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μ-CONOTOXINS: SPECIFIC BLOCKERS OF MUSCLE SODIUM CHANNEL

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ABSTRACT

The voltage sensitive sodium channel is one of the key components of excitable membranes involved in the transmission of impulses in nerve and muscle. It is a medically important macromolecule not only because of its central physiological role but also because it is the target of a number of toxins including the paralytic shellfish poisons (PSP), puffer fish toxin (tetrodotoxin) and μ-conotoxins from the venomous marine snails, *Conus*. Although derived from very different biological sources, the three toxins have the same mechanism of action, they block the sodium channel and compete for binding at the same site on the protein.

μ-Conotoxins are the most potent peptide toxins known to block sodium channels; saxitoxin and tetrodotoxin are both complex polycyclic guanidinium compounds. Thus, given present technology, μ-conotoxins are much more amenable to chemical synthesis, chemical modification and radiolabeling than the two other toxins. In fact, μ-conotoxin GIIIA has recently been chemically synthesized and a biologically active, radioiodinated derivative prepared (Cruz *et al.* Biochemistry 28:3437, 1989; LeCheminant *et al.*, Trans. Nat. Acad. Sci. Tech. Phil., 10:423-430, 1989). The radiolabeled derivative binds specifically to a membrane protein with molecular weight in the size range of sodium channels and it competes with tetrodotoxin for binding to an elextroplax membrane preparation. The binding competition between tritiated derivative of μ-conotoxin and saxitoxin has similarly been demonstrated by Yanagawa *et al.* (J. Neurosci 7: 1498, 1987) This specific competition between saxitoxin and μ-conotoxins is now being developed as the basis for a sensitive biochemical assay to analyze paralytic shellfish poisons produced by the toxic dinoflagellates responsible for red tide.

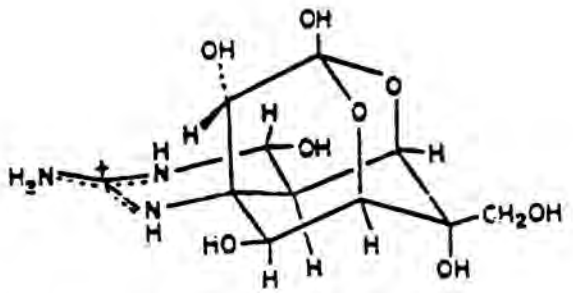
In contrast to saxitoxin and tetrodotoxin which block both nerve and muscle sodium channels to about the same extent, μ-conotoxins are highly specific for the muscle channel subtype. This unique specificity has made μ-conotoxins the choice pharmacological agents for applications in medicine and physiology requiring a quiescent muscle system while maintaining normal synaptic activity.

Introduction

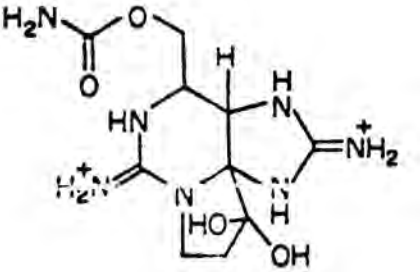
The voltage sensitive sodium channel is one of the key elements of excitable membranes. In most multicellular organisms, it plays a fundamental role in the generation and propagation of action potentials. This protein is a medically im-

portant macromolecule not only because of its central physiological role but also because it is the target of a number of lethal neurotoxins which either inhibit or enhance sodium ion transport (1,2,3). The most potent sodium channel blockers are the paralytic shellfish poisons (PSP) such as saxitoxin, the puffer fish toxin (tetrodotoxin) and μ-conotoxins from the venomous marine snails, *Conus*. Although chemically different and derived from unrelated biological sources (Fig. 1), μ-conotoxins are the only peptides (7); tetrodotoxin and the paralytic shellfish poisons, such as saxitoxin, are all polycyclic guanidinium compounds (8). Thus, given present technology, μ-conotoxins are much more amenable to chemical synthesis, chemical modification and radiolabeling than the two other toxins (9).

