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PHOTOACTIVABLE NEUROTOXINS IN STUDYING MEMBRANE RECEPTORS

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Abstract—Seven monomodified photoactivable derivatives of neurotoxin M_{10} from the scorpion *Buthus eupeus* venom and neurotoxin II from the cobra *Naja naja oxiana* venom have been obtained and characterized. By means of these toxin analogs fast sodium channels and the acetylcholine receptor have been studied.

Generation and transmission of nerve excitation is inseparably connected with functioning of specialized transport and receptor systems in the membrane. Nowadays the detailed investigation of the most important components of excitable membranes, ion channels and receptors of neuromediators, is quite urgent. It demands development of effective procedures for identification, isolation and structural analysis of these very complicated membrane systems. One of the most productive ways is the application of natural neurotoxins which selectively interact with certain functionally significant components of the excitable membrane and so disturb the normal process of nerve impulse propagation.[1,2]

Native toxins are suitable as a rule only for pharmacological and electrophysiological experiments. Isolation of some receptors can be performed by using neurotoxin radioactive analogs which are also of great help in binding studies. However, with potential-dependent receptor systems or receptors which are inactivated on solubilization, application of photoactivable toxin analogs is the best way for labeling the membrane receptors.

In addition photosensitive derivatives of polypeptide neurotoxins can be utilized for identification and isolation of receptors, study of their topography, elucidation of the nearest environment of the receptors, etc.

Neurotoxins from the scorpion venom selectively act on the gating mechanism of sodium channels reducing the rate of their inactivation,[3] therefore the receptors of these toxins are considered as functionally important components of the system providing the transmembrane transport of sodium ions across the electrically excitable membrane. Toxic polypeptides selectively blocking the postsynaptic membrane receptors are contained in cobra venoms.[4] These neurotoxins are successfully applied for studying the molecular organization of the choline receptor. In our work the membrane receptors were investigated with the aid of neurotoxin M_{10} from the scorpion *Buthus eupeus* venom[5] and neurotoxin II (NT-II) from the cobra *Naja naja oxiana* venom,[6] their amino acid sequences are shown in Figs. 1 and 2.

RESULTS AND DISCUSSION

To study scorpion neurotoxin receptors the toxin derivative with a photoactivable label in the vicinity of the "active site" of the toxic polypeptide is much desired. To obtain this derivative of neurotoxin M_{10} use was made of 2,4-dinitro-5-fluorophenylazide.[7] The choice of this reagent is due to the fact that it is 2000-fold more active relative to free amino groups than 3-nitro-4-fluorophenylazide usually applied for this purpose. Therefore the modification could be

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                                                    10
VAL-ARG-ASP-GLY-TYR-ILE-ALA-ASP-ASP-LYS-ASP-CYS-
                                        20
ALA-TYR-PHE-CYS-GLY-ARG-ASN-ALA-TYR-CYS-ASP-GLU-
                            30
GLU-CYS-LYS-LYS-GLY-ALA-GLU-SER-GLY-LYS-CYS-TRP-
                    40
TYR-ALA-GLY-GLN-TYR-GLY-ASN-ALA-CYS-TRP-CYS-TYR-
      50                                        60
LYS-LEU-PRO-ASP-TRP-VAL-PRO-ILe-LYS-GLN-LYS-VAL-

SER-GLY-LYS-CYS-ASN
```

Fig. 1.

```
                                        10
LEU-GLU-CYS-HIS-ASN-GLN-GLN-SER-SER-GLN-PRO-PRO-THR-THR-LYS-THR-
            20                                          30
CYS-SER-GLY-GLU-THR-ASN-CYS-TYR-LYS-LYS-TRP-TRP-SER-ASP-HIS-ARG-
                                40
GLY-THR-ILE-ILE-GLU-ARG-GLY-CYS-GLY-CYS-PRO-LYS-VAL-LYS-PRO-GLY-
      50                                        60
VAL-ASN-LEU-ASN-CYS-CYS-ARG-THR-ASP-ARG-CYS-ASN-ASN
```

Fig. 2.

carried out under mild conditions that undoubtedly favoured the preservation of the biological activity of the neurotoxin. From the reaction mixture the monosubstituted derivative of neurotoxin M_{10} (DNPA-M_{10}) was isolated by means of ion-exchange chromatography.

