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Synthesis of fluorescent analogs of α -conotoxin MII

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Abstract

α -Conotoxins (α -CTxs) are small peptides that are competitive inhibitors of nicotinic acetylcholine receptors (nAChRs) and have been used to study the kinetics of nAChRs. α -CTx MII, from the venom of *Conus magus*, has been shown to potently block both rat $\alpha 3\beta 2$ and rat chimeric $\alpha 6/\alpha 3\beta 2\beta 3$ cloned nAChRs expressed in *Xenopus* oocytes. Tetramethylrhodamine (TMR), Bodipy[®] FL, Alexa Fluor[®] 488 and terbium chelates (TbCh) are fluorescent molecules that can be reacted with the N-terminus of the conopeptide to produce fluorescent conjugates. TMR and Bodipy[®] FL were individually conjugated to α -CTx MII using different succinimidyl ester amine-labeling reactions resulting in the formation of carboxamide conjugates. Alexa Fluor[®] 488 succinimidyl ester conjugation reaction yielded low amounts of conjugate. TbCh was also individually reacted with the N-terminus of MII using the isothiocyanate conjugation reaction resulting in the formation of a thiourea conjugate. The conjugates were purified using reverse phase high pressure liquid chromatography (RP-HPLC) and their masses verified by matrix-assisted laser desorption-ionization with time of flight mass spectroscopy (MALDI-TOF MS). When tested on target nAChRs expressed in *Xenopus* oocytes, TMR-MII, Bodipy[®] FL-MII and TbCh-MII potently blocked the response to acetylcholine with slow off-rate kinetics. These fluorescent conjugates can be used to localize specific subtypes of neuronal nAChRs or ligand-binding sites within receptors in various tissue preparations; additionally they may also be used to study conformational changes in receptors using fluorescence or lanthanide-based resonance energy transfer.

Keywords

α -Conotoxins; α -CTx MII; TMR; Bodipy[®] FL; Terbium chelates; Alexa Fluor[®] 488

INTRODUCTION

The venom of *Conus* (cone snail) contains diverse pharmacologically active peptides (1). Conotoxins are disulfide-rich peptide toxins that act at ligand-gated ion channels and voltage-gated ion channels. α -Conotoxins (α -CTxs) are small peptides that competitively inhibit nicotinic acetylcholine receptors (nAChRs) and have been used extensively to study and differentiate specific subtypes of nAChRs. Some α -CTxs also bind with different affinities to the individual acetylcholine-binding sites on nAChRs. α -CTxs are thus ligands of choice to study localization, differentiation and kinetics of specific subtypes of nAChRs (2–5). α -CTxs

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Supporting information available

The Supplementary Material is composed of additional information about (a) synthesis of Alexa Fluor[®] 488-MII and (b) testing of TMR-MII on *X. laevis* oocytes expressing $\alpha 3\beta 2$ nAChRs.

may act on either muscle or neuronal nAChRs (2–5). Most α -CTxs generally have four cysteines, with disulfide connectivities between the first and third cysteines and between the second and fourth cysteines. Based on the number of residues in between successive cysteine residues in a two-loop framework, the α -CTxs that act on neuronal nAChRs are further classified into α 4/7, α 4/3 or α 4/4 framework peptides (5). α -CTx MII, isolated from the venom of *Conus magus*, is a peptide that has 16 amino acid residues with four residues in the first loop and seven residues in the second loop making it a prototypical α 4/7 framework peptide (6). The peptide sequence and characteristic disulfide linkage of α -CTx MII are shown in figure 1. α -CTx MII specifically blocks α 3- and α 6-containing cloned nAChRs expressed in *X. laevis* oocytes (6–9). ^{125}I - α -CTx MII has been used to label α 3- and α 6-containing nAChRs in autoradiography assays (10,11). Striatal α 6-containing nAChRs have been implicated in Parkinson's disease (12,13). Fluorescent analogs of α -CTx MII would thus provide a novel tool to explore the functional neuroanatomy of α 3- and α 6-containing nAChRs in the central nervous system.

