Identification and Isolation of ω-conotoxin Binding Protein from Rabbit Brain.

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Abstract. N-type of calcium channels belongs to a family of voltage-dependent calcium channels and occurs predominantly in neuronal tissue. One of the basic characteristics of this channel type is binding of ω-conotoxin GVIA – a potent blocker of the calcium current through this type of channel.

We have found that two proteins with Mw 170–175 kD and 45–60 kD possess ω-conotoxin GVIA binding sites, while the only protein with Mw 170–175 kD was able to transport calcium after reconstitution into the phospholipid vesicles. The same results were obtained from human embryonic brain. Our results suggest that protein with Mw 170–175 kD corresponds to the α_1-subunit of N-type of voltage-dependent calcium channel.

Key words: N-type calcium channel — ω-conotoxin binding — Dihydropyridine binding — Calcium transport

Introduction

Voltage-dependent calcium channels (Ca-channels) play a crucial role in a variety of cellular pathways. Majority of these channels belongs to L-type subgroup, which is sensitive to calcium antagonists (phenylalkylamines, benzodiazepines and dihydropyridines) (for review see Krizanová et al. 1993). Besides these, another type of Ca-channel has been found predominantly in those areas of brain, which are rich in synaptic connections (Sher and Clementi 1991). ω-Conotoxin GVIA (ω-CgTx) is a potent blocker of these channels.

Conotoxins are venoms from marine snails *Conus*, and they are targeting several different receptors – voltage-sensitive Ca^{2+} channel, acetylcholine receptor,

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voltage-sensitive Na^+ channel, vasopressin receptor and NMDA receptor (Olivera et al. 1991). ω-CgTx from *Conus geographus* has been used for investigation neuronal N-type Ca-channels. First experiments were focused to estimate molecular weight of ω-CgTx-binding protein. Several different molecular weights were obtained, especially those of 310 kD, 230 kD and 34 kD (Abe and Saisu 1987). However, cloning and sequencing revealed protein of 232 amino acids (Dubel et al. 1992; Horne et al. 1993). Therefore, based on all these data, we attempt to utilize a biochemical approach for identification of N-type Ca-channel protein.

Materials and Methods

Membrane preparations: Brain membrane fractions were prepared as described in Takemura et al. (1987) with slight modifications. Male rabbits (weighing approximately 2.5 kg) were decapitated and the whole brains were removed and immediately homogenized (10 ml/g tissue) in ice cold homogenization buffer (0.32 mol/l sucrose, 5 mmol/l Na-phosphate buffer (pH 7.4), phenylmethylsulphonylfluoride, iodacetamide, phenantroline in the final concentration 100 nmol/l).

The homogenate was centrifuged at 1200 × g for 10 minutes and supernatant was centrifuged at 1900 × g for 60 minutes. This supernatant was used as postsynaptosomal fraction and pellet (synaptosomal fraction) was resuspended in the small volume of homogenization buffer and applied to discontinuous sucrose density gradient of concentrations 0.4, 0.6, 0.8 1.0 and 1.2 mol/l. Gradient was centrifuged at 120,000 × g at 4 °C for 100 min. Fractions from each gradient interface were collected, diluted 10-fold with homogenization buffer and centrifuged at 120,000 × g for 60 minutes at 4 °C. Pellets were resuspended in 50 mmol/l HEPES-NaOH pH 7.4, 1 mmol/l $CaCl_2$, so that the protein concentration reached 5 mg/ml. The same procedure was used for the same isolation from 12 weeks old male embryonic fetus (with hemophilia defect).

Binding assays: [^{125}I] ω-CgTx was purchased from Amersham (U.K.). The incubation buffer contained 20 mmol/l sucrose in HEPES/$CaCl_2$ buffer. The binding assay was carried out in final volume of 500 μl, which contained 200 μg of protein and 0.5 nmol/l (78 TBq/mmol/l) of labeled toxin. For the estimation of specific binding, unlabeled ω-conotoxin in the final concentration of 2 μmol/l was added. The incubation was carried out at room temperature for 30 min and free toxin was separated through Sephadex G-50 column (5 × 150 mm).

[^{3}H] PN 200–110 (obtained from Amersham (U.K.)) binding assay was determined as described previously (Križanová et al. 1990).

