# Synthesis and characterization of an N-terminal-specific $^{125}$I-photoaffinity derivative of $\mu$-Conotoxin GIIIA which binds to the voltage-dependent sodium channel

Stefan Becker[1], Reinhardt Liebe[2] and Robert D. Gordon[1]

[1]*Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Straße 7, D-6000 Frankfurt am Main, FRG* and [2]*Hoechst AG, Abt. Radiochemisches Labor, Postfach 800320, D-6023 Frankfurt am Main, FRG*

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An N-terminal, iodinatable photoaffinity derivative of $\mu$-Conotoxin GIIIA, 4-Azido-salicylyl-$\mu$-Conotoxin GIIIA (CTXASA), was synthesized by solid phase peptide synthesis. The binding of $^{125}$I-CTXASA to the voltage dependent sodium channel from electroplax of *Electrophorus electricus* was specific, as demonstrated by saturation binding experiments. Using autoradiography, $^{125}$I-CTXASA labeled a protein with a molecular mass of 260 kDa, consistent with the apparent molecular mass of the sodium channel. This labeling could be suppressed by excess of tetrodotoxin and $\mu$-Conotoxin GIIIA.

$\mu$-Conotoxin GIIIA; Photoaffinity derivative; Sodium channel

## 1. INTRODUCTION

$\mu$-Conotoxins (also called geographutoxins) are a group of peptide neurotoxins from the piscivorous sea snail *Conus geographus* [1]. They bind to skeletal muscle and electric eel electroplax voltage-dependent sodium channels, but not brain, peripheral nerve or heart muscle sodium channels [2]. These toxins, together with tetrodotoxin (TTX) and saxitoxin (STX), bind to a common site on the sodium channel, thus blocking the sodium ion flux [3]. This binding site is one amongst five different neurotoxin binding sites on the sodium channel [3]. Therefore derivatives of $\mu$-Conotoxins are valuable tools for structure-function investigations at the sodium channel.

In this study we have concentrated on the derivatization of a particular $\mu$-Conotoxin, $\mu$-Conotoxin GIIIA (CTX). Up to now there have been a few reports on derivatives of CTX (also called Geographutoxin 1) [4–6]. The basis behind all these derivatizations was the nonspecific insertion of the derivatizing reagent into the natural toxin. These reports were unable to demonstrate binding to the sodium channel protein. Our group, as others [5], has recently worked out a chemical synthesis of CTX [8]. In this study we have used our synthetic approach to synthesize an N-terminal, specific, iodinatable [16] photoaffinity derivative of CTX, 4-azido-salicylyl-$\mu$-Conotoxin GIIIA (CTXASA). This site-specific photoaffinity derivative of CTX exhibit a highly specific binding to the sodium channel that can be blocked by excess TTX or CTX.

*Correspondence address.* R.D. Gordon, Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Straße 7, D-6000 Frankfurt am Main, FRG

*Abbreviations:* STX, saxitoxin; TTX, tetrodotoxin; CTX, $\mu$-Conotoxin IIIA; CTXASA, 4-azidosalicylyl-$\mu$-Conotoxin IIIA; trityl, triphenylmethyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; tBu, *tert*-butyl; Boc, *tert*-butyloxycarbonyl; NHS-ASA, *N*-hydroxysuccinimido-4-azidosalicylylic acid; HOBT, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; NBS, *N*-bromsuccinimide

## 2. MATERIALS AND METHODS

The following side-chain protection groups were used: Cys, trityl; Arg, Pmc; Hyp, tBu; Lys, Boc; Asp, tBu; Thr, tBu. The peptide amide was assembled on a polystyrene resin with the 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-linker at a substitution of 0.6 mmol/g (Nova Biochem, Switzerland). *N*-Hydroxysuccinimido-4-azidosalicylic acid (NHS-ASA) was purchased from Sigma.

### 2.1. *Synthesis of CTXASA*

Peptide synthesis was performed by Fmoc based solid phase synthesis in a similar manner to our recent protocol for CTX [8] except that we used the unactivated amino acids in 3-fold excess (30 $\mu$mol) with equimolar amounts of the coupling reagents 1-hydroxybenzotriazole (HOBT) and dicyclohexylcarbodiimide (DCC). Both HOBT and DCC were also used for the final coupling of the derivatizing reagent NHS-ASA to the deprotected N-terminus of CTX, which was performed in dim light. The raw peptide was then oxidized as previously described [8] without prior purification of the reduced peptide. The main peak of the oxidation procedure was separated by reversed-phase HPLC on a 250 × 10 mm Vydac $C_{18}$-column (5 $\mu$m particle size, 300 Å pore size) using the same gradient and solvent system as previously described [8]. FABMS of the purified peptide gave a protonated molecular ion at $m/z$ 2769.9 consistent with a calculated weight of 2769.98. The yield of the oxidized, purified peptide as a percentage of the raw peptide was 16.8%.