The physiological activity and chemical properties of μ-conotoxins make them very suitable for development as biochemical reagents for the detection and quantitation of PSP. The basis for such a method is the specific competition among the sodium channel blockers. However, in order to develop μ-conotoxin GIIIA as a convenient reagent, relatively large amounts of the toxin must be available. To remedy the current shortage of pure toxin, we have devised a quicker method for the isolation of μ-conotoxins. (Although μ-conotoxin GIIIA has recently been chemically synthesized, the method for folding of the synthetic peptide to give the active conformer needs improvement. Moreover, facilities for peptide synthesis are not yet available in the Philippines.) Radioiodinated μ-conotoxin GIIIA with high specific activity has also been prepared recently. This paper describes our preliminary binding studies and cross-linking experiments to ascertain specificity of radiolabeled toxin derivatives for the voltage sensitive sodium channel in preparation for their development as tools for the quantitation of PSP levels in food and biological samples.



A. Tetrodotoxin



B. Saxitoxin

Arg.Asp.Cys.Cys.Thr.Hyp.Hyp.Lys.Lys.Cys.Lys.Asp.Arg.Gln.Cys.Lys.Hyp.Gln.Arg.Cys.Cys.Ala*

C. μ-Conotoxin GIIIA

Figure 1. Structures of potent blockers of voltage sensitive sodium channels. Saxitoxin is one of the paralytic shellfish poisons which are closely related guanidinium compounds. The asterisk at the end of the peptide indicates an amidated carboxyl end.

Materials and Methods

μ-Conotoxin GIIIA was isolated from *Conus geographus* according to the method of Cruz *et al.* (7) or as described below. Radiolabeled μ-conotoxin GIIIA, ^{125}I-3[4-hydroxyphenyl] propionyl GIIIA (^{125}I-HPP-GIIIA, 0.44 mCi/nmole) was prepared as described previously (9). The membrane preparation from the electric organ of *Electrophorus electricus*, a rich source of muscle type sodium channels, was generously provided by Dr. Edward Moczydlowski of Yale University, Connecticut, U.S.A. All biochemicals were from Sigma Chemical Corporation, St. Louis, Missouri.

Modified method for the preparation of μ-conotoxins. Lyophilized crude venom from *Conus geographus* was extracted several times with 0.1 M ammonium acetate, pH 6.8 and then several times with 1.1% acetic acid by repeated suspension in the solvents, sonication and centrifugation at 10,000 rpm using a Sorvall SS-34 rotor. Ammonium acetate extracts were pooled and used directly for high pressure liquid chromatography but pooled HAc extracts were lyophilized and resuspended in the minimum amount of 0.1M ammonium acetate, pH 6.8 prior to chromatography. The isolation procedure involves fractionation through a molecular sieve followed by a series of runs through C18 reverse phase columns. As a second step, 5-ml aliquots of the extracts were chromatographed through a preparative BioSil TSK-125 HPLC column (Biorad) eluted with 0.1M ammonium acetate, pH 6.8. Fractions were bioassayed by intraperitoneal injection to 10-gram mice (Swiss Webster strain) and peaks which caused paralysis and death were further fractionated using a semi-preparative Vydac C18 reverse phase HPLC column eluted with a 1.2%/min gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA). Pure toxin was obtained by rechromatography of active peaks on an analytical Vydac C18 reverse phase HPLC column eluted with shallower gradients (0.06 to 0.3%/min) of acetonitrile in 0.1% TFA.

Binding assay. Assay for the binding of μ-conotoxin to the receptor site on the sodium channel was done as developed previously (6) using electroplax membrane preparation from *E. electricus.* The reaction mixture in a total volume of 200 μL contained 0.32M sucrose, 5mM HEPES/Tris buffer, pH 7.4, 45 mM KCl and about 50 μg protein of membrane preparation. Pre-incubation of control samples with unlabeled toxin (GIIIA or tetrodotoxin) and other unlabeled ligands was done for 30 minutes on ice. The final concentration of unlabeled toxins and ligands was 5 μM, except for lysozyme which was 0.2 mg/mL. Radiolabeled toxin was then added to all tubes and reaction mixtures were incubated for 30 minutes at room temperature. Samples were filtered through Whatman GF/C under vacuum and washed three times with 2.0 mL of wash medium containing 16 mM choline chloride, 15 mM $CaCl_2$, 5 mH HEPES/Tris, pH 7.4 and 0.2 mg/mL BSA. A Packard Multi-Prias gamma counter was used to determine radioactivity of filters.

