Characterization of the ω-Conotoxin Target. Evidence for Tissue-Specific Heterogeneity in Calcium Channel Types†

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ABSTRACT: ω-Conotoxin GVIA (ω-CgTx-VIA) is a 27 amino acid peptide from the venom of the fish-hunting snail, *Conus geographus*, that blocks voltage-activated Ca channels. The characterization of a biologically active, homogeneous ^{125}I-labeled monoiodinated Tyr^{22} derivative of ω-conotoxin GVIA and its use in binding and cross-linking studies are described. The ^{125}I-labeled toxin is specifically cross-linked to a receptor protein with an apparent M_r of 135 000. The stoichiometry between ω-conotoxin and nitrendipine binding sites in different chick tissues was determined. Skeletal muscle has a high concentration of [^{3}H]nitrendipine binding sites (>1000 fmol/mg) but no detectable ω-conotoxin sites (<7 fmol/mg). Brain microsomes have both binding sites, but ω-conotoxin targets are in excess. These results, combined with recent electrophysiological studies (E. W. McCleskey, A. P. Fox, D. Feldman, L. J. Cruz, B. M. Olivera, R. W. Tsien, and D. Yoshikami, unpublished results), define four types of Ca channels in chick tissues, N, T, L_n (ω sensitive), and L_m (ω insensitive), and are consistent with the hypothesis that the α-subunits of certain neuronal Ca^{2+} channels (L_n, N) are the molecular targets of ω-conotoxin GVIA.

Voltage-activated calcium channels are a key component of the membranes of all excitable cells since they transduce electrical signals into biochemical events (Hille, 1984; Hagiwara & Byerly, 1981; Tsien, 1983; Janis & Triggle, 1983). In the presynaptic termini of neurons, neurotransmitter release is dependent on these channels. Thus, modulation of synaptic activity can be effected by regulating Ca channel activity.

Our laboratories have described a novel class of peptide toxins from fish-hunting snails (Olivera et al., 1984, 1985) that inhibit voltage-activated calcium channels (Kerr & Yoshikami, 1984; Feldman and Yoshikami, unpublished results; McCleskey et al., 1986). These Ca^{2+} channel antagonists have been purified from two species of piscivorous marine gastropods, *Conus geographus* and *Conus magus*. To date, seven homologous ω-conotoxins have been characterized. In addition to the ω-conotoxins, the venoms of these predatory mollusks also contain α-conotoxins which inhibit the acetylcholine receptor and μ-conotoxins which block voltage-activated sodium channels of muscle [see Olivera et al. (1985) and Cruz et al. (1985a) for reviews].

Most of the biochemical work that has been done with voltage-activated calcium channels to date has employed pharmacological agents generally referred to as "organic blockers" that fall into one of three chemical classes [for reviews, see Miller (1986) and Triggle (1982)]. These are (1) the 1,4-dihydropyridines, such as nitrendipine and nifedipine; (2) phenylalkylamines, including verapamil and D-600, and (3) the benzothiazepines, including such antiarrhythmic drugs as diltiazem. Of these, the dihydropyridines have been employed most frequently; recently these drugs were used to purify voltage-activated calcium channels from T-tubules (Curtis & Catterall, 1984; Borsotto et al., 1984). A large literature exists characterizing dihydropyridine receptors.

The relationship between ω-conotoxin and dihydropyridine receptors needs to be defined. In a previous report (Cruz & Olivera, 1986), we showed that there is no competition between ω-conotoxins and dihydropyridines at either frog muscle or chick brain sites. Two extreme possibilities are the following: (1) the dihydropyridines and the ω-conotoxins inhibit the same voltage-activated calcium channels but compete for different sites on the same molecule, and (2) the dihydropyridines inhibit one class of voltage-activated calcium channels, while the ω-conotoxins inhibit an entirely separate class of voltage-activated calcium channels. In this report, we have carried out a number of experiments to define the ω-conotoxin target and to explore the relationship between the ω-conotoxin target and the dihydropyridine receptor.