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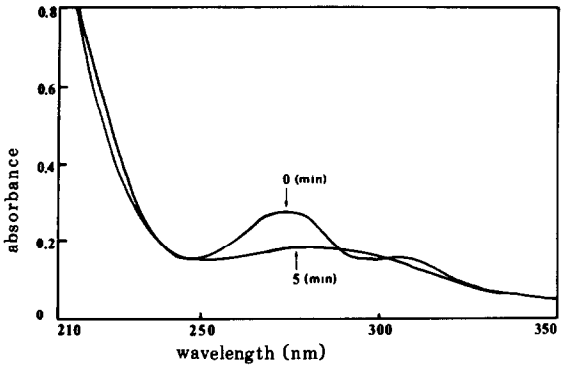


Fig. 1. Spectral change of CTXASA upon photoactivation in aqueous solution at room temperature (10 $\mu$M).

### 2.2. *Iodination*

The iodination of CTXASA was performed according to Sinn et al. [9]. The molar ratio of *N*-bromsuccinimide (NBS)/$Na^{125}I$ was 7.5 with $Na^{125}I$ containing 80 mol% of unlabeled iodine. The molar ratio of CTXASA/$Na^{125}I$ was 5. After a reaction time of 2 min the reaction mixture (44 $\mu$l) was applied on a reversed-phase HPLC column (see Fig. 3). The radioactive peaks following the fraction of noniodinated CTXASA were collected separately and lyophilized.

### 2.3. *Preparation of electric eel membranes and binding assays*

The competitive [$^3$H]STX binding assays were performed as previously described [11]. To characterize $^{125}$I-CTXASA, electric eel membrane vesicles, prepared as in [10], were incubated with $^{125}$I-CTXASA (63.4 Ci/mmol). The incubation medium (500 $\mu$l) consisted of 10 mM Hepes/KOH (pH 7.4), 100 mM choline chloride and 0.1% bovine serum albumin with or without 5 $\mu$M of unlabeled TTX and reacted at 0°C for 30 min. Finally the samples (200 $\mu$l) were rapidly filtered through GF/C filters that had been presoaked in 0.3% poly(ethyleneimine).

### 2.4. *Autoradiography*

Electric eel membrane vesicles (50 $\mu$l) were diluted 5-fold with binding buffer which consisted of 10 mM Hepes/KOH (pH 7.4), 100 mM choline chloride and with or without an excess (20 $\mu$M) of unlabeled TTX or CTX. Each sample contained a 4-fold excess of $^{125}$I-CTXASA as compared to the total binding sites for [$^3$H]STX. After an incubation time of 30 min at 0°C the samples were centrifuged at 35 000 rpm (in TL 100.3 rotor) for 25 min and then washed with the same buffer. After this step the pellet was suspended in 250 $\mu$l of binding buffer and irradiated for 5 min with an XBO lamp (150 W) at a distance of 10 cm. After another centrifugation the pellet was solubilized in 50 $\mu$l of 0.125 M Tris-HCl (pH 6.8), 1% SDS for 30 min. After a final centrifugation at 35 000 rpm the supernatant was applied to a 7.5% SDS PAGE. An autoradiography of the dried gel was developed for 12 h at −70°C using a Kodak X-OMAT AR film with intensifier screen.

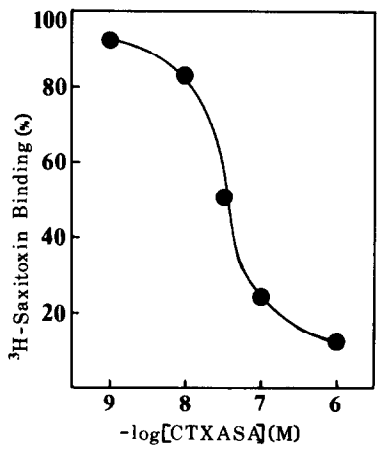


Fig. 2. Inhibition of specific [$^3$H]STX binding (8 nM) to electric eel electroplax membrane by unlabelled CTXASA at various concentrations.

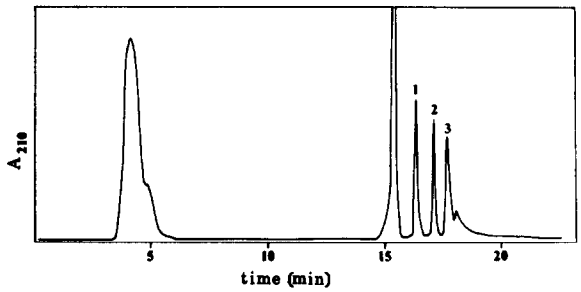


Fig. 3. HPLC chromatogram (Vydac $C_{18}$: 250 mm × 4 mm; 5 $\mu$m particle size; 300 Å pore size) of the iodination mixture immediately after reaction. After a 5 min wash with distilled water/0.05% TFA, elution was achieved by a linear gradient (0–60%) of acetonitrile/distilled water/TFA (55:45:0.05) in distilled water/0.05% TFA. The peak of the unlabelled CTXASA emerged at 15.3 min. Radioactivity was only detected in the three following peaks 1, 2 and 3, the presumptive mono-, di- and triioderivatives. No radioactivity was measured in the broad salt peak at 4 min elution time.