Fluorophores are functional fluorescent groups that absorb energy of a specific wavelength and emit the energy at a different specific wavelength. Fluorescence labeling, when used in conjunction with a sensitive imaging device (such as a confocal microscope) provides a direct method to visualize molecular interactions with high precision and resolution. An external energy source (like a laser) excites photons and a photon's energy can be absorbed by a fluorophore creating an excited, unstable electronic singlet state, following which the excited molecules drop to the lowest vibrational energy level within the electronic excited state and then to the ground state. When a fluorophore molecule falls from the excited state to the ground state, the energy of the emitted photon is often emitted at a characteristic wavelength (14). A fluorophore can be chemically attached to a nonfluorescent molecule like a peptide (15) to produce novel fluorescent conjugate molecules (Scheme 1). Tetramethylrhodamine (TMR) (16), Bodipy[®] FL (17) Alexa Fluor[®] 488 (18) and terbium chelates (TbChs) (19) are fluorescent molecules (available as succinimidyl ester, sulfosuccinimidyl ester, carboxylic acid-succinimidyl ester and isothiocyanate respectively) that can be reacted with the N-terminus of the conopeptide to produce fluorescent conjugates (figure 1). Herein we report the successful synthesis and purification of three different fluorescent analogs of α -CTx MII, namely TMR-MII, Bodipy[®] FL-MII and TbCh-MII. The use of conjugation reaction to synthesize fluorescent analogs of conotoxins is particularly appealing because the bond between the fluorophore and peptide is stable to routine storage conditions. The masses of these fluorescent analogs were measured and verified. They were then tested on *X. laevis* oocytes that heterologously expressed rat α 6/ α 3 β 2 β 3 nAChRs.

EXPERIMENTAL PROCEDURES

Materials

TMR, Bodipy[®] FL and Alexa Fluor[®] 488 (Catalog no. C2211, D6140 and A20000 respectively) were obtained from Molecular Probes, Inc. (Eugene, OR). TbChs (Catalog no. P3055) was obtained from PanVera LLC (Madison, WI).

Synthesis and folding of α -CTx MII

α -CTx MII was synthesized as described earlier (6). Briefly, the synthesis was done on an amide resin using Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) chemistry and standard side protection, except on cysteine residues, that were protected in pairs with *S*-trityl on the first and third cysteine residues and *S*-acetamidomethyl on the second and fourth cysteine residues. Following removal from the resin and precipitation, the peptides were folded using a two-step oxidation process using potassium ferricyanide and iodine.

Conjugation reaction

The dye and the conopeptide were mixed in fixed ratios in the presence of an alkaline buffer, like sodium bicarbonate or sodium borate, to enable the conjugation process. The following measures helped in obtaining a higher labeling yield:

- i. The reaction had 3 to 4 molar excesses of dye per toxin peptide.
- ii. The pH of the reaction was kept alkaline (pH range 7.0–8.5).
- iii. The reaction was carried out at room temperature for one hour and stored overnight at 4°C.

Acylating reagents are susceptible to degradation in the presence of water molecules, with the rate increasing as the pH increases. Since most of the buffers do contain water molecules, the three measures listed above help in reducing the degradation of the reactants (14). The photosensitive dye was protected from UV light throughout the experiments (15). There was minimal light exposure during the conjugation reaction and the purification of the conjugated peptides.

RP-HPLC

Analytical reverse phase high pressure liquid chromatography (RP-HPLC) with a diode array detector was used to separate the conjugated products from the unconjugated reactants. The analysis and separation of the conjugated products was done on an analytical RP-HPLC using buffers A (0.1% trifluoroacetic acid) and B (0.092 % trifluoroacetic acid, 60% acetonitrile) on a Vydac C18 column. The samples tested included the unconjugated parent peptide, unconjugated dye and the conjugation reaction mixture. The buffer gradients used to purify TMR-MII, Bodipy® FL-MII, Alexa Fluor®-MII and TbCh-MII were 25% to 60% B60 in 35 minutes, 2% to 95% B60 in 40 minutes, 2% to 70% B60 in 60 minutes and 0% to 80% B60 in 40 minutes respectively at a flow rate of 1 ml/min.

