Characterization of α -conotoxin interactions with the nicotinic acetylcholine receptor and monoclonal antibodies

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The venoms of predatory marine cone snails, *Conus* species, contain numerous peptides and proteins with remarkably diverse pharmacological properties. One group of peptides are the α -conotoxins, which consist of 13–19 amino acids constrained by two disulphide bonds. A biologically active fluorescein derivative of *Conus geographus* α -conotoxin GI (FGI) was used in novel solution-phase-binding assays with purified *Torpedo californica*

nicotinic acetylcholine receptor (nAChR) and monoclonal antibodies developed against the toxin. The binding of FGI to nAChR or antibody had apparent dissociation constants of 10–100 nM. Structure–function studies with α -conotoxin GI analogues composed of a single disulphide loop revealed that different conformational restraints are necessary for effective toxin interactions with nAChR or antibodies.

INTRODUCTION

During the last 50 million years, predatory marine cone snails (*Conus* species) have developed a wide array of biologically active peptides which are found in their venom and possess exquisite specificities for many different receptors [1–3]. Some venoms contain over a 100 different peptides [2], thereby giving these animals a substantial arsenal for subjugating various prey. The α -conotoxins represent just one group of structurally related neurotoxic peptides [1,2]. These toxins, the smallest known polypeptide inhibitors of nicotinic acetylcholine receptor (nAChR), compete with acetylcholine and block signal transmission at the neural synapse [4]. The nAChR isolated from *Torpedo californica* electric organ and skeletal muscle consists of five protein subunits ($\alpha_2\beta\gamma\delta$) which form a cylindrical ion channel within the postsynaptic membrane [5]. Acetylcholine and other ligands interact with the extracellular domain of both α -subunits, but adjacent subunits contribute significantly to some unique binding properties [6,7]. For example, the α/δ and α/γ regions of nAChR have strikingly different binding affinities for the α -conotoxins [8–10].

The α -conotoxins have potent biological activities that correlate well with their binding affinities for *T. californica* nAChR [8,9,11]. α -Conotoxins GI and MI, isolated from the respective venoms of *Conus geographus* and *Conus magus*, cause a rapidly lethal muscle paralysis in mice at submicrogram quantities. In contrast, no signs of intoxication are observed in mice given relatively large doses of *Conus striatus* α -conotoxin SI [11], which also binds very weakly to the *T. californica* nAChR [8]. Selective binding of α -neurotoxins to the nAChR from different animal species is also evident for α -bungarotoxin, a snake postsynaptic neurotoxin purified from *Bungarus multicinctus* venom [12].

The α -conotoxins are structurally simpler than α -bungarotoxin and other snake venom α -neurotoxins, which usually consist of 60–70 residues plus four or five disulphide bridges. Subsequently, the α -conotoxins are likely to have fewer contact sites necessary for efficient binding to nAChR, thus making these molecules an invaluable tool for analysing ligand–nAChR interactions. Recent crystallographic studies with α -conotoxins provide further information on toxin structure and may lead to a better understanding of how these toxins differentially interact with subtypes

of neuronal and muscle nAChR [13,14]. The natural variations among α -conotoxin sequences and use of these molecules for studying receptor subtypes may result in pharmaceutically useful compounds [3,10,11,15–17].

This study describes the use of non-radioactive solution-phase-binding assays to quickly measure specific receptor–ligand and antibody–antigen interactions of small peptides such as the α -conotoxins. These novel methods enabled us to investigate the structure–function properties of these toxins by using single-loop α -conotoxin GI analogues. Finally, we characterized the binding specificity and epitopes recognized by two unique monoclonal antibodies developed against α -conotoxin GI.

EXPERIMENTAL

Toxins, peptides and nAChR

Synthetic α -conotoxins GI (ECCNPACGRHYSC-NH₂), MI (GRCCHPACGKNYSYSC-NH₂), SI (ICCNPAACGPKYSYSC-NH₂) and μ -conotoxins GIIIB (RDCCTP*P*RKCKDRCKP*MKCCA-NH₂; P* = hydroxyproline) and GIIIA (RDCCTP*P*KKCKDRQCKP*QRCCA-NH₂) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Homologues of α -conotoxin GI containing the natural C-terminal amide group and various cysteine to serine substitutions, GIp1 (ESSNPASGRHYSS-NH₂), GIp2 (ECSNPACGRHYSS-NH₂) and GIp3 (ESCNPASGRHYSC-NH₂), were obtained from Quality Controlled Biochemicals (Hopkinton, MA, U.S.A.). These peptides were characterized by the manufacturer using HPLC plus MS. The primary sequence of each α -conotoxin GI homologue was kindly verified by Dr. James Schmidt (USAMRIID) with an Applied Biosystems 470A sequencer (Foster City, CA, U.S.A.). Affinity-purified nAChR was prepared from frozen *T. californica* electric organ (Pacific Biomarine, Venice, CA, U.S.A.) as previously described [18,19].