Protein extraction from gels: Proteins were separated on 6% polyacrylamide gels according to Laemmli (1970). Each sample approximately 20 μg/lane has been applied in two identical lanes. Afterwards, gel was divided into two analogous parts. One part was stained by silver to show protein profile. Then, other part of the gel (which remained unstained) was cut into small pieces (0.5 × 0.15 × 0.2 cm). Each piece was extracted separately on Extraphor equipment (LKB) in running buffer (50 mmol/l Tris-HCl, pH 8.9, 50 mmol/l glycine, 0.1% SDS) for 1 hour at 100V. Proteins were concentrated into 1 mol/l sodium chloride. Afterwards, fractions were desalted by centrifugation through Whatman ultrafilter and diluted in 50 mmol/l HEPES-NaOH, pH 7.4.

Reconstitution and calcium transport: Both these procedures are described in detail in

Križanová et al. (1990). Briefly, reconstitution was done with an azolectin-protein ratio of 40 : 1. The mixture was solubilized in 1% CHAPS. After solubilization, CHAPS was removed on a Sephadex G-50 column. The vesicles were eluted with 20 mmol/l Tris-HCl, pH 7.4. Proteoliposomes were sonicated 2 × 30 s to obtain unilamellar particles.

Table 1. Comparison of [^{3}H] PN 200–110 and [^{125}I]ω-CgTx GVIA binding to the rabbit brain fractions

Fraction	PN 200–110 K_D (nmol/l)	PN 200–110 B_{max} (fmol/mg)	CgTx GVIA K_D (nmol/l)	CgTx GVIA B_{max} (fmol/mg)
Crude membranes	0.26 ± 0.05	330.0 ± 70.0	2.40 ± 0.25	3.32 ± 0.30
Synaptosomal	0.14 ± 0.07	183.0 ± 25.0	1.11 ± 0.06	7.28 ± 0.31
Postsynaptosomal	0.31 ± 0.09	250.0 ± 25.0	2.73 ± 0.28	2.43 ± 0.45

Values are expressed as means ± S.D. $n = 6$
Concentration of [^{3}H] PN 200–110 *is 10 nmol/l and concentration of* [^{125}I] ω-CgTx *is 0.5* nmol/l.

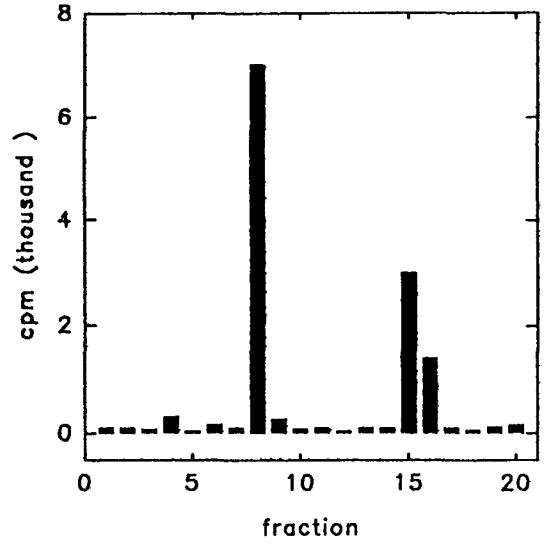

Figure 1. ω-Conotoxin GVIA specific binding to synaptosomal proteins from rabbit brain extracted from 6% polyacrylamide gel. Approximately 30 μg of each fraction (1–20) was extracted from polyacrylamide gel, as described in Materials and Methods. ^{125}I ω-CgTx was binding specifically to fraction 8 containing proteins with Mw 170–175 kD and to fractions 15 and 16 containing proteins with Mw 45–65 kD. Figure represents a typical profile from 3 independent experiments.

Proteoliposomes were incubated with $^{45}Ca^{2+}$ in 0.5 mol/l K^+ and/or Na^+ solution for defined periods of time (1, 5, 20 and 30 min). Then, free calcium was removed on CM-Sepharose and the radioactivity of proteoliposomes was subsequently measured on a Rackbeta counter (LKB) in Bray's scintillation cocktail (Spolana Neratovice).

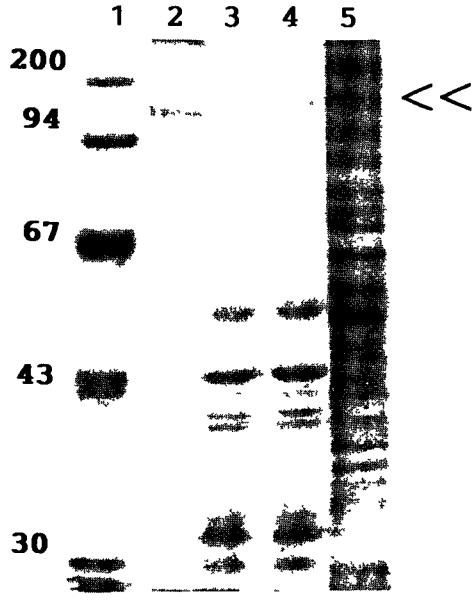

Figure 2. Electrophoretic profiles of ω-GVIA binding fractions isolated from rabbit brain after extraction from 6% denaturating gel. Fractions 8 are from human embryonic brain (lane 2) and from rabbit brain (lanes 3,4). Lane 1 represents molecular weight kit, which indicates molecular weight in kD. Double arrows indicate the position of protein with Mw 175 kD. For comparison, in the lane 5 is the crude synaptosomal fraction.