The eluents corresponding to the various peaks in the chromatogram were collected, quantitated and lyophilized. The absorbance of the eluent peak corresponding to the conjugate at 220 nm (A_{220}), 280 nm (A_{280}) and the emission maximum of the fluorophore (A_{EM}) were measured. Following purification of TbCh-MII using RP-HPLC, the terbium center of the chelate was reconstituted by adding 1.1 equivalents of $TbCl_3$ to the purified conjugate.

Mass spectrometry

The conjugation process was verified by measuring the mass of the conjugates using matrix-assisted laser desorption-ionization with time of flight mass spectroscopy (MALDI-TOF MS).

Oocyte expression and electrophysiology

When injected with appropriate β nAChR subunits, rat $\alpha 6$ nAChR subunits do not express successfully in *X. laevis* oocytes (7,9), but they can be functionally expressed as a chimeric receptor with amino acids 1 to 237 of rat $\alpha 6$ nAChR subunit protein linked to amino acids 233 to 499 of the rat $\alpha 3$ nAChR subunit protein (7). cRNA was transcribed *in vitro*, injected in *X. laevis* oocytes and electrophysiological recording using two-electrode voltage-clamp configuration (model OC-725B; Warner Instrument, Hamden, CT) was done as described previously (7). Briefly, 5 ng cRNA of each subunit was injected into stage V *X. laevis* oocytes and incubated at 18° C. After 3 days, the oocytes were voltage-clamped at –70 mV in a 30 μ l cylindrical Sylgard recording chamber perfused with ND96A buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1 μ M atropine and 5 mM HEPES, pH 7.1–7.5) and pulsed with acetylcholine (ACh) every minute. The fluorescent analogs were applied for 5 minutes in static bath followed by monitoring of responses to ACh pulses. Nonspecific adsorption of

peptide was reduced by including 0.1 mg/ml bovine serum albumin in buffer and toxin solutions.

RESULTS AND DISCUSSION

Fluorophores

The use of fluorophores to label ligand peptide molecules produces a novel ligand analog that can functionally help in understanding and visualizing receptor-ligand interactions (14). TMR, Bodipy[®] FL and Alexa Fluor[®] 488 are organic fluorophores; TMR has an absorbance maximum of 555 nm and emission maximum of 580 nm, Bodipy[®] FL has an absorbance maximum of 505 nm and emission maximum of 513 nm and Alexa Fluor[®] 488 has an absorbance maximum of 494 nm and emission maximum of 519 nm. TbChs (a kind of lanthanide chelates) are good alternatives to conventional organic fluorophores because of their distinct structural and spectral characteristics. Structurally, the amine-reactive TbChs used to label peptides are made up of a diethylene triamine pentaacetate (DTPA) chelate with a carbostyryl 124 (CS124) antenna and an isothiocyanate functional group. The CS124-DTPA-based terbium chelates have an absorbance maximum of 340 nm and the emission spectrum has four distinct peaks at 490, 546, 585 and 620 nm. The reaction between the TbCh isothiocyanate and the peptide N-terminus free α -amine results in the formation of a thiourea conjugate.