The absorption spectrum of DNPA-M_{10} shows two bands, an intensive one at 355 nm and a weak one at 410–415 nm. According to the data on the amino acid analysis this derivative contains one modified lysine residue per molecule, the N-terminal valine residue being preserved free. In order to localize the modified lysine residue, a synthesis of the neurotoxin analog containing 2,4-dinitroaniline instead of 2,4-dinitrophenylazide was performed. It is reasonable to assume that the similar derivative of toxin M_{10} contains the same modified lysine residue. Modification of the neurotoxin with 2,4-dinitro-5-fluoroaniline was carried out under conditions analogous to those for the DNPA-M_{10} synthesis, and from the reaction mixture a monosubstituted derivative of the toxin with one modified lysine residue was isolated by ion-exchange chromatography. CD spectra of native and modified neurotoxin M_{10} show that the incorporation of 2,4-dinitroaniline is not accompanied by a noticeable change in the structural characteristics of the polypeptide chain. The difference observed in the CD spectra within the region 285–295 nm can probably be assigned to some change of the asymmetric environment of aromatic amino acid residues, mainly of tryptophan caused by the proximity of the incorporated label. Apparently with DNPA-M_{10} the substantial changes also do not occur. Upon investigation of tryptic hydrolysate of carboxymethylated toxin M_{10} containing the residue of 2,4-dinitroaniline it was established that the label is present only in one peptide. The analysis of the structure of this peptide allows to conclude that lysine 57 is a modified residue. Thus the neurotoxin photosensitive derivative probably contains the label on the lysine 57 residue.

Binding of scorpion neurotoxins to electrically excitable membranes is characterized by a high level of nonspecific sorption that apparently depends on the toxin molecule interaction with the membrane lipid matrix. Such an assumption means the existence of a clearly defined hydrophobic region in the toxin molecule localized in fragment 46–56 in case of M_{10}. It seems very important that this fragment contains almost half the conserved amino acid residues. Analysis of the tertiary structure of the variant 3 toxin from the scorpion *Centruroides sculpturatus* showed that practically all conserved residues are clustered together on the surface of the molecule, forming a large continuous patch, and the hydrophobic region is situated in the vicinity of this patch.[8] In neurotoxin M_{10} the lysine 57 resudue is situated near the similar patch of conserved and hydrophobic amino acid residues. Consequently, at the moment of interaction with fast sodium channels the photosensitive toxin analog labeled at Lys 57 should modify mainly receptor components of electrically excitable membranes.

DNPA-M_{10} action on sodium channels was investigated by the voltage clump technique on neuroblastoma cell membranes. Similar to the native neurotoxin DNPA-M_{10} slows down the inactivation of the sodium current and as a rule makes it incomplete.[7] The values of the binding constants for native and modified toxins are $(1.64 \pm 0.4) \times 10^7\,M^{-1}$ and $(0.30 \pm 0.1) \times 10^7\,M^{-1}$, respectively. Therefore, modification of neurotoxin M_{10} results in some decrease of its affinity to Na-channels, i.e. apparently connected with slight decrease of the total positive charge of the whole neurotoxin molecule as well as with the insertion of a quite bulky radical.

