtained with nifedipine, D 600, or verapamil whereas the divalent cations Ca^{2+} and Co^{2+} strongly

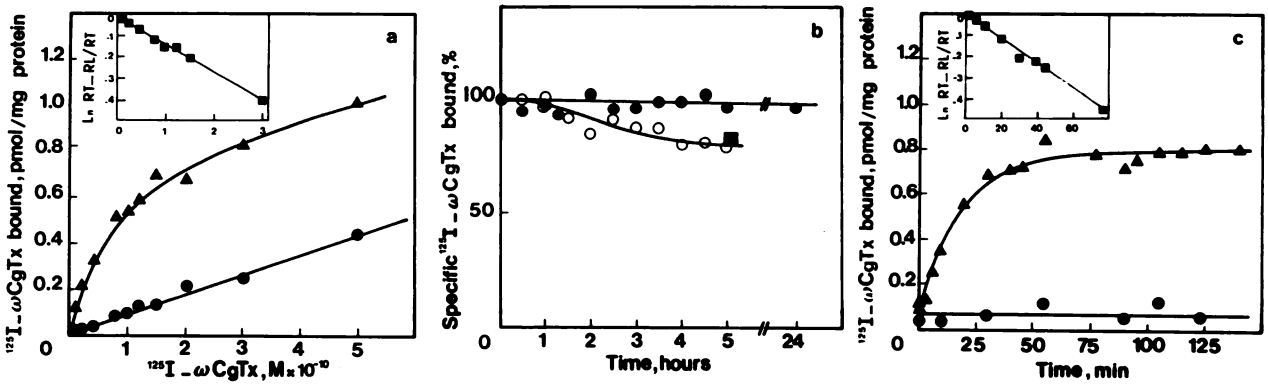

Fig. 2. Binding characteristics of ^{125}I-ω-CgTx to rat brain synaptosomal membranes. Membranes (5 μg of protein) were incubated at 37° with ^{125}I-ω-CgTx in a final volume of 300 μl of incubation buffer. a, Saturation experiment. Membranes were incubated for 40 min with increasing concentrations of ^{125}I-ω-CgTx in the presence (●) or absence (▲) of 0.1 μM native toxin. *Inset*, the specific binding was linearized as indicated in the text. b, Dissociation kinetics. A total of 26 pM ^{125}I-ω-CgTx was incubated at 37° with membranes. After 45 min, 0.1 μM native toxin was added and the remaining specific membrane-bound radioactivity was measured at the indicated time after incubation at 4° (●) or at 37° (○). In a control experiment, membranes were preincubated at 37° for 255 min without toxin and then 45 min with 26 pM ^{125}I-ω-CgTx (■). c, Association kinetics. Membranes were incubated for the indicated time with 0.1 nM ^{125}I-ω-CgTx in the presence (●) or absence (▲) of 0.1 μM native toxin. *Inset*, the specific binding was linearized as indicated in the text.

depressed the specific binding of ^{125}I-ω-CgTx. Co^{2+} was more active than Ca^{2+}, inasmuch as half maximum effect was obtained with 0.7 mM Co^{2+} and with 1.8 mM Ca^{2+}. Batrachotoxin and veratridine, which are well known Na^+ channel activators but which have also been shown to specifically block voltage-sensitive calcium channels in neuronal cells (17), did not modify the ^{125}I-ω-CgTx binding to brain synaptosomes. α and β scorpion toxins and apamin were also inactive.

^{125}I-ω-CgTx binding to cultured embryonic neurons. As with synaptosomal membranes, the interaction of ^{125}I-ω-CgTx with cultured embryonic neurons was completely irreversible (data not shown). Fig. 3a shows ^{125}I-toxin binding as a function of concentration at a given time and Fig. 3b shows binding as a function of time at a given concentration. The association kinetic constant k was calculated to be 3.6×10^6 (Fig. 3a) and 2.6×10^6 $M^{-1}s^{-1}$ (Fig. 3b). The binding capacity was 60 fmol/mg of protein in cells cultured for 13 days (Fig. 3a), which corresponds approximately to 10,000 sites per cell, assuming that all cells bear binding sites. K^+ depolarization of cell membranes did not modify ^{125}I-ω-CgTx binding.