Cross-Linking of radiolabeled GIIIA to sodium channels (9). A reaction mixture containing about 150 μg of membrane protein and 0.2 pmol of radioiodinated GIIIA in 200 μL of 20 mM NaHEPES, pH 7.5 was incubated for 30 minutes at room

temperature. To determine nonspecific binding, the same amount of membrane protein in buffer was preincubated with 0.1 nmol of unlabeled native GIIIA for 30 minutes at 0°C before radioiodinated GIIIA was added. Toxin receptor complexes were cross-linked according to the procedure of Pilch *et al.* (10) then analyzed by SDS-PAGE electrophoresis by the method of Laemmli (11) using 4-15% gradient gels. Pellets were dissolved with PAGE sample buffer containing 80 mM DTT just before electrophoresis.

Results

Until recently, the method used for isolating μ-conotoxins from extracts of *C. geographus* venom consisted of molecular sieving through a very long column of Sephadex G-25 followed by a series of HPLC runs through reverse phase columns (7). For the first step alone, it took a week to standardize the column and chromatograph the venom extract. We therefore devised a quicker way for purifying

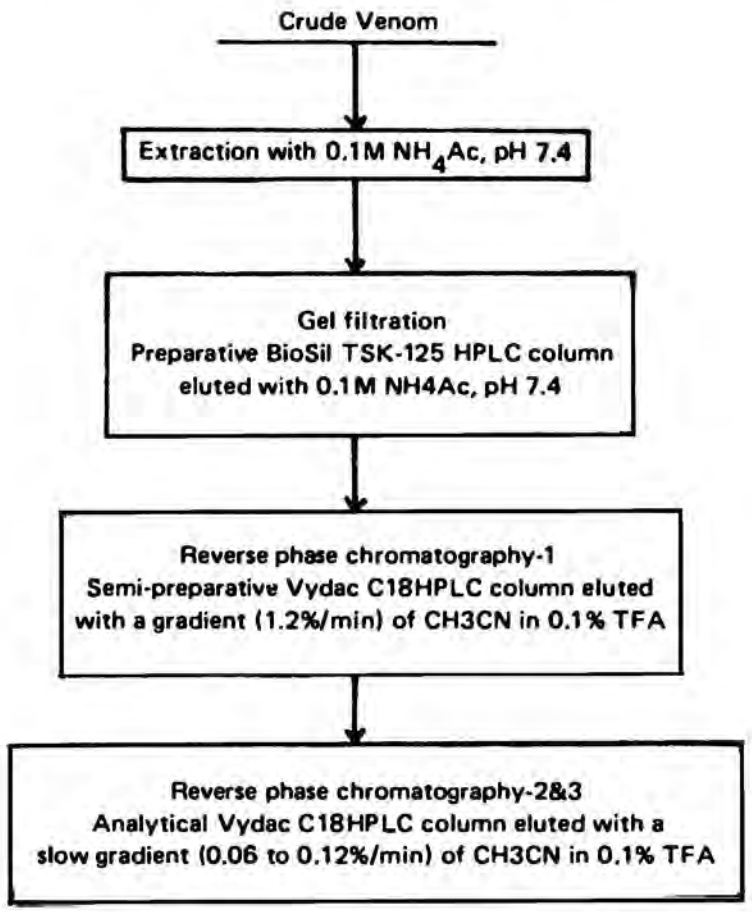


Figure 2. Schematic diagram of revised method for isolation of μ-conotoxins from *C. geographus* venom.

μ-conotoxins as shown in Fig. 2. By substituting an HPLC run for the regular gel filtration chromatograph in the second step, molecular sieving of crude venom extracts can be accomplished in a couple of hours. Subsequent steps involving reverse phase HPLC columns were also modified; a quick subfractionation on a semi-preparative C18 column using a fast gradient (1.2%/min) of acetonitrile in 0.1% TFA is followed by one or two purification steps on analytical C18 column eluted with shallower gradients (0.06 to 0.3%/min) of acetonitrile in 0.1% TFA. This method has now been adapted as a general strategy for the isolation of not only μ-conotoxins but also of other biologically active compounds from *Conus* venom. With the availability of more μ-conotoxins through a faster purification method, supply of the toxin will no longer be limiting.