Fluoresceinated α -conotoxin GI (FGI)

A 1 ml solution containing 0.35 mg of α -conotoxin GI in 0.1 M phosphate buffer, pH 8.0, was mixed for 30 min with 100 μ l of DMSO containing 0.5 mg of FITC (Molecular Probes, Eugene,

Abbreviations used: FGI, fluoresceinated α -conotoxin GI; mAb, monoclonal antibody; nAChR, nicotinic acetylcholine receptor.

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OR, U.S.A.). FGI was purified by reverse-phase HPLC with a PepRPC HR5/5 column (Pharmacia, Piscataway, NJ, U.S.A.) and represented 82% of the initial peptide used for conjugation. The N-terminal glutamic acid was modified [20], as evidenced by unsuccessful Edman degradation of FGI (500 pmol). After lyophilization, FGI was dissolved in sterile PBS and stored in a foil-covered tube at 4 °C. There was no detectable decrease in fluorescence or receptor-binding activity of FGI over several months when stored at 4 °C. An absorption coefficient of $66800 \text{ cm}^{-1} \cdot \text{M}^{-1}$ at $\lambda = 495 \text{ nm}$ [21] was used to determine the FGI concentration. The fluorescence spectra, obtained from a Perkin-Elmer model 650-40 spectrophotometer (Norwalk, CT, U.S.A.), showed characteristic excitation and emission maxima at $\lambda = 492 \text{ nm}$ and $\lambda = 517 \text{ nm}$ respectively. With 2 nm slits, the detection sensitivity in PBS containing 0.1% Triton X-100 (PBST) was 20 fluorescence units/nmol of FGI.

Spin-column assays

FGI binding to *T. californica* nAChR or monoclonal antibody (mAb) was determined by spin-column experiments at room temperature. Affinity-purified nAChR or mAb, with protein concentrations measured by a microBCA assay (Pierce Chemical Co., Rockford, IL, U.S.A.) using BSA standards, were equilibrated with PBST on a PD-10 column (Pharmacia). Direct binding studies employed various concentrations of FGI incubated for 60 min with the nAChR or mAb. For competitive ligand-displacement assays, FGI was premixed with various dilutions of the unlabelled ligands and then incubated with nAChR or mAb. An aliquot (200 μl) of the incubation mixture was applied to a 1 ml bed of Bio-Gel P10 (Bio-Rad Laboratories, Richmond, CA, U.S.A.), and the protein-bound fraction of FGI was rapidly separated using centrifugal flow methods [22,23]. The void-volume samples from receptor-binding assays were diluted with PBST containing 10 mM carbamoylcholine to dissociate the ligand-receptor complex. Fluorescence was measured in ratio mode using 1 cm^2 cuvettes with excitation and emission monochromators set at $\lambda = 490 \text{ nm}$ and $\lambda = 525 \text{ nm}$ respectively. Optimum sensitivity in the linear range of the detector was obtained by adjusting the optical slit widths to between 5 and 15 nm. Direct binding results from the spin-column assays were analysed by using eqn. (1) [24], which describes the fractional saturation of a known concentration of receptor (R_T) as a function of the total FGI (L_T) concentration:

$$\Delta F / \Delta F_{\max} = [(K_D + R_T + L_T) - (K_D + R_T + L_T)^2 - 4R_T L_T] / (2R_T) \quad (1)$$

The best-fit parameters for the dissociation constant (K_D) and the maximum fluorescence change ($\Delta F_{\max}$) were determined from the non-linear curve-fitting routines of SigmaPlot. Curve-fitting and data from competition assays were analysed with the program LIGAND [25]. As this program requires input parameters in the form of specific radioactivity ($\mu\text{Ci/mol}$), appropriate numerical conversions were made for the non-radioactive data values.