Results

ω-Conotoxin binding occurs preferentially in synaptosomal fraction (B_{max} = 7.28± 0.31 fmol/mg prot.), while binding of PN 200–110 was detectable in both, synaptosomal and postsynaptosomal fraction (Table 1). Proteins from synaptosomal fraction were separated on 6% polyacrylamide gel electrophoresis and twenty protein fractions ranging from 200 to 35 kD were extracted from gel and tested for ω-conotoxin binding. Specific binding has been obtained in three fractions: 8, 15 and 16 (Fig. 1). Protein profiles of these fractions are shown on 6% SDS polyacrylamide gel (Fig. 2). One peak corresponds to proteins in the range of Mw 170–175 kD (fraction 8) (Fig. 2) and other peak (fractions 15 and 16) corresponds to proteins in the range of Mw 45–60 kD. Based on these results we proposed that in brain synaptosomal fraction two proteins with a binding site for ω-CgTx exist. This is in agreement with Scatchard plot transformation, which reveals two binding sites for ω-CgTx of 6 pmol/l and 0.25 pmol/l (Fig. 3). Fraction 8 extracted from the gel was reconstituted into phospholipid vesicles and tested for calcium transport (Fig. 4).

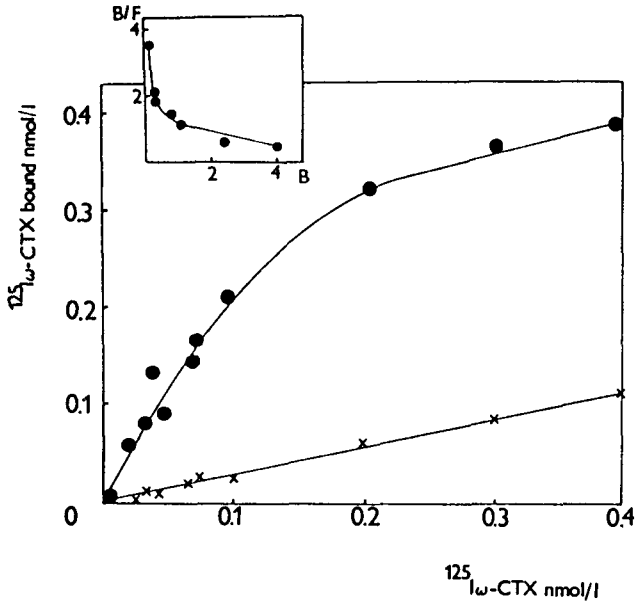

Figure 3. Equilibrium saturation isotherm of [^{125}I] ω-CgTx GVIA binding to the 0.1 mol/l sucrose gradient fraction isolated from rabbit brain synaptosomes. Filled circles represent total binding, crosses represent nonspecific binding. Inset: Scatchard plot analysis reveals two binding sites: high affinity binding site (with K_D 0.25 mol/l and B_{max} 3.3 pmol/mg prot.) and low affinity binding site (with K_D 6.0 pmol/l and B_{max} 0.08 pmol/mg prot).

This transport reached maximum in 5th minute and was completely blocked by 4 nmol/l ω-conotoxin, while PN 200–110 (10 nmol/l) blocked this transport only in very moderate way. Similar results were obtained in fraction from human brain (Fig. 5), where the calcium transport was significantly blocked by ω-CgTx.