For binding and immunological assays, radioiodinated reagents have been preferred because of the very high specific activities one can obtain with ^{125}I and the ease in determining radioactivity of gamma-emitters. Scintillation cocktails are not necessary and quenching is not a problem. However, since μ-conotoxins do not have tyrosine or histidine residues which can be radioiodinated the Bolton-Hunter reagent has been used to prepare 3-[4-hydroxyphenyl] propionyl derivatives of GIIIA. Five derivatives were found to be biologically active but as a first attempt, the most abundant one was iodinated to give ^{125}I-HPP-GIIIA (9). This derivative was used for binding and cross-linking studies. To biochemically characterize the receptor, radiolabeled μ-conotoxin GIIIA was bound to membrane preparations and cross-linked to the receptor using a bivalent crosslinker, disuccinimidylsuberate. As shown in Figure 3, electrophoresis of cross-linked material under denaturing conditions indicated a specifically labeled band with a relative molecular weight greater than 200,000 which is the expected result if cross-linking of radiolabeled GIIIA were occurring at the sodium channel.

Table 1 shows the effect of various unlabelled ligands on the binding of radioiodinated μ-conotoxin GIIIA. It is clear that the two sodium channel blockers, GIIIA and tetrodotoxin completely inhibit binding of GIIIA to the receptor or ion channel. On the other hand, the other positively charged conotoxins which have different physiological action do not significantly affect the binding of labeled GIIIA; lysozyme, a positively charged protein, similarly does not significantly affect the binding of μ-conotoxin. These data indicate that radioiodinated μ-conotoxin GIIIA binds specifically to the guanidinium site on the sodium channel which is not bound by other positively charged peptides or protein. Although we did not test competition of GIIIA with saxitoxin (due to a very limited supply of standard saxitoxin), the binding competion between tritiated derivative of μ-conotoxin and saxitoxin has recently been demonstrated by Yanagawa *et al.* (5). The above findings indicate the feasibility of using μ-conotoxin GIIIA as a research tool for the guanidinium binding site on the sodium channel and as a biochemical reagent for the quantitation of PSP levels in food and biological samples.