Photoaffinity labeling of ω-CgTx-binding molecule. Mono[^{125}I]iodo-ω-CgTx was reacted as described in Experimental Procedures with ANPAA succinimidyl ester and immediately incubated in the dark with synaptosomal membranes. After irradiation, washing, denaturation, and reduction of disulfide bridges, membranes were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography (Fig. 4). Among the major labeled bands detected in Fig. 4, *lane 1*, only one at 218 kDa was not present when the experiment was done in the presence of an excess of native toxin (Fig. 4, *lane 2*). The M_r of this specifically labeled component was analyzed by using the method of Ferguson and Wallace (18), revealing a normal electrophoretic behavior of this component after reduction of disulfide bridges. Omission of disulfide bridge reduction did not change the migration (data not shown). The mean value of the apparent M_r was 225,000 ± 9,000 (five experiments) after reduction and 222,000 ± 4,000 (eight experiments) without reduction. A single band was also obtained when experiments were done with intact synaptosomes (P2 fraction) prepared and incubated in the presence of protease inhibitors at 1° instead of 37°, i.e., conditions in which proteolysis was minimized. In some experiments, shown in Fig. 4, a low molecular weight component of 25,000–35,000 also appeared to be specifically labeled, but this labeling was not systematically observed and was absent when membranes were prepared in the presence of protease inhibitors.

Similar experiments were carried out on cultured embryonic neurons and the results are illustrated in Fig. 4. A single band

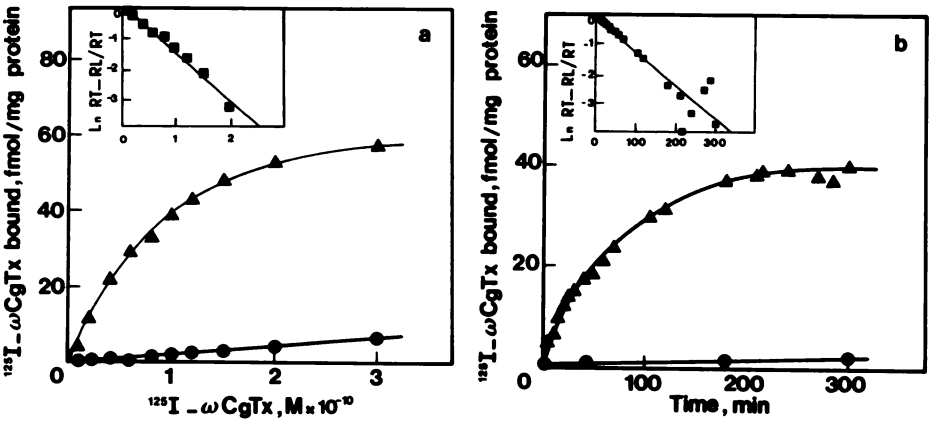

Fig. 3. Binding characteristics of ^{125}I-ω-CgTx to cultured brain neurons. Cells were incubated at 37° with ^{125}I-toxin in 400 μl of binding buffer. a, Saturation experiment. After 13 days in culture, cells were incubated for 75 min with increasing concentrations of ^{125}I-ω-CgTx in the presence (●) or in the absence (▲) of 0.1 μM native toxin. *Inset*, the specific binding was linearized as indicated in the text. b, Association kinetics. After 14 days in culture, cells were incubated for the indicated time with 74 pM ^{125}I-ω-CgTx in the presence (●) or absence (▲) of 0.1 μM native toxin. *Inset*, the specific binding was linearized as indicated in the text.