Fluorescence quenching assays

FGI was added to a temperature-controlled cuvette at 23 °C and titrated with small aliquots of *T. californica* nAChR. Fluorescence measurements taken at 1 min intervals after ligand addition were stable within 3–5 min. After a 5 min incubation between each successive addition, the fluorescence was measured and compared with control samples containing only FGI. Slight decreases in control fluorescence were probably due to cumulative photobleaching, which was minimized by using a narrow excitation slit width and limiting light exposure. Subsequent addition of excess

unlabelled α -conotoxins MI or GI in each experiment verified that quenching caused by the addition of nAChR was specifically reversible. After a dilution correction, the binding reaction was analysed with a modified form of eqn. (1) where the observed fluorescence change ($F_{\text{obs}} - F_{\text{min}}$) was normalized relative to the initial unquenched fluorescence value (F_{obs}) and the terms for L_T and P_T were interchanged because FGI was titrated with receptor.

The specificity of FGI binding to receptor and relative displacement efficiencies of several competitive ligands were also measured with a reverse fluorescence quenching assay. FGI was incubated with excess nAChR, so that the initial ligand was predominantly bound to receptor. Preformed complexes were titrated with multiple aliquots of unlabelled ligand, and the fluorescence measured at 5 min intervals. The data were corrected for dilution and analysed using the curve-fitting routines of SigmaPlot and eqn. (2) [26]:

$$(F_{\text{obs}} - F_{\text{min}}) / (F_{\text{max}} - F_{\text{min}}) = L / (L + C_{1/2}) \quad (2)$$

L represents the total molar concentration of unlabelled ligand, F_{obs} is the observed fluorescence, F_{min} is the initial quenched fluorescence value, F_{max} is the maximum fluorescence corresponding to free ligand at final titration, and $C_{1/2}$ is the best-fit midpoint in the titration curve.

Characterization of single disulphide-loop analogues of α -conotoxin GI

Freshly prepared solutions of GI_{p2} and GI_{p3} contained 2 mol of cysteine/mol of tyrosine, as shown by reaction with excess 5,5'-dithiobis-(2-nitrobenzoic acid). The peptides were diluted to 50 μM with PBS and oxidized slowly at room temperature for 48 h with continuous mixing. Analysis with 5,5'-dithiobis-(2-nitrobenzoic acid) revealed that the free cysteine concentration gradually decreased to the limit of thiol detection. After oxidation, the peptides were concentrated tenfold by lyophilization and a 120 μl aliquot of each was added to a calibrated Bio-Gel P10 size-exclusion column. Chromatograms for each oxidized peptide consisted of a single symmetrical peak that was not indicative of high-molecular-mass aggregates caused by intermolecular disulphide bonds.

Generation of mAbs against α -conotoxin GI

Methods described for conjugate synthesis, immunization and ELISA analysis of antisera to α -conotoxin GI [27], plus the generation of stable hybridomas [28], were used to produce mAbs against α -conotoxin GI. Ascites fluids from two subcloned cell lines, 5A1 and 8D2, were used to purify each mAb by Protein G column chromatography. Subisotyping analysis (Mouse Typer Kit; Bio-Rad Laboratories) revealed that mAb 5A1 and 8D2 were an IgG_{2a} and IgG₁ respectively. *In vivo* neutralization of α -conotoxin GI by each mAb was tested in a mouse lethality assay [27]. All experiments with animals were performed in a facility fully accredited by the American Association for Accreditation of Laboratory Animal Care according to protocols approved by the USAMRIID Laboratory Animal Care and Use Committee, and is in compliance with the Guide for Laboratory Animal Facilities under the directions of the Committee on the Guide for Laboratory Animal Resources/NRC.

RESULTS

Binding of FGI to *T. californica* nAChR: spin-column assay

The molecular-mass ratio of nAChR/ α -conotoxin is $> 100:1$, suggesting that free and receptor-bound fractions of FGI could

Figure 1 Binding of FGI to *T. californica* nAChR in a solution phase; spin-column assay

Data represent the mean $\pm$ S.D. fluorescence reading for duplicate samples obtained with various concentrations of FGI plus nAChR (80 nM). Background fluorescence data ($\circ$) were obtained by chromatography of FGI alone.

be separated by gel-filtration chromatography. Preliminary experiments indicated that FGI slowly dissociated from nAChR during gravity-flow chromatography. Therefore further studies were carried out with a rapid spin-column technique [22,23] to decrease the resolution time. The microBCA assay verified that nAChR (10–50 μ g/ml) was completely recovered after a 200 μ l application to a spin column. Triton X-100, which maintained nAChR solubility in the receptor-binding assays, probably prevented adsorptive losses of protein during chromatography.