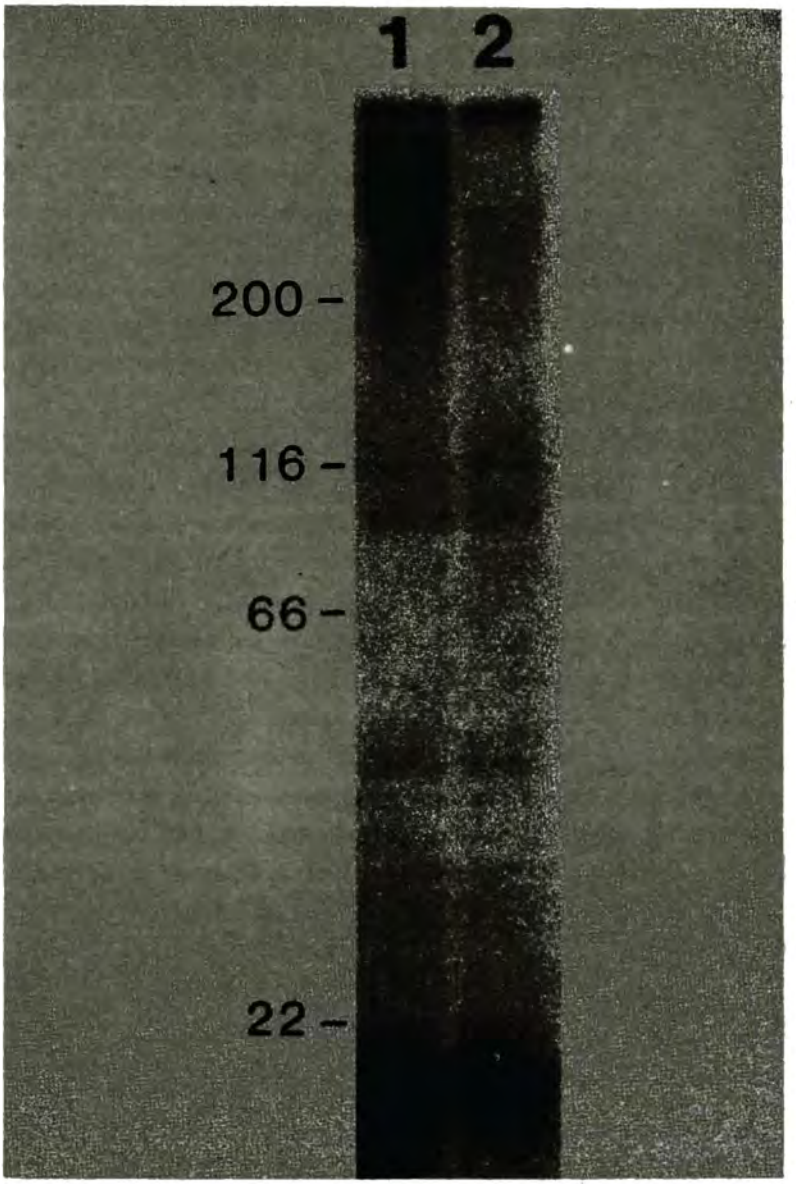


Figure 3. Cross-linking of radiolabeled μ-conotoxin GIIIA to membrane preparation from eel electroplax. Lane 1: Membrane incubated with ^{125}I-3[4-hydroxyphenyl] propionyl GIIIA. Lane 2: Membrane preincubated with unlabeled GIIIA before addition of labeled toxin.

Table 1. Competition between radiolabeled μ-conotoxin GIIIA and various unlabeled ligands

Unlabeled Ligand	*% Binding of 125I-HPP GIIIA*	*Target*
None	100	
μ-Conotoxin GIIIA	0	Voltage sensitive sodium channel
Tetrodotoxin	0	Voltage sensitive sodium channel
ω-Conotoxin GVIA	96	Voltage sensitive calcium channel
α-Conotoxin MI	80	Acetylcholine receptor
α-Conotoxin GI	92	Acetylcholine receptor
Lysozyme	83	Bacterial cell wall

Discussion

So far, the following have already been accomplished toward the development of μ-conotoxin GIIA as an analytical tool for saxitoxin and other paralytic shellfish poisons: 1. Alternative methods for the preparation of pure toxin have been established, one a quicker way for the isolation of μ-conotoxins from crude *C. geographus* venom and another, the chemical synthesis of μ-conotoxin GIIIA 2. A biologically active radiolabeled derivative of μ-conotoxin GIIIA has been prepared by first making a 3[4-hydroxyphenyl] propionyl derivative using the Bolton-Hunter reagent and then labeling one of the derivatives with iodine-125; 3. A radioiodinated derivative of μ-conotoxin GIIIA has been demonstrated to compete specifically with the sodium channel blocker, tetrodotoxin. Tritiated GIIIA has been demonstrated by Yanagawa *et al* (5) to also compete with saxitoxin To pursue the development of this promising reagent we plan to radioiodinate the four other biologically active hydroxyphenyl-propionyl derivatives of GIIIA and compare their binding characteristics. Relative affinities of the labeled derivatives for the ion channel/receptor complex will be determined. The one with the highest affinity will be chosen for standardizing binding curves of radiolabeled. GIIIA and its displacement from the receptor by saxitoxin. Conditions to maximize sensitivity and to ensure linearity of the assay will be studied. The assay based on the competition between guanidinium toxins and μ-conotoxins promises to be a very sensitive and specific one for PSP.

μ-Conotoxin, saxitoxin and tetrodotoxin differ in their tissue specificities. The guanidinium toxins have high affinities for skeletal muscle and nerve sodium channels but low affinities for heart type sodium channels. On the other hand, μ-conotoxin GIIIA is very active only on skeletal muscle type; it is at least 1000-fold less active on nerve versus muscle sodium channels and it also has a low affinity for cardiac muscle sodium channels (7,9). This unique specificity has made μ-conotoxins the choice pharmacological agents for applications in medicine and physiology requiring a quiescent muscle system while maintaining normal synaptic activity.

Acknowledgments

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