Experimental Procedures

Materials. ω-Conotoxin GVIA (ω-CgTx-VIA)[1] was purified as described by Olivera et al. (1984). [*5-methyl*-^{3}H]-nitrendipine was obtained from New England Nuclear (Boston, MA), and Na ^{125}I was from Amersham Corp. (Arlington Heights, IL). Iodogen was purchased from Pierce Chemical Co. (Rockford, IL) and TPCK–trypsin from Cooper Biomedical.

Iodination of ω-CgTx-VIA. The toxin was iodinated according to the method of Fraker and Speck (1978) as previously described (Cruz & Olivera, 1986), with Na ^{125}I (40–150 μCi/nmol) as label. The more abundant iodinated species were found to have biological activity. The major iodinated derivative was used for practically all experiments described here except for earlier trials of the cross-linking experiment where

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[1] Abbreviations: ω-CgTx-VIA, ω-conotoxin GVIA; DRG, dorsal root ganglion; DSS, disuccinimidyl suberate; DTT, dithiothreitol; EGS, ethylene glycol bis(succinimidyl succinate); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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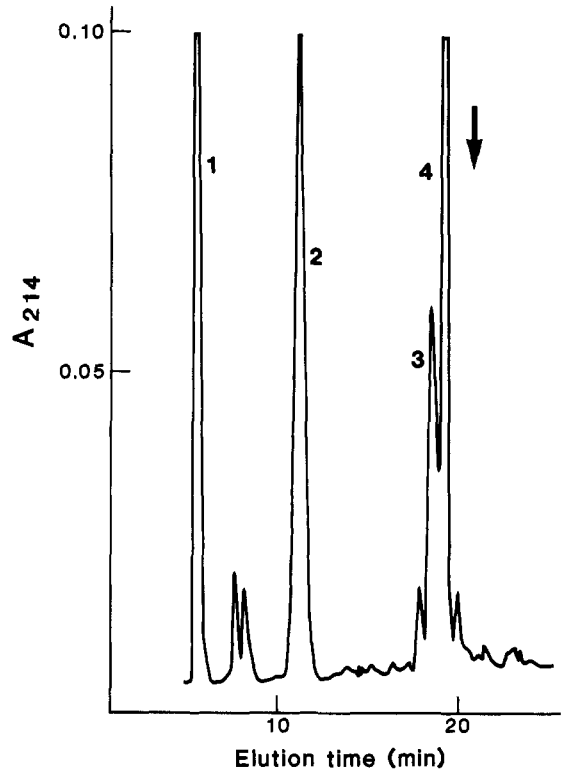

FIGURE 1: HPLC analysis of tryptic digest of ω-conotoxin GVIA. ω-Conotoxin GVIA and the major ^{125}I-labeled ω-conotoxin GVIA derivative (Cruz & Olivera, 1986) were digested with trypsin (see Experimental Procedures). The sequence of the four major tryptic fragments was determined (Figure 2). Three of the four major tryptic fragments were also found in the labeled toxin digest; however, peak 2 was missing. A new radiolabeled peak was found at the position of the arrow. Since Tyr^{22} is the only tyrosine residue in peak 2 (see Figure 2), monoiodination must have occurred at this position. This assignment was confirmed by direct sequencing of the radiolabeled peak. Since all tryptic peptides containing Tyr were accounted for by the major peptide peak, the minor components (which may be incomplete digestion products) were not further analyzed.

a mixture of the two most abundant iodinated ω-CgTx derivatives was used.

Structure Determination of the Major Iodinated Derivative. Trypsin/Pronase digestion of ^{125}I-ω-CgTx and high-voltage electrophoresis of the digest were done as previously described by Gray et al. (1984) to determine if the derivative contained mono- or diiodotyrosine. The toxin (2.5 nmol) was reduced and carboxymethylated as described previously (Cruz et al., 1985b) and then digested for 3 h at 37 °C with TPCK–trypsin (0.4 nmol) in 104 μL of 0.3 M NH_4OAc, pH 8.0. Fragments were separated by HPLC as shown in Figure 1. Sequence analysis of peptides was carried out in a Beckman Model 890D sequencer (Edman & Begg, 1967) using a 0.1 M Quadrol program. PTH-amino acids were analyzed by HPLC as previously described (Gray et al., 1981).