The binding of FGI to *T. californica* nAChR yielded a saturable dose-response curve (Figure 1) characterized by a single class of binding sites ($K_D = 41.3 \pm 8.2$ nM). Interactions of FGI with a second low-affinity site on nAChR [8,9] could not be measured because FGI concentrations above 5 μ M had high background signals.

Specific binding of FGI to *T. californica* nAChR

Although FGI bound to nAChR in a saturable dose-dependent manner, we had to establish the specificity of this interaction. Competition assays were performed with various concentrations of unlabelled α -conotoxins as competitors (Figure 2). The two-ligand/single-binding-site model of LIGAND was used to determine the best-fit equilibrium dissociation constants of α -conotoxins MI ($K_D = 19.3 \pm 4.4$ nM), GI ($K_D = 95 \pm 27$ nM) and SI ($K_D = 334 \pm 90$ nM). The binding of FGI to nAChR was also inhibited effectively by α -bungarotoxin (results not shown). However, μ -conotoxin GIIIA, a specific blocker of muscle Na^+ channels and consisting of 22 residues plus three disulphide loops, was not a competitor for α -conotoxin binding to nAChR (results not shown).

Measurement of FGI binding to *T. californica* nAChR by fluorescence quenching

Preliminary experiments revealed that the fluorescence signal from FGI, when added to a fivefold molar excess of nAChR, was quenched by 20 % compared with a buffer control. This finding was unique to nAChR interactions with FGI, as neither α -conotoxin GI mAb had the same effect. The reduced signal from FGI–nAChR complex was not caused by adsorption because

Figure 2 Unlabelled α -conotoxins compete with FGI binding to *T. californica* nAChR

FGI (25 nM) was incubated with various concentrations of α -conotoxins MI, GI or SI and 80 nM nAChR. The receptor-bound fluorescent ligand was resolved by spin-column chromatography and the data represent means $\pm$ S.D. for duplicate samples normalized relative to controls lacking competitor.

Figure 3 *T. californica* nAChR quenches the fluorescence of FGI

Successive aliquots of receptor (top line with error bars) or PBST ($\circ$) were added to cuvettes containing FGI (initial concentration 100 nM). Brief fluorescence measurements were taken after 5 min intervals at 23 $^{\circ}\text{C}$. The data, presented as means $\pm$ S.D. for duplicate samples, were corrected for dilution and normalized relative to the initial unquenched fluorescence value (F_0). The cumulative photodecomposition data ($\bullet$) were obtained from a second control sample of non-titrated FGI. The dashed line is a corrected binding curve, derived by subtracting the photodecomposition component from the experimental data.

there was no further decrease in fluorescence when the quenched sample was added to a fresh cuvette. Addition of α -conotoxins GI or MI (10 μ M) to the FGI–nAChR mixture restored the fluorescence levels to 100 %, indicating that quenching was due to specific and reversible binding interactions. This information led to the use of 10 mM carbamoylcholine to dissociate FGI from the receptor after spin-column chromatography and also formed the basis for a direct solution-phase-binding assay (Figure 3), which measures changes in FGI fluorescence during titrations with nAChR. Cumulative photodegradation of FGI controls

Figure 4 Reversed quenching of FGI complexed to *T. californica* nAChR by α -conotoxins

Quenched complexes of FGI (25 nM) and excess *T. californica* nAChR (160 nM) were titrated with α -conotoxins MI, GI, SI and a single-loop (Cys2–Cys7) analogue of GI (Glp2). Titrations with Glp1 or Glp3, the Cys3–Cys13 single-loop version of α -conotoxin GI, did not reverse quenching. Each data point represents the mean reading of duplicate samples normalized relative to the range between initial quenched fluorescence and the final unquenched value verified by the addition of 10 μ M α -conotoxin MI or GI.