The analysis of efficiency for irreversible modification of sodium channels from the neuroblastoma cell membrane under voltage clump conditions lies in checking reversibility of the DNPA-M_{10} effect before and after irradiation. It was established here that photoinduced modification affects the channel fragment responsible for the inactivation process.[7]

The radioactive photosensitive analog of neurotoxin M_{10} was obtained upon DNPA-M_{10} iodination with ^{125}I isotope by means of lactoperoxidase. The modified neurotoxin (^{125}I-DNPA-M_{10}) was separated from impurities by ion-exchange chromatography on CM-cellulose. The radioactive and photosensitive analog of the toxin preserved a high level of biological activity according to electrophysiological tests under voltage clump technique on neuroblastoma cell membranes.[7]

The data on the competition of the photosensitive toxin analog for the membrane receptors of rat brain synaptosomes with native toxin M_{10} were used for determining the portion of its specific binding. Covalent attachment of the modified toxin to the membrane was carried out by irradiation at a wavelength above 320 nm, since, according to electrophysiological data, properties of fast sodium channels were not disturbed under these conditions. According to the obtained results the level of binding of ^{125}I-DNA-M_{10} decreased to 50% with the increase of native toxin concentration (Fig. 3). The apparent equilibrium dissociation constant of the toxin-receptor complex for toxin M_{10} is approx. 10^{-7} M thus closely coinciding with the data on electrophysiological experiments. Under the condition that the ratio of the toxin binding to the receptor occurs in a 1:1 ratio the number of binding sites is within the range of 80–120 fmole/mg of synaptosomal protein.[9]

Toxin II from the sea anemone *Anemonia sulcata* also suppresses binding of ^{125}I-DNPA-M_{10} by synaptosomes (the apparent dissociation constant ~ 200 nm) (Fig. 4). Calcium ions practically completely inhibit specific binding of the photosensitive

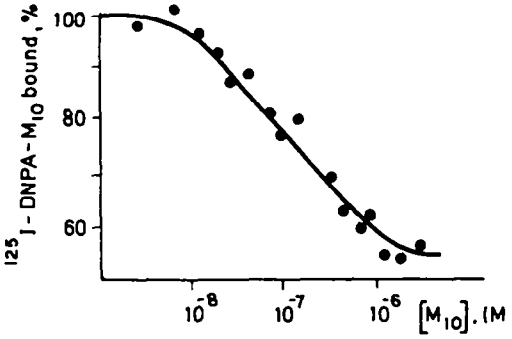

Fig. 3.

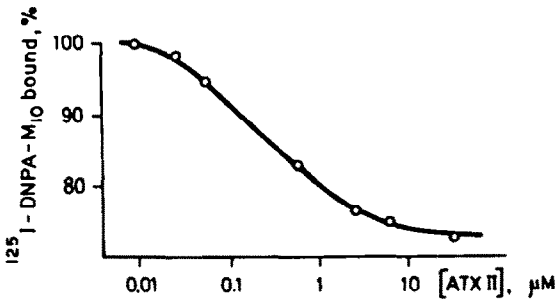

Fig. 4.

analog of the scorpion toxin, within an 0–10 mM range of concentrations but simultaneously increase the level of nonspecific binding of the labeled neurotoxin. Such an effect can be determined by increasing nonspecific interactions of the toxin with the lipid phase, the state of which depends on the presence of calcium ions.

The data obtained clearly indicate a direct attachment of the scorpion toxin photosensitive derivative to the receptor components of fast sodium channels. Hence, it seems possible to characterize receptors of the scorpion neurotoxin as well as to carry out the comparative analysis of these components for membranes from various sources.

The nature of scorpion neurotoxin receptors was revealed by means of electrophoresis of preparations of electrically excitable membranes covalently bound to M_{10} in polyacrylamide gel with sodium dodecylsulfate. Binding membrane components were identified using difference in radioactivity profiles of electrophoregrams obtained in the absence and in the presence of excess of native neurotoxin M_{10} necessary for the suppression of specific binding of the label in the control run. Reproducibility of the results obtained was shown by statistical analysis. Figure 5 shows a densitogram and the corresponding profile of the radioactivity distribution for the specifically bound labeled neurotoxin after electrophoresis of the modified synaptosomes. The main radioactivity zone covers molecular weights up to 20,000 and its level ranges within dozens of pmole/mg of synaptosomal protein. Extraction of modified membranes with acetone or a chloroform–methanol mixture does not decrease noticeably the radioactivity level of this zone. Apparently, within this low molecular weight range the modified toxin, bound to lipid components of the membrane, is contained. Probably it is that type of interaction which determines the high level of nonspecific binding of scorpion toxins to membrane preparations.

At the same time in numerous parallel experiments two binding sites of its photosensitive analog (M.W. 84,000 and 58,000) specifically suppressed by the excess of the toxin were observed. Radioactivity values exceeded more than 3 times the radioactivity base line of the electrophoregram profile. For each of the bands found the binding level of the photosensitive toxin is 10–30 fmole/mg of the protein. So, the total binding of the modified toxin to both components is 20–60 fmole/mg of synaptosomal protein. This value completely corresponds to the binding capacity of scorpion toxin receptors of the membrane, if one keeps in mind that for displacement of ^{125}I–DNPA–M_{10} in the control run we used 50-fold excess of the native toxin, suppressing 50–60% of specific binding of the modified neurotoxin.