Conjugation reaction

α -CTx MII is a prototypical α 4/7 α -conotoxin that is composed of 16 amino acid residues. Other important structural characteristics of α -CTx MII are (a) the C-terminal α -carboxyl group of α -CTx MII is amidated, (b) the backbone structure conformation of α -CTx MII is determined by the disulfide bonds, and (c) there are no lysines in the α -CTx MII peptide sequence (6). Amine-reactive fluorescent moieties are ideal functional groups that can be conjugated to the N-termini of small peptides. Most commercially available amine-reactive fluorophore moieties are acylating reagents that form carboxamides, thioureas or sulfonamides upon reaction with amines (15). Amine reactive moieties generally react with (a) the free α -amine at the N-terminus or (b) lysine's ϵ -amino group in a peptide. The free α -amine at the peptide N-terminus usually has a pK_a of ~ 7 making it selective to modification at near neutral pH. If the peptide contains lysine in its sequence, the ϵ -amino group of lysine will also be labeled at pH of 8.5 to 9.5 (14). Succinimidyl and sulfosuccinimidyl esters of dyes are commonly used in conjugation reactions to label peptides because they form carboxamide products containing stable amide bonds between the dye molecules and the peptides. An alternative labeling strategy involves the use of thiol reactive fluorescent moieties, which would be unsuitable for labeling α -CTxs because of the characteristic disulfide connectivities between the cysteines.

Since there are no lysines in the peptide sequence of α -CTx MII, only the N-terminus free α -amine reacts with the succinimidyl esters of TMR and Alexa Fluor[®] 488, the sulfosuccinimidyl ester of Bodipy[®] FL or the isothiocyanate group of TbCh to form fluorescent α -CTx MII analogs. The amount of conjugate yield from the conjugation reaction, using only a few nanomoles of peptide, depends on (i) reaction buffer pH (ii) reaction temperature (iii) duration of reaction and (iv) dye to peptide molar ratio (15). All of the above factors were altered to determine optimal conditions for each conjugation reaction.

Purification and quantification of the fluorescent conjugates

Analysis of the RP-HPLC chromatogram indicated four distinct peaks, two of which corresponded to the unreacted dyes, one of which corresponded to the unreacted toxin and the other peak corresponded to the fluorescent conjugate. The peak areas for the conjugates provide estimates of the efficiency of the labeling reaction. When calculating the concentration of the

fluorescent conjugates, the contribution of the dye to the absorbance of the conjugate (A_{280}) was corrected using published values of correction factors and extinction coefficients for each of the fluorophores. The conjugate yield was 28–40% of the parent peptide added in the reaction. The TMR-MII conjugation reaction was the most efficient with the conjugate yield close to 40% under optimal conditions. The Bodipy[®] FL-MII conjugate was easier to purify because the unconjugated reactants showed distinct peaks on the RP-HPLC chromatogram and could be more discernibly differentiated based on their absorbance analysis (Figure 2). Alexa Fluor[®] 488 conjugation reaction produced a very low conjugate yield that could not be further improved by altering the reaction conditions (see Supplementary Material). The other fluorescent analogs were purified and tested on cloned nAChRs expressed in *X. laevis* oocytes.

Verification of masses of fluorescent conjugates

Since α -CTx MII has a mass of 1710.6 Da (6) and the molecular masses of TMR, Bodipy[®] FL and TbCh are 527.53, 491.2 and 909.14 Da (16,17,19) respectively, the fluorescent conjugates must have masses approximately in the range of 1900–2500 Da (equal to the sum of the mass of a single molecule of each fluorophore and the molecular mass of α -CTx MII minus the mass of the by-product, if present, after accounting for the new bonds being formed during the conjugation reaction). The monoisotopic masses of TMR-MII, Bodipy[®] FL-MII and TbCh-MII were observed to be 2122.52 (calculated 2122.79), 1984.86 (calculated 1984.75) and 2465.17 (calculated 2464.86) respectively.

Activity of the fluorescent conjugates

The lyophilized eluent fractions that were confirmed by MALDI-TOF MS to be fluorescent α -CTx MII conjugates were then tested on rat $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs expressed in *X. laevis* oocytes using two-electrode voltage clamping. All fluorescent analogs of α -CTx MII blocked rat $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs with slow off-rate kinetics similar to that of α -CTx MII (Figure 3). 2 μ M of Bodipy FL-MII was found to be more potent on rat $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs than the same concentration of TMR-MII or TbCh-MII. TMR-MII was further investigated by testing its potency on $\alpha 3\beta 2$ nAChRs expressed in *X. laevis* oocytes (see Supplementary Material). When compared to the parent peptide, the fluorescent analogs of α -CTx MII exhibited ~45–60 fold decrease in affinity towards the target receptors. The fluorescent analogs of α -CTx MII have not exhibited dissociation following long storage times ($n = 60$ days).