Discussion

Introduction of ω-CgTx as a blocker of neuronal calcium channels of N-type has a great impact on their study. The first experiments with iodinated, purified native ω-CgTx demonstrated specific binding of the toxin to frog and chicken brain, but not to frog muscle (Cruz and Olivera 1986). Other authors have further characterized ω-CgTx binding sites from chick, rat and bovine brain preparations (Abe and Saisu 1987, Barhanin et al. 1988, Marqueze et al. 1988). Unfortunately, Mw of the proteins labeled differed in these papers from 34 kD to 310 kD. Possible causes of

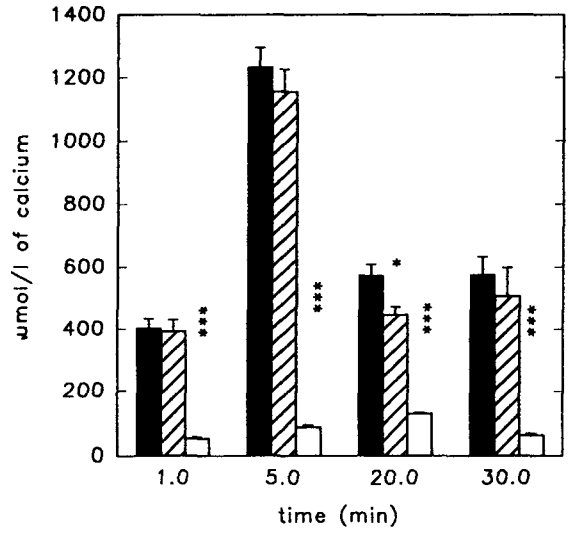

Figure 4. Effect of ω-CgTx and PN 200–110 on calcium transport into the azolectin vesicles with reconstituted protein of Mw 170–175 kD (fraction 8). Protein was incorporated into azolectin vesicles and proteoliposomes were subjected to calcium transport. Incorporated protein was able to transport calcium in time-dependent way (black bars). Dihydropyridine PN 200–110 in concentration 10 nmol/l blocked this transport only in a very moderate way (hatched bars), while ω-CgTx (0.5 nmol/l) was able to block this transport almost completely (empty bars) in all time range measured. Mean values S.E.M. are from 8 measurement.

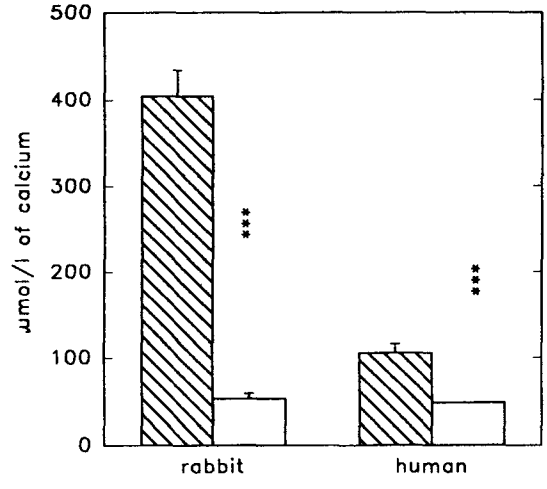

Figure 5. Comparison of ^{45}Ca transport into the azolectin vesicles with reconstituted fraction 8 from rabbit brain and from embryonic human brain (hatched bars). Calcium transport was measured after 1 minute and in both cases could be blocked by ω-CgTx GVIA (empty bars).

these differences may be diversity of toxin receptors in different animals, different degree of posttranslational processing and cross-linking of multiple subunits to form larger protein aggregates. Some of the labeled proteins resembled those of the subunits of sodium channels (approximately 260 kD and 37–39 kD). However, this proposal needs to be proved. Therefore, in order to determine the protein(s) of N-type calcium channel, we correlated the binding assay with calcium transport. Our experiments have shown, that ω-conotoxin binds specifically to two proteins with Mw 45–60 kD and 170–175 kD. These molecular weights correlate with the ones published by Horne et al. (1991). Here, three proteins with molecular masses of approximately 170, 60 and 42 kD, which bind ω-CgTx were reported (Horne et al. 1991). Both proteins labeled were cut out from the gel, reconstituted into azolectin vesicles and tested for calcium transport. Protein of Mw 45–60 kD did not transport calcium, while protein of Mw 170–175 kD was transporting calcium after induction with KCl in time dependent manner (Fig. 4). To confirm that calcium transport is due to the N-type Ca-channel protein, we blocked this transport with the dihydropyridine antagonist PN 200-110 and/or ω-CgTx. As it was already shown, L-type of calcium channel in brain does not bind ω-CgTx (Takemura et al. 1987) and *vice versa*. Calcium transport of 170–175 kD protein was almost completely blocked by ω-CgTx, while it was almost insensitive to PN 200–110. This experiment strongly suggests that protein with Mw 170–175 kD is the N-type of calcium channel, or at least its important part. Obviously, smaller protein cannot belong to Ca-channels, because of the lack of Ca-transport ability. It can be either a proteolytic fragment of larger protein, or, which is more probable, other protein with unknown function. The function of this protein of Mw 45–60 kD remains to be tested.

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