If the binding ratio of ^{125}I–DNPA–M_{10} to the receptor is 1:1 and the molecular weight of the neurotoxin analog is 7000, true molecular weights of the found components are 77,000 and 51,000.[9]

In spite of the considerable difference in the protein composition of neuroblastoma cells and synaptosomes, during their photomodification with

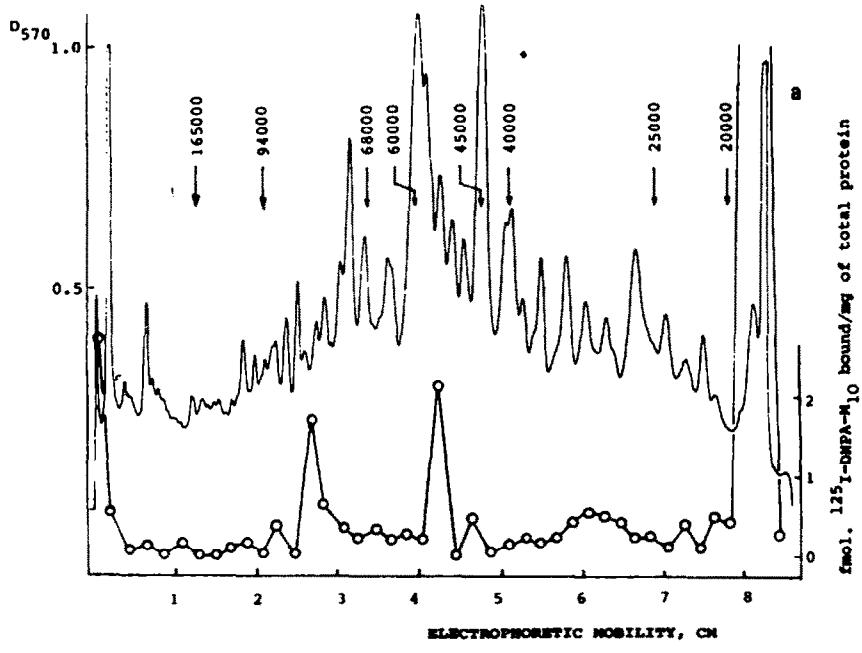

Fig. 5.

^{125}I-DNPA-M_{10} one can also observe the presence of two specifically binding components with molecular weights of 73,000 and 52,000. For neuroblastoma cells of the clone N-18 A5 the overall level of binding of the toxin by both components is 55 fmole/mg of the total protein.[9]

Though binding of scorpion neurotoxins depends on the level of the membrane potential, one can achieve the detected level of covalent modification of depolarized membranes in the presence of high concentrations of the photoaffinity analog under equilibrium conditions. Upon photomodification of plasma membrane preparations from crab nerves two components were observed with molecular weights of 78,000 and 51,000, specifically binding ^{125}I-DNPA-M_{10}.[9] Consequently the availability of two components specifically binding the scorpion toxin is characteristic of excitable membranes of both mammals and crustaceans.

It is noteworthy that given values of molecular weights are "apparent", the measurement error being about 3000 dalton. Therefore, if we assume that the mentioned receptor components are identical for all three types of the investigated membranes, apparent molecular weights of large and small subunits are 76,000 and 51,000, respectively. The modification of these two membrane components can be explained by their spatial proximity relative to the fixation point of the scorpion toxin in the membrane.

At the interaction of the photosensitive analog of the scorpion toxin from the *Leiurus quinquestriatus* venom with rat brain synaptosomes its covalent binding with two proteins of molecular weights 270,000 and 38,000[10] occur. Differences in characteristics of scorpion toxin receptors are difficult to explain now. Noteworthy that neurotoxins from the scorpion venom can possess principally different modes of action,[11,12] and for the toxin from *Leiurus quinquestriatus* the possibility of interaction even with a choline receptor was shown.[13] Further investigations of receptors of various neurotoxins will apparently shed light on structures and functioning of fast sodium channels.