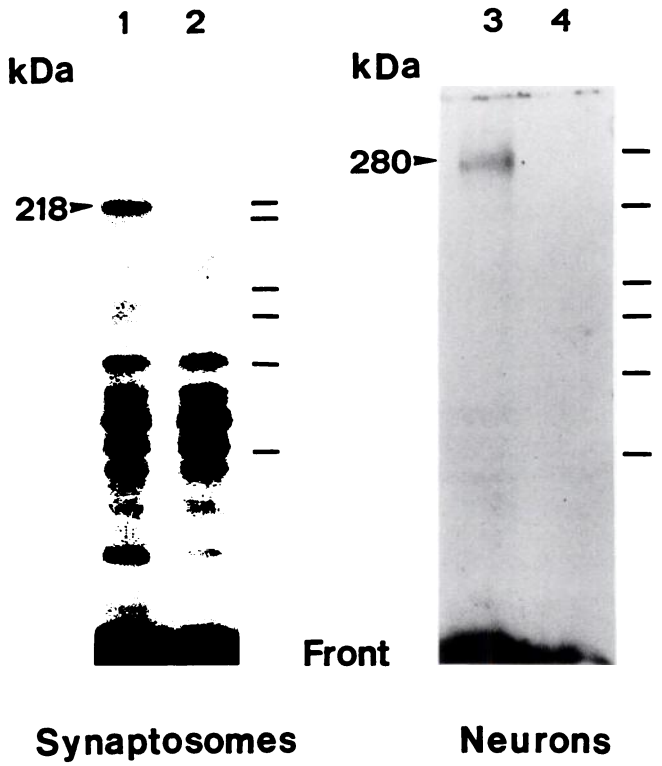

Fig. 4. Photoaffinity labeling of ω-CgTx binding molecule. Synaptic membranes or neurons in culture were photolabeled and then analyzed by SDS polyacrylamide gel electrophoresis in 5–15% acrylamide gel gradients as described under Experimental Procedures. Nonspecific labeling was determined in the presence (*lanes 2–4*) of 0.1 μM ω-CgTx. The migration of standard proteins is indicated by *horizontal bars*: ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase B (92,500), β-galactosidase (116,250), myosin (200,000), and ferritin (220,000) were used for synaptosomes, whereas ^{125}I-thyroglobulin (330,000) from cultured porcine thyroid cells were used instead of ferritin in experiments on neurons.

was specifically labeled (Fig. 4, *lane 3*). Its molecular weight was difficult to determine with precision and fluctuated between 245,000 and 300,000, although it was always higher than the specific band detected on synaptosomes. No clear change in the migration was observed when disulfide reduction was omitted.

Our results obtained with synaptosomal membranes are in part similar to those published by Abe and Saisu (7), who have described three different bands at 310, 230, and 34 kDa in the same preparation. Our band at 222 kDa might correspond to their 230-kDa polypeptide and the component at 25–35 kDa that we observed in the absence of protease inhibitors is reasonably close to 34 kDa. On the contrary our results are clearly different from the data of Cruz *et al.* (8) in which a 135-kDa component was labeled by ^{125}I-ω-CgTx after cross-linking with bifunctional reagents in chick brain synaptosomes. This discrepancy might be due to species differences or to differences in the methodology used.

The molecular weight of our labeled component is very similar to the molecular weight of the dihydropyridine receptor from rabbit skeletal muscle (212,000) as calculated from the amino acid sequence obtained from the cDNA (19) but is higher than the molecular weight (175,000–170,000) of the α_1 subunit of the same protein evaluated by purification and SDS polyacrylamide gel electrophoresis (20, 21).

Finally, the disparity between the molecular weight observed for the ω-CgTx receptor in adult synaptic terminal membranes and in cultured embryonic neurons may be due to developmental differences. The higher molecular weight in cultured neurons could result from a longer translation product or a greater degree of glycosylation. However, we cannot definitively exclude the possibility that the band obtained in synaptosomes is the product of proteolysis of a higher molecular weight component that is not cleaved in the membrane preparation from cultured neurons.

Acknowledgments

We are grateful to E. Loret for assistance in high performance liquid chromtography analysis, to J.-M. Bottini for help in the preparation of the manuscript, to P. Deprez for technical assistance, and to Dr. M. Seagar for helpful discussion.

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Send reprint requests to: Dr. F. Couraud, Laboratoire de Biochimie, Faculté de Médecine Nord, Bd P. Dramard, F-13326 Marseille Cedex 15, France.