Membrane Preparations. Synaptosomes and crude microsomes were prepared from the brain of 19–20-day-old chick embryos and 3-day-old chicks as previously described (Cruz & Olivera, 1986; Catterall et al., 1979). Crude microsomes were obtained from the legs and heart of 20-day-old chick embryos and 3-day-old chicks according to the method of Glossmann et al. (1983) except that the following protease inhibitors were included in all solutions used: 1 μM pepstatin A, 2 μM leupeptin, 1 mM phenylmethanesulfonyl fluoride, and 1 mM 1,10-phenanthroline. All final pellets were resuspended in 0.32 M sucrose and 5 mM HEPES/Tris, pH 7.4, plus the inhibitors (SHTI buffer).

Protein Determination. The protein concentration of membrane preparation was determined according to the Lowry method as modified by Peterson (1977).

Binding Assays. The binding of ^{125}I-ω-CgTx to brain, leg, and heart preparations was determined as previously described (Cruz & Olivera, 1986). Measurement of [^{3}H]nitrendipine binding to the membrane preparations was done as described by Curtis and Catterall (1983).

Cross-Linking of ^{125}I-ω-CgTx-VIA to Its Target. ^{125}I-ω-CgTx-VIA (0.25 mL, 10 μCi/nmol, 0.31 nM in SHTI buffer) was incubated with 1.75 mL of chick brain synaptosomes (8.4 μg/μL in SHTI buffer) for 60 min at room temperature. A control reaction was also carried out except for a 30-min preincubation with an 84× excess of unlabeled ω-CgTx-VIA. Specific binding was checked by the filter assay (Cruz & Olivera, 1986); 60% of the toxin was bound. Reaction mixes were centrifuged at 30900*g* and the pellets washed twice with a 0.1 M sodium phosphate buffer, pH 7.4. After the second wash, both pellets were resuspended in 0.1 M sodium phosphate, pH 7.4, plus protease inhibitors. Toxin–receptor complexes were cross-linked with disuccinimidyl suberate (Pilch et al., 1980). Cross-linked samples and controls were analyzed on SDS–PAGE (discontinuous gel, Tris/glycine buffer system) (Laemmli et al., 1970); the running gel was either 5% or 8% acrylamide. In some experiments either 5% β-mercaptoethanol or 5% DTT was added to the sample before electrophoresis. The gel was stained with Coomassie blue G-250 and dried. Autoradiography was for 1 week with two Du Pont Cronex Lightning Plus screens on Kodak XAR-5 film.

RESULTS

Structure of Homogeneous ^{125}I-ω-Conotoxin GVIA. In order to characterize the ω-conotoxin receptor, a single molecular species of ^{125}I-labeled ω-conotoxin GVIA was prepared. ω-Conotoxin GVIA was iodinated, and the major radioactive biologically active ω-conotoxin derivative was characterized. The purified iodinated ω-conotoxin yielded monoiodotyrosine and no diiodotyrosine after complete proteolysis with Pronase and trypsin (data not shown). The ^{125}I-labeled ω-conotoxin and the unmodified ω-conotoxin were digested with trypsin, and the digestion products were analyzed by HPLC. A comparison of the tryptic fragments of the unmodified and iodinated peptides reveals that a single tryptic peak was shifted to a later eluting position (see Figure 1 and Experimental Procedures); the shifted peak was labeled with ^{125}I. The amino acid sequence of the original peak and that of the shifted ^{125}I-labeled peak were consistent with the assignments in Figure 2. All other tryptic fragments were identical in the unmodified and iodinated conotoxins. Thus, the structure of