Figure 5 Specific binding of mAbs 5A1 and 8D2 to α -conotoxins

Various concentrations of α -conotoxins GI, MI, SI and Glp3 were used in the spin-column assay as competitive ligands to displace 25 nM FGI from 5A1 or 8D2 (20 μ g/ml). Glp1 or Glp2 did not compete with either mAb for binding to FGI. Data represent the mean reading for duplicate samples normalized relative to a control mAb against staphylococcal enterotoxin A.

represented less than 5% of the initial fluorescence. Analysis of the quenching experiments indicated that FGI bound to a single class of sites ($K_D = 31 \pm 10$ nM). Subtraction of the decomposition component yielded a corrected binding curve ($K_D = 17 \pm 7$ nM) that extrapolated to a saturation binding limit of $[(F_0 - F)/F_0 = 0.20 \pm 0.01]$.

Fluorescence quenching of FGI was also used to evaluate the binding of other α -conotoxins and single-loop analogues of α -conotoxin GI. Titrations with unlabelled α -conotoxins MI ($C_{1/2} = 0.20$ μ M), GI ($C_{1/2} = 0.48$ μ M) and SI ($C_{1/2} = 13$ μ M) clearly revealed that quenching was reversed with various concentrations of specific ligands (Figure 4). The dose–response curves of these toxins in quenching assays were consistent with the effective concentration ranges previously demonstrated for the spin-column assays. α -Conotoxin SI bound less avidly to *T. californica* nAChR, perhaps because of one major structural difference not

found in α -conotoxins GI and MI. A proline (Pro9) is located in the middle of the α -conotoxin SI large loop, whereas α -conotoxins GI and MI respectively contain a positively charged residue, arginine and lysine, which probably influences toxin conformation and ultimately receptor binding [8].

Besides investigating the effects of α -conotoxins on FGI binding to *T. californica* nAChR in quenching experiments, a single-loop analogue (Cys2–Cys7) of α -conotoxin GI (designated Glp2) also proved to be an effective competitor of FGI binding to receptor (Figure 4). This structural constraint apparently stabilized Glp2 conformations that specifically recognized nAChR, albeit with sevenfold lower avidity than native toxin. Freshly prepared solutions of Glp2 did not bind nAChR before oxidation, thus emphasizing the importance of this loop structure for receptor interactions. However, 10 μ M concentrations of another single-loop analogue of α -conotoxin GI, Glp3 (Cys3–Cys13), or a reduced version of the native toxin, Glp1, did not reverse quenching of FGI (results not shown).

Solution-phase binding of FGI with mAbs

To help to identify immunodominant regions (i.e. epitopes) on the α -conotoxins, we developed mAbs against α -conotoxin GI. Previous studies with polyclonal antibodies developed against α -conotoxin GI suggest that the small-loop sequence CCXPAC, where X = N in α -conotoxins GI and SI and X = H in α -conotoxin MI, represents an immunodominant homologous region within these molecules [27].

The two mAbs (5A1 and 8D2) generated against α -conotoxin GI did not recognize linear epitopes, as determined by studies with overlapping peptides spanning the α -conotoxin GI sequence (results not shown). Both mAbs bound to FGI in spin-column experiments (Figure 5), unlike a control mAb against an unrelated antigen-like staphylococcal enterotoxin A (results not shown). Competitive displacement assays demonstrated that 5A1 ($K_D = 10 \pm 1$ nM) and 8D2 ($K_D = 46 \pm 4$ nM) specifically bound α -conotoxin GI with high avidity (Figure 5).

Neither mAb to α -conotoxin GI recognized the other homologous toxins (MI or SI) in a direct ELISA. However, the spin-column assay revealed cross-reactivity between mAb 5A1 and α -conotoxins SI and MI, but this interaction was 100–300-fold less avid than binding to the homologous antigen (Figure 5). This mAb did not bind any of the loop analogues of α -conotoxin GI.

Unlike mAb 5A1, there was no cross-reactivity between 8D2 and the other α -conotoxins. However, the 8D2 mAb did interact with a single-loop analogue of α -conotoxin GI, Glp3 (Cys3–Cys13), but not the short-loop analogue (Cys2–Cys7; Glp2) or reduced version of α -conotoxin GI (Glp1). The competitive binding data for Glp3 and mAb 8D2 revealed a tenfold less avid interaction than the binding of mAb 8D2 with native α -conotoxin GI (Figure 5). These findings suggest that the large loop stabilizes an immunoreactive conformation similar to that found on the native toxin.