Application of photoactivable neurotoxins pursued different aims in cases of sodium channels and acetylcholine receptor (AchR). Not long ago the views on the subunit composition and molecular weight of AchR were rather contradictory, however, at present these characteristics are exactly known: M.W. ~ 250,000, 5 subunits ($\alpha_2\beta\gamma\delta$). In the membrane these can form the so-called dimeric AchR via a disulfide bond between δ-subunits.[14,15]

To clarify the mechanism of neurotoxin action, it was of interest to establish the regions of their contact with the AchR. In its turn, this could shed light on topography of the corresponding binding site of the AchR.

Earlier in our laboratory a series of derivatives of neurotoxin II from *Naja naja oxiana* (NT-II), each of them containing one spin or fluorescence label, was obtained. Their binding with the purified AchR from electric organs of *Torpedo marmorata* was studied by EPR and fluorescence methods, and multipoint character of the toxin-receptor interaction was established.[16,17] It opens perspectives for the elucidation of AchR topography by using derivatives with one photoactivable group in various regions of the neurotoxin.

In NT-II amino groups (Leu 1, Lys 15, Lys 25, Lys 26, Lys 44 and Lys 46) located in 3 of 4 disulfide-confined loops are suitable points for modification. The conditions elaborated for obtaining mono-derivatives of NT-II at its modification with N-hydroxysuccinimide esters of spin labels[17] were also used to modify this neurotoxin with N-hydroxysuccinimide ester of p-azidobenzoic acid.[18]

Singly labeled *p*-azidobenzoyl derivatives of NT-II were separated by ion-exchange chromatography on Bio-Rex 70 column (Fig. 6). To identify the modified residues, the azide group and S–S bonds were reduced, thus completely escaping intramolecular crosslinking. The reduced carboxymethylated derivatives were subjected to trypsinolysis and the hydrolysates were separated on Sephadex G-25sf. The presence of label in the peptide of the known structure and blocking the cleavage of bonds formed by the corresponding modified lysine residues pointed to the position of the modified residue. In such a way the labels were localized in all 6 derivatives (Fig. 6).

Competition of photoactivable NT-II derivatives and [^{3}H]-acetylated NT-II or toxin 3 *Naja naja siamensis* provided data on their binding (both covalent and noncovalent) to purified solubilized AchR. The experiments demonstrated high efficiency of binding for all the photoactivable derivatives upon their incubation in the dark. Thus, insertion of *p*-azidobenzoyl groups in the indicated positions (Fig. 6) (as well as earlier described insertion of spin labels or acetylation[16]) does not prevent the neurotoxin from binding to AchR.

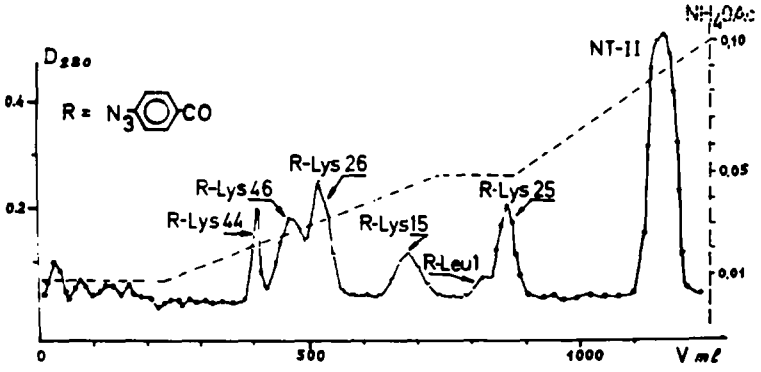

Fig. 6.

Control tests showed no changes in the spectral characteristics of the receptor and its activity under chosen conditions of irradiation.

Upon irradiation all photoactivable derivatives, except one, form crosslinks with AchR with efficiency 70–80%. The derivative modified at lysine 44, which effectively binds to AchR in the dark but forms no covalent bonds upon irradiation, can be considered as an "internal reference" at evaluation of the specificity of the crosslinking. Binding of photoactivable labels located in different parts of the NT-II enables the contact points of neurotoxin with AchR to be identified.