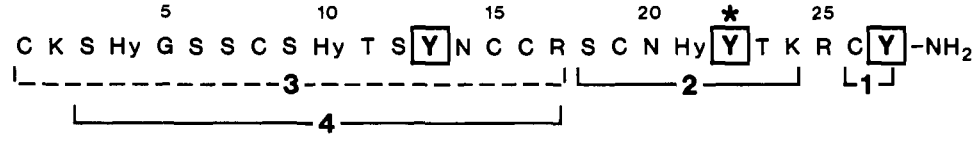

FIGURE 2: Sequence of ω-CgTx-VIA and mono-^{125}I-ω-CgTx-VIA, showing fragments (1–4) resulting from trypsin digestion. The numbers also correspond to the HPLC peaks shown in Figure 1. Hy is hydroxyproline. The asterisk over Tyr^{22} indicates that monoiodination has occurred in this position. The C terminus is amidated.

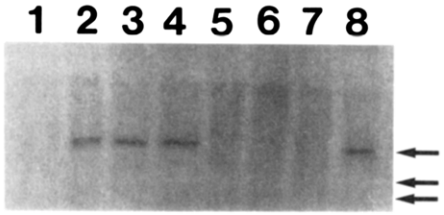

FIGURE 3: Cross-linking of ^{125}I-ω-CgTx-VIA to its target. The toxin was cross-linked to membrane preparations as described under Experimental Procedures. Lane 1, 40 μL of toxin–receptor complex, no DSS; lanes 2–4 and 8, 40 μL of toxin–receptor complex, increasing concentrations of DSS (0.4, 1.0, 2.0, and 4.0 μM); lane 5, 40 μL of reaction mixture preincubated with excess unlabeled toxin (control), no DSS; lanes 6 and 7, 40 μL of reaction mixture preincubated with excess unlabeled toxin (control), with 1.0 and 4.0 μM DSS, respectively. In this experiment, an 8% running gel was used for electrophoresis and no β-mercaptoethanol or DTT is present. Thus the apparent molecular weight of 210000 is anomalously high (see text). The arrows indicate markers: from the top, they are myosin (M_r 200000), β-galactosidase (M_r 116250), and phosphorylase B (M_r 92500).

the ^{125}I-labeled ω-conotoxin used for these experiments is the [^{125}I-Tyr^{22}]-ω-conotoxin GVIA. This derivative had 30–50% of the biological activity of unmodified toxin when tested on mice (Olivera et al., 1984). A detailed description of the structure and biological activity of iodinated derivatives of ω-conotoxins will be presented elsewhere (D. Johnson and W. Gray, unpublished results).

Cross-Linking of Iodinated ω-Conotoxin to the Receptor. We previously demonstrated that iodinated conotoxin would bind specifically to chick brain synaptosome preparations (Cruz & Olivera, 1986). The homogeneous [^{125}I-Tyr^{22}]-ω-conotoxin was used for cross-linking to chick brain synaptosomes. The toxin–receptor complex was formed (see Experimental Procedures), and cross-linking of the toxin to the receptor complex was carried out with either disuccinimidyl suberate (DSS) or ethylene glycol bis(succinimidyl succinate) (EGS).

After cross-linking, the toxin–receptor complex was solubilized with SDS and dithiothreitol. The solubilized toxin–receptor complex was analyzed by SDS–polyacrylamide gel electrophoresis, and cross-linked toxin was visualized by autoradiography as described under Experimental Procedures. The results of such a cross-linking experiment using DSS are shown in Figure 3. In the lanes where radioactive toxin–receptor complex is expected, a single sharp band is seen. If the receptor were pretreated with an excess of unlabeled toxin or if the cross-linking agent were omitted, this band is not found. Although other faint bands are visible in the gel, these do not show the specificity expected of the cross-linked receptor–toxin complex (i.e., they are also present when the receptor is pretreated with unlabeled toxin or in gel lanes where cross-linker was not added). Similar results were obtained with EGS as the cross-linking agent.