Neutralization of α -conotoxin GI with mAbs

An antibody/ α -conotoxin GI molar ratio of 1:1 (120 μ g antibody: 1.1 μ g toxin) afforded complete protection in mouse lethal assays ($n = 10$ mice per antibody) when either mAb was pre-incubated with toxin at 37 °C for 30 min before injection. These results were not surprising, since the determined affinities of 8D2 and 5A1 for α -conotoxin GI in the spin-column assay were comparable with values obtained for α -conotoxin GI binding to *T. californica* nAChR. A control mAb against staphylococcal enterotoxin A did not protect mice against the same lethal dose of α -conotoxin GI (1.1 μ g/animal), resulting in 90% lethality.

As reported with polyclonal antibodies against α -conotoxin GI [27], protection was also afforded by either mAb which effectively prevented α -conotoxin GI binding to nAChR *in vivo*.

DISCUSSION

Snake venom α -neurotoxins, adsorbed on to microtitre plates, specifically bind *T. californica* nAChR in a simple non-radioactive assay [29,30]. The nAChR molecule contains two high-affinity (nanomolar) binding sites for snake venom α -neurotoxins, but the same receptor possesses a high- and low-affinity (micromolar) binding site for the α -conotoxins [8,9]. Therefore concentrations of α -conotoxin that saturate only the high-affinity site do not effectively inhibit the binding of snake venom α -neurotoxins to receptor [29]. As with most receptor-binding studies for snake venom α -neurotoxins, those for the α -conotoxins have included displacement of a radiolabelled postsynaptic neurotoxin like α -bungarotoxin [8,9]. Evidently α -conotoxin GI can also be radioiodinated and subsequently bind to murine nAChR *in vivo*, but there is a noticeable loss of biological activity [31].

α -Conotoxins (GI, MI or SI) adsorbed on to microtitre plates react with polyclonal antibodies in a direct ELISA [27], demonstrating that these small molecules can provide a solid-phase target for immunoglobulins. However, adsorbed α -conotoxins do not bind to *T. californica* nAChR [29]. Various derivatives of α -conotoxin GI were tested in solid-phase assays, but neither an α -conotoxin GI-BSA conjugate linked to an Affigel-10 affinity matrix nor α -conotoxin GI biotinylated via the N-terminus bound to *T. californica* nAChR (J. D. Ashcom and B. G. Stiles, unpublished work). These results prompted us to explore further and develop a simple, non-radioactive solution-phase assay for measuring the binding of small neuroligands, such as the α -conotoxins, to nAChR.

By attaching fluorescein to the N-terminus of α -conotoxin GI, we discovered, using a quick and inexpensive spin-column technique, that this toxin derivative (FGI) bound to *T. californica* nAChR in a specific high-affinity dose-dependent manner. Previous work suggests that this modification does not affect the receptor-binding properties of α -conotoxin GI [32], as the N-terminal glutamic acid can be deleted without diminishing receptor-binding activity. The N-terminal isoleucine of α -conotoxin SI can also be deleted without decreasing biological activity [11]. Our results with the spin-column or quenching experiments revealed saturable high-affinity binding of FGI to *T. californica* nAChR that was effectively competed for by unlabelled α -conotoxins GI, MI or SI. The determined affinities of α -conotoxins GI, MI and SI from our study agreed with results from an ^{125}I - α -bungarotoxin-displacement assay [8]. Since the binding affinities of α -conotoxins for *T. californica* nAChR are consistent with the relative toxicities in mice [11,27], FGI displacement in either non-radioactive assay might be useful for evaluating the potential toxicity of other α -neurotoxins *in vitro*. In addition, for postsynaptic binding studies, it is much easier to extract sufficient quantities of nAChR from *Torpedo* electroplax than from mammalian tissue [18].

Based on the displacement of FGI from *T. californica* nAChR and two protective mAbs, the conformational constraints provided by both disulphide bonds were important for optimal α -conotoxin GI-binding activity. Free rotation about the amide bonds in GIp1, which lacked any disulphide bridges, probably produced a large number of peptide conformations unfavourable for binding to nAChR or either mAb. A single disulphide bond between Cys2-Cys7 provided a receptor-binding structure (GIp2) not recognized by either mAb. However, the 8D2 mAb bound the other constrained peptide (GIp3) containing a single Cys3-

Cys13 loop, but this peptide did not interact with nAChR. The 8D2 mAb was very specific for α -conotoxin GI or GIp3 and did not bind to either α -conotoxins MI or SI. The other mAb, 5A1, failed to recognize any of the single-loop α -conotoxin analogues, although this antibody did cross-react with α -conotoxins MI and SI with approx. 100-fold less binding avidity than the homologous antigen. Our results suggest that different three-dimensional requirements are necessary for effective binding of α -conotoxin GI to *T. californica* nAChR and antibodies.