## 3. RESULTS AND DISCUSSION

The purified CTXASA was shown to be a single peak by HPLC and had the correct molecular weight in

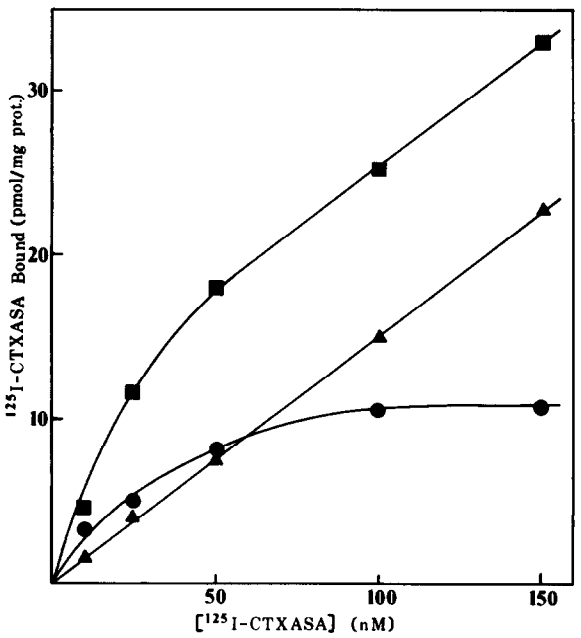


Fig. 4. Binding of $^{125}$I-CTXASA to eel electroplax membranes. Eel electroplax membranes were incubated with various concentrations of $^{125}$I-CTXASA and the bound $^{125}$I-CTXASA was measured. Total binding (■) and nonspecific binding determined in the presence of 5 $\mu$M TTX (▲) are represented. Specific binding (●) was calculated as the difference between total and nonspecific binding.

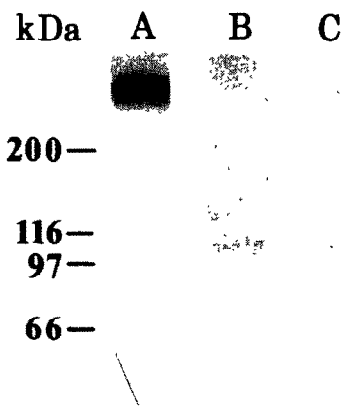


Fig. 5. Analysis of the covalently labeled protein of electric eel membrane vesicles by SDS-PAGE. The vesicles were incubated with $^{125}$I-CTXASA (lane A) alone or with additional 20 $\mu$M CTX (lane B) or TTX (lane C).

FABMS analysis, demonstrating that the 4-azido-2-hydroxysalicylyl group is stable under the conditions of our synthesis and that the derivatized toxin is pure. The spectrum of CTXASA shows a pronounced decrease of the absorption at 275 nm after irradiation (see Fig. 1). Thus the photoaffinity group in the CTXASA is readily photoactivatable. When CTXASA was used to block [$^3$H]STX in binding experiments the $K_i$ of CTXASA as calculated from the $IC_{50}$ was $6 \pm 1.2$ nM (mean $\pm$ SEM, $n = 3$) (see Fig. 2), indicating that this substance binds with high affinity to the sodium channel, comparable to that of the parent CTX and with values from the literature [5,8].

We next labeled CTXASA with $^{125}$I using NBS as a mild oxidizing reagent [9]. The resulting products were separated by reversed phase HPLC (see Fig. 3), delivering three major radioactive fractions. Peak 2 exhibited the highest affinity with a $K_D$ of 37.8 nM and a $b_{max}$ of 13.1 pmol/mg protein (see Fig. 4). These values are in good agreement with those obtained for [$^3$H]propionyl-CTX [4]. We subsequently irradiated the $^{125}$I-CTXASA in the presence of solubilized electric eel membrane protein, separated the proteins by SDS-PAGE and subjected the gel to an autoradiography (see Fig. 5). The experiment showed that only one band in the SDS-PAGE was labeled with a molecular mass of 260 kDa. The labelling of this band could be totally blocked by excess TTX and CTX in the incubation medium. Thus, the iodinated CTXASA specifically binds to the binding site 1 on the sodium channel protein from eel electroplax. This is, to our knowledge, the first report on radiolabelling the sodium channel protein via a binding site 1 complex.

Noda et al. [13] constructed a TTX-insensitive single point mutant of the $\alpha$-subunit by site-directed mutagenesis. In their hypothetical model of the sodium channel $\alpha$-subunit, for which there is now growing evidence [12], the amino acid exchange had taken place on the loop between segments 5 and 6 of domain 1. They interpreted this single amino acid as the binding site of TTX. TTX-insensitive sodium channels from heart muscle and skeletal muscle have now been sequenced [14,15], and the corresponding glutamate residue that was mutated in Noda et al.'s work has been found to be conserved; however, an adjacent asparagine is mutated to arginine. Noda et al. propose that the interaction of the charges on the glutamate residue and the arginine causes TTX insensitivity in heart muscle. It is an attractive hypothesis; however, doubts must surely remain until the appropriate mutants are constructed and the point is proved.

Our approach to label the sodium channel at site 1 with a specific photoaffinity derivative of CTX will allow us to identify this binding site more thoroughly by fragmentation of the labeled sodium channel.

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