Of interest is comparison of the data on photolabeling with previous spectroscopic results. Scheme 7 summarizes data on the presence (+) or absence (−) of contacts between AchR and the label in the corresponding monolabeled derivative. Spin labels bound to residues Leu 1, Gln 2, Lys 15, Lys 25, Lys 26, His 31 and Lys 46 are attached to AchR with different efficiency, whereas a spin label on Lys 44 in the toxin–AchR complex remains exposed into solution.[17] Dansyl groups on residues Lys 25, Lys 26 and Lys 46 are under direct influence of the AchR groupings; however, dansyl groups in positions Leu 1 and Lys 15 do not contact AchR.[17] So it became clear that amino groups of the Leu 1 and Lys 15 residues can contact AchR; apparently there are regions of multiple contact between AchR and NT-II, for instance, in the region of binding residues Lys 26, His 31 and Lys 46, as well as weaker single contacts (possibly of less functional role), for example, in the region of Leu 1 and Lys 15 binding. For the latter the inserted group can contact AchR or lie outside the receptor molecule.

On the whole, data on photolabeling support results of spectral studies on topography of interacting surfaces of neurotoxin and AchR. This, in particular, concerns evaluation of the binding area size on AchR.[17]

High conformational stability of NT-II and its mono-derivatives towards the pH, polarity and temperature changes suggests that upon binding to AchR neurotoxin molecules do not undergo substantial conformational rearrangements. Consequently, distances between toxin groups interacting with AchR can give an idea about sizes of the binding area of AchR itself. These distances determined by physicochemical investigations in solutions or inferred from the crystal structure of homologous neurotoxin[19] are up to 30 Å. The size of the AchR molecule on the outer membrane surface (where neurotoxins are bound) is roughly 80 Å, so it follows that two neurotoxin molecules cannot be confined within the two α-subunits (as was thought until recently) but should contact the neighbouring subunits. Noteworthy, formation of photoinduced crosslinks with various subunits was observed for the long-type neurotoxins,[20–23] however, the contacting site of the neurotoxin was never identified.

To describe neurotoxin location in the binding site of AchR in detail, one should know what subunits bind photoactivable groupings attached to certain sites of the neurotoxin surface. With this aim in view ^{14}C-analogs of the above photoactivable derivatives were obtained. At present identification of neurotoxin contacts with AchR subunits is carried out using polyacrylamide gel electrophoresis. Radioactive photoactivable derivatives of NT-II can be useful for establishment of the mutual disposition of AchR subunits which is only hypothesized till now.

The examples of application of neurotoxins with photosensitive groups presented in this report illustrate potentialities of photoaffinity modification. A thorough characterization of the structural and biological properties of photoactivable compounds, comparison with the results for related substances having labels of different nature in the same regions of the molecule enables convincing interpretation of the obtained results on the organization of the corresponding receptor systems.

EXPERIMENTAL

Neurotoxin M_{10} *and neurotoxin II* were isolated from the venoms of scorpion *Buthus eupeus*[5] and cobra *Naja naja oxiana*, respectively. Rat brain synaptosomes were obtained according to the known procedure.[24] The acetylcholine receptor was isolated from electric organs of *Torpedo marmorata* by using Triton X-100 and affinity chromatography on NT-II-Sepharose 4B; the obtained preparations (here and further in 0.3 M Na-phosphate buffer, pH 7.5, 0.025% Triton X-100, 0.02% sodium azide) possess specific activity equal to 7–10 nmol of toxin-binding sites per mg of the receptor protein. 2,4-Dinitro-5-fluorophenylazide (DNPA) was obtained by the modified method of Wilson *et al.*[7,25] and

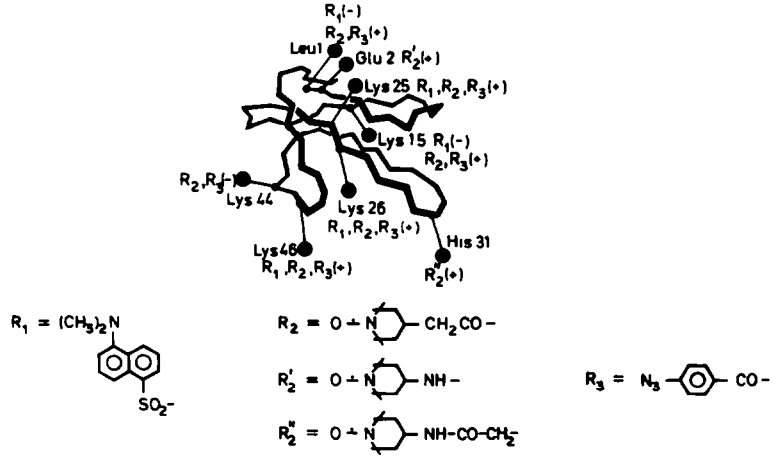

Fig. 7.