The apparent molecular weight of the labeled band varies, depending on the denaturation and electrophoresis conditions. Apparent molecular weights of 135000–210000 have been obtained. The minimum value (M_r 135000) is reproducibly obtained in the presence of a reducing agent. In the absence of a reducing agent, the apparent molecular weight is a function of the gel concentration, with a higher apparent molecular weight at higher gel concentrations. Similar anomalous behavior under nonreducing conditions has been observed for the large subunit of the purified T-tubule Ca channel (Curtis & Catterall, 1984).

Tissue Quantitation of ω-Conotoxin Binding Sites: Lack of Correspondence to Dihydropyridine Binding Sites. It was previously shown that the dihydropyridines and ω-conotoxin GVIA do not compete for binding, although both agents act as voltage-activated Ca channel antagonists. One interpretation of these results is that the two types of calcium blockers bind to different antagonist sites on the same voltage-activated calcium channels. A quantitative comparison of the number of binding sites in different tissues was carried out to test this hypothesis (Figure 4 and Table I).

Table I: Comparison of Nitrendipine and ω-CgTx Binding Sites in Crude Microsome Preparations from Brain, Heart, and Legs of 3-Day-Old Chicks

tissue	no. of binding sites (pmol/mg)[a] ω-CgTx	nitrendipine
brain	0.56	0.11
heart	<0.007	0.30
leg	<0.007	1.17

[a] The number of binding sites was estimated as described in the legend to Figure 4. The amounts of protein from heart preparations used in the nitrendipine and ω-CgTx binding assays were 151 and 499 μg, respectively. These data are typical of many measurements. In all experiments with heart and leg, the level of ω-CgTx is below the present sensitivity of the assay. However, the ratio of ω-CgTx to nitrendipine sites in brain is a function of how synaptosomes are prepared; we have obtained preparations that are either richer or more depleted in nitrendipine sites (relative to ω-CgTx sites).

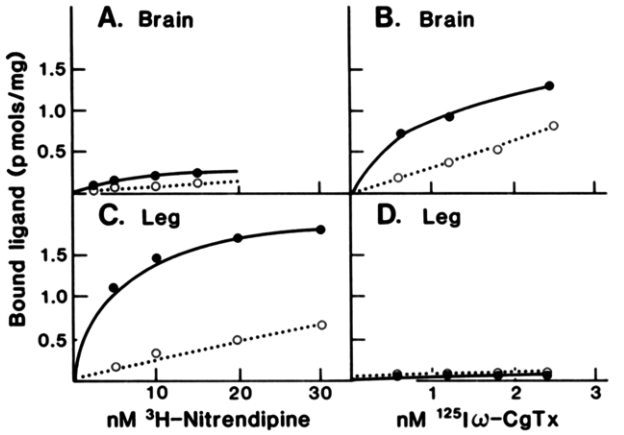

FIGURE 4: Binding of ω-CgTx and nitrendipine to crude membrane preparations from brain and legs of 3-day-old chicks. Binding assays were done as described under Experimental Procedures. The amounts of protein used for [^{3}H]nitrendipine binding reactions were 169 and 25.3 μg from brain and leg membrane preparations, respectively. For ^{125}I-ω-CgTx binding, the reaction mixtures contained 50.8 and 506 μg of protein from brain and leg preparations, respectively. The range of concentrations of labeled nitrendipine and ω-CgTx used should be sufficient to reach saturation, allowing an estimate of the number of binding sites. The filled circles and solid lines show total binding, while the open circles and dotted lines show nonspecific binding. In this experiment, the two tissues are directly compared for binding by the two Ca channel antagonist ligands; the results are typical of values obtained from many different preparations.