Limitations

There are certain limitations in the above described synthesis of fluorescent conjugates of α -CTxs. The conjugation reaction is an efficient method of labeling the N-termini of α -CTxs that have no lysines in their peptide sequences. In the presence of lysine(s) in the peptide sequence of an α -CTx, the lysine can be selectively labeled by increasing the pH of the conjugation reaction buffer. Alternatively, selective deprotection of the lysine residue after fluorophore conjugation could be utilized. If lysine is not critical to toxin activity, a mutant version of the toxin (where the lysines have been substituted by alanines) may be used for labeling. The other limitation in the function of these conjugates is the ~45–60 fold loss in affinity towards the target receptors, which might be overcome by the insertion of a spacer between the fluorophore and the peptide.

CONCLUSION

We have synthesized three different fluorescent analogs of α -CTx MII after optimizing the conditions for each conjugation reaction. These fluorescent analogs have the expected masses and retain activity against target receptors with off-rates comparable to that of the parent peptide. They can be used to localize target neuronal nAChRs including $\alpha 3\beta 2$ and $\alpha 6^*$ nAChRs in the central nervous system.

There may be other applications of these fluorescent α -CTx MII analogs. Both Bodipy[®] FL-MII and TMR-MII are organic fluorophore-conjugated molecules with the emission spectrum of Bodipy[®] FL overlapping the absorbance spectrum of TMR making them an ideal donor-acceptor pair for fluorescence resonance energy transfer (FRET). FRET has been used to quantify distances in the range of 1–10 nm between two fluorophore molecules and study conformational changes following interaction between receptors and ligands (20). TbCh-MII is a conjugate that holds the terbium ion in the chelate and acts as a donor to conventional organic fluorophores like TMR in lanthanide-based resonance energy transfer (LRET) (21). LRET is a modification of the FRET technique that has advantages of reducing problems of background luminescence and accurately measuring larger distance ranges (>10 nm). LRET has been used to study conformational changes in *Shaker* potassium channels (22), interactions of proteins dystrophin and actin in muscle cells (23) and conformational changes following DNA-protein interactions (24).

Fluorescent analogs of α -CTx MII have been used in preliminary studies to localize $\alpha 6$ -containing receptors in specific anatomical regions of mouse brain using confocal microscopy (25,26). Other α -conotoxins could also be fluorescently labeled to increase the number of fluorescent ligand probes for specific subtypes of nAChRs. Thus, fluorescent analogs of α -conotoxins represent a novel class of functional tools that may aid in our understanding of receptor localization and in the study of receptor conformational changes, kinetics and neuroanatomy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. The chemical structures of TMR, Bodipy[®] FL, Alexa Fluor[®] 488 and TbCh fluorophores (left) and the peptide sequence and disulfide connectivity of α -CTx MII (right). The conjugation reaction involves interaction of the fluorophores with the free α -amine of the α -CTx MII N-terminus. **B.** Three dimensional structure of α -CTx MII with fluorophore being conjugated to the free α -amine of the N-terminus of α -CTx MII.

Figure 2. Analysis of the TMR (A), Bodipy[®] FL (B), TbCh (C) conjugation reaction samples at wavelength 220 nm. Black arrows indicate respective conjugates in each HPLC chromatogram.

Figure 3.

Kinetics of toxin block. 2 μ M fluorescent analogs of α -CTx MII were applied to *X. laevis* oocytes expressing rat $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs. TMR-MII (A), TbCh-MII (B) and Bodipy[®] FL-MII (C) at 2 μ M were bath-applied for 5 minutes and the toxin unblock kinetics were monitored by applying 1 sec pulses of ACh every minute. C, control pulses.

Scheme 1.

Experimental design: synthesis of fluorescent analogs of α -CTx MII.