The marked differences between the single-loop analogues of α -conotoxin GI and binding to receptor or antibody are not surprising considering the results of a recent crystallographic study of the native toxin [14]. The short loop forms a left-handed spiral and the large loop contains a right-handed spiral. Native α -conotoxin GI is a compact triangle of 4.6–7.2 Å width, with Glu1, Arg9 and Cys13 representing the corners. The lone negative charge is the carboxy group of Glu1, oriented 17.9 Å opposite the positive side chain of Arg9. Evidently Glu1, which does not participate in binding to nAChR [32] and is not conserved among the α -conotoxins, adopts various spatial positions with few stabilizing interactions via adjacent residues. These previous findings further support our results showing that a fluorescein group attached to Glu1 does not affect receptor- or antibody-binding properties of the native toxin.

The importance of a conserved small-loop structure among α -conotoxins was previously shown, since substitution of the proline residue in this region eliminates the biological activities of α -conotoxins MI and GI in frog abdominal muscle [33] and mouse diaphragm assays [32] respectively. These reports also revealed that single-loop analogues of α -conotoxins MI or GI lack paralytic activity. Our single-loop peptides of α -conotoxin GI (GIp2 and GIp3) were not lethal in mice when tested at a peptide concentration (10 µg/20 g mouse) equivalent to 45 LD₅₀ of native toxin (J. D. Ashcom and B. G. Stiles, unpublished work). Such findings apparently contradict our receptor-binding studies with GIp2, but a possible interpretation is that GIp2 does not bind to nAChR of some animal species because of significant differences in the receptor structure versus *T. californica* nAChR. Similar results with different animal nAChRs have been reported for the snake postsynaptic neurotoxin, α -bungarotoxin [12]. Since the affinity of GIp2 for *T. californica* nAChR was approximately tenfold lower than that of the native toxin, some receptor-binding determinants that stabilize the toxin-receptor complex were probably not present in this single-loop peptide.

The inability of GIp3 to bind nAChR does not prove that the large loop of α -conotoxin GI is unable to facilitate receptor recognition. GIp3, composed of 11 residues within the disulphide loop, probably lacks the correct conformation for effective interactions with *T. californica* nAChR. Specific amino acid substitutions within the large loop decrease the receptor-binding properties of α -conotoxins MI (Tyr12/D-Tyr) [33] and GI (Tyr11/D-Tyr) [32]. However, replacement of Pro9 with Lys in α -conotoxin SI generates a molecule that structurally mimics α -conotoxin MI with a concomitant higher affinity for nAChR [34].

Although α -conotoxin GI consists of only 13 amino acids, the immune system obviously recognizes different epitopes on such a small molecule when conjugated to a carrier protein. Our repeated attempts to develop antibodies against sublethal doses of native unconjugated α -conotoxin GI were not productive. However, sublethal doses of various snake venom α -neurotoxins have been very effective for the generation of mAbs [28,35].

To our knowledge, this study is the first to describe mAbs for any α -conotoxin. Unlike mAbs against snake α -neurotoxins, which only delay the time to death [28,35], either α -conotoxin GI

mAb was completely protective at a 1:1 molar ratio of α -conotoxin to antibody. There is one report of mAbs developed against ω -conotoxin GVIA [36], a Ca^{2+} channel antagonist found in *C. geographus* venom and consisting of 27 residues plus three disulphide bonds. Some of these mAbs prevent binding of ω -conotoxin GVIA to rat brain synaptosomes. Like the α -conotoxin mAbs, the ω -conotoxin mAbs recognize conformational epitopes.

With a fluorescein derivative of α -conotoxin GI and two assay systems, we were able to monitor effectively the binding of these small neuroligands to receptor and antibody. Relative to native toxin, neither partially constrained (single-loop) peptide of α -conotoxin GI possessed complete binding activity. Multiple conformational restraints evidently contribute to the essential structure-function of α -conotoxins, and a scaffold consisting of two disulphide loops may provide the best design for other α -conotoxin analogues. It appears that Nature has provided us with a pharmaceutical cornucopia in the form of *Conus* peptides. Further studies will only enhance our understanding, and use, of these molecules in medicine and science.

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