T-Tubule membranes are a rich source of nitrendipine binding sites (Figure 4C). However, these dihydropyridine binding sites are not ω-conotoxin binding sites (Figure 4D and Table I). Heart muscle shows similar specificity: a significant level of nitrendipine binding sites, but no ω-conotoxin sites, is detected (Table I). In chick brain both dihydropyridine and ω-conotoxin binding sites occur (Figure 4A,B). However, in this tissue, there is an excess of ω-conotoxin binding sites compared to nitrendipine targets. These data indicate that there is not a one-to-one correspondence between dihydropyridine and ω-conotoxin binding sites in any of the chick tissues tested. Indeed, within the sensitivity of the assay,

Table II: Proposed Characterization of Different Ca Channel Types Using ω-Conotoxin and Dihydropyridines

Ca^{2+} channel type	dihydropyridine sensitivity and high-affinity binding	persistent ω-conotoxin sensitivity and high-affinity binding
T	−	−
N	−	+
L_n	+	+
L_m	+	−

ω-conotoxin binding sites appear to be absent from both heart and skeletal muscle.

Discussion

The results described in this paper demonstrate that the ω-conotoxin target and the dihydropyridine target are distinct from each other. While skeletal muscle T-tubules are rich in dihydropyridine receptors (specific activity, >1000 fmol/mg of protein), the same tissue has essentially no ω-conotoxin receptors (<7 fmol/mg of protein). Although voltage-activated calcium channels can presumably bind dihydropyridines in this tissue, these same channels are refractory to both ω-conotoxin binding and ω-conotoxin inhibition. Qualitatively similar results were obtained with chick heart, although the concentration of nitrendipine binding sites is not as high as in skeletal muscle T-tubules. Thus, in both skeletal and cardiac muscle, we conclude that dihydropyridine binding complexes do *not* contain ω-conotoxin binding targets. Electrophysiological data are consistent with the binding results: ω-conotoxin does not block cardiac or skeletal muscle voltage-activated calcium channels in either system (McCleskey et al., 1987).

Recent electrophysiological studies on embryonic chick dorsal root ganglion cells in culture have shown that there are at least three types of calcium channels in this tissue (Nowycky et al., 1985), designated L-, N-, and T-type Ca^{2+} channels. The dihydropyridines affect the activity of L-type channels. In chick DRG cells, it has been demonstrated that ω-conotoxins can inhibit both L- and N-type voltage-activated calcium channels (McCleskey et al., 1987). Thus, the electrophysiological results suggest that ω-conotoxins have wider specificity than dihydropyridines, since there is a class of calcium channels in embryonic DRG cells that can be inhibited by the peptide neurotoxins but not by the dihydropyridines.

In contrast, the data in this paper, in conjunction with a number of physiological studies, suggest that ω-conotoxin tissue specificity is narrower than the nitrendipine tissue spectrum since the ω-conotoxins do not bind nonneuronal Ca^{2+} channels. The dihydropyridines bind skeletal muscle T-tubule calcium channels and can inhibit voltage-activated entry of calcium into skeletal muscle. By this criterion, therefore, the T-tubule channels are "L" channels. Thus, ω-conotoxins do not bind the putative L channels in T-tubules but do bind the L channels in chick embryo DRG cells, indicating that ω-conotoxins can differentiate between neuronal and nonneuronal L channels. This suggests that L-channel complexes are not identical in nerve and muscle. We propose to call the ω-sensitive and ω-insensitive channels L_n and L_m, respectively, to reflect their predominant tissue distribution. In addition, the ω-conotoxins appear to be the only agents so far discovered that block N channels. Thus, in brain there are more ω-conotoxin binding sites (N + L_n) than dihydropyridine binding sites (L_n alone). In heart and skeletal muscle, there are apparently no N channels with high affinity for ω-conotoxin. Present evidence indicates that neither the ω-conotoxins nor the dihydropyridines persistently inhibit the third type of voltage-activated calcium channel, the T channel (however, transient inhibition of T channels was observed in chick DRG cells in culture). A list of all the presently distinguishable calcium channel types is summarized in Table II.

It should be emphasized that all of the results above were obtained with a single iodinated derivative of one of the seven natural ω-conotoxins, [^{125}I-Tyr^{22}]-ω-conotoxin GVIA. Preliminary data suggest that the discriminatory properties of ω-conotoxins for different Ca channel subtypes may vary, depending on the specific ω-conotoxin derivative used.

The experiments in which ^{125}I-labeled ω-conotoxin was cross-linked to chick brain synaptosomes show that the ω-conotoxin target is a large protein subunit with a molecular weight of ca. 135 000. The purified voltage-activated calcium channel from T-tubules (which is not an ω-conotoxin binding site) has three subunits (Curtis & Catterall, 1984; Borsotto et al., 1984). The largest of these, the α-subunit, has an apparent molecular weight of 132 000–145 000. A number of cross-linking studies with dihydropyridines have been carried out. Most studies suggest that the dihydropyridine acceptor protein has a molecular weight of 30 000–50 000 (Ventner et al., 1983; Campbell et al., 1984; Sarmiento et al., 1985), although one study suggested that cross-linking occurred to a subunit of M_r 145 000 (Ferry et al., 1984). It is of interest that, analogous to the results above on the cross-linked ω-conotoxin receptor, the purified α-subunit of the T-tubule channel showed anomalous mobilities in gel electrophoresis experiments under nonreducing conditions. This was interpreted as evidence for an internal disulfide bond (Curtis & Catterall, 1984).

A reasonable interpretation of present biochemical and electrophysiological data on calcium channels is that different Ca channel complexes may have homologous but distinct subunits. The homologous subunits presumably differ in their electrophysiological characteristics; in addition, there are different tissue types. Our results are consistent with the suggestion that the α-subunits of certain neuronal voltage-activated Ca channel subtypes (L_n, N) are the molecular targets of ω-conotoxin GVIA.

Acknowledgments

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Registry No. Ca, 7440-70-2; ω-conotoxin, 92078-76-7; nitrendipine, 39562-70-4.

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Interaction of Cholera Toxin with Ganglioside G_{M1} Receptors in Supported Lipid Monolayers†

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ABSTRACT: Lipid monolayers formed at the air-water interface containing the ganglioside G_{M1} in egg yolk phosphatidylcholine have been transferred according to the Langmuir-Blodgett technique to (a) glass cover slips coated with octadecyl- or hexadecyltrichlorosilane and (b) carbon-coated electron microscope grids. Monolayer transfer has been demonstrated with fluorescence microscopy, by the transfer of a fluorescent phospholipid analogue, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine or Lucifer yellow labeled G_{M1} (LY-G_{M1}), incorporated into the lipid monolayer. Incubation of supported monolayers with solutions of fluorescein-labeled cholera toxin (FITC cholera toxin) resulted in specific binding of the toxin to monolayers containing G_{M1}, as revealed by fluorescence microscopy. Lateral diffusion coefficients were measured for both the receptor (LY-G_{M1}) [$(3.9 \pm 2.1) \times 10^{-8}$ cm^2/s] and the receptor-ligand complex (G_{M1}-FITC cholera toxin) [$(8.9 \pm 3.2) \times 10^{-9}$ cm^2/s] according to the technique of fluorescence recovery after photobleaching. In separate studies, G_{M1}-containing monolayers transferred to electron microscope grids were incubated with solutions containing unlabeled cholera toxin, followed by negative staining with uranyl acetate. Electron microscopy revealed patches of stained cholera toxin molecules (diameter ~70 Å) in crystalline, two-dimensional hexagonal arrays. Optical diffraction and image reconstruction showed the arrangement of the cholera toxin molecules in a planar hexagonal cell, a = 81 Å. These initial reconstructions give structural information to a resolution of ~30 Å and indicate a doughnut-shaped molecule with a central aqueous channel.

Cholera toxin is produced by the Gram-negative bacterium *Vibrio cholerae*. The toxin molecule (M_r ~84 000) is considered to be an assembly of five identical B subunits (protomer M_r ~11 600) and a single A subunit (M_r ~27 000). Cell surface binding is associated with the B subunits, and the cell surface receptor is the ganglioside G_{M1}. The biological effect is mediated by penetration of the A subunit [a disulfide-linked A1:A2 (M_r ~22 000:~5400) heterodimer] through the host membrane. Following reduction of the disulfide bond, the A1

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