N-hydroxysuccinimide ester of *p*-azidobenzoic acid as in the paper.[26]

Preparation of DNPA–M_{10}. To a soln of 0.5 μmole of neurotoxin M_{10} in 0.3 ml of 0.1 M phosphate buffer, pH 8.6, was added 0.51 μmole of DNPA in 35.6 μl of ethanol. The mixture was stirred in the dark at 35° for 12 hr, another 0.51 μmole of DNPA was added, and stirring was continued for another 12 hr at 30°. The mixture was desalted on a column (1.5 × 40 cm) of Bio-Gel P-2 (100–120 mesh) equilibrated with 0.2 M AcOH. The protein fraction was freeze-dried, dissolved in 2 ml of 0.01 M ammonium acetate buffer, pH 7.6, and applied on a column (1 × 6.5 cm) of Bio-Rex 70 (200–400 mesh) equilibrated with the same buffer. Elution was carried out with 30 ml of 0.01 M $AcONH_4$, pH 7.6 and then with a linear concentration gradient (from 0.01 to 0.1 M) of the same buffer. The fraction volume was 15.4 ml and the rate of elution 37 ml/hr. The separation was monitored at 280 nm. The fractions corresponding to a molarity of the buffer of 0.017 M were combined and were freeze-dried twice. The yield of DNPA–M_{10} was 11.5%. UV spectrum (water) λ_{max}: 278, 355, 412 nm.

Iodination of DNPA–M_{10}. A mixture of DNPA–M_{10} (2.8 nmole) in 20 μl of 0.05 M phosphate buffer, pH 7.3, 20 μl (2.5 mCi, 1.6 nmole) of Na ^{125}I ("Izotop", U.S.S.R.), and 2 μl of an aqueous suspension of lactoperoxidase, 10 mg/ml (Boehringer-Mannheim) was stirred and at 1-min intervals 0.68 mM H_2O_2 was added to the mixture (5 × 5 μl). Then it was diluted with 100 μl of 0.05 M $AcONH_4$ buffer, pH 5.5, and was deposited on a polyethylene column containing 3 ml of CM-cellulose CM-32 (Whatman) equilibrated with the same buffer, and the column was washed with 0.05 M acetate buffer, pH 5.5, until the level of radioactivity in the eluate fell to a constant value (~ 30 ml). The detection of the radioactivity of fractions with a volume of about 3 ml was carried out in a "Thyac III" Geiger counter (Victoreen). The ^{125}I–DNPA–M_{10} was eluted in a 0.05–0.3 M linear gradient of ammonium acetate, pH 5.5. The fraction corresponding to the peak of radioactivity was collected and was freeze-dried twice. From the results of measurements on an Ultrogamma gamma counter (LKB) the specific radioactivity of the ^{125}I–DNPA–M_{10} was 38 Ci/mole, without taking into account the efficiency of measurement. To prevent the sorption of the modified toxin on the walls of the vessels, 1 mg of bovine serum albumin (Serva) was added to the radioactive preparation. Unlabeled I–DNPA–M_{10} for testing the biological activity in experiments with the recording of the potential was obtained by the method described above using nonradioactive NaI (os.Ch. ["very pure"], "Soyuzreaktiv") in a molar ratio to the DNPA–M_{10} of 1.5:1. The concentrations of the toxins were determined from the results of the amino acid analysis.

Modification of NT-II *with N-hydroxysuccinimide ester of p-azidobenzoic acid.* To a soln of 69.3 mg (10.3 μmol) of NT-II in 7 ml of 0.2 M AcONa buffer (pH 8.08) with 6 M guanidinium hydrochloride was added 2.87 mg (11 μmol) of N-hydroxysuccinimide ester of *p*-azidobenzoic acid in 0.38 ml of dioxane in the dark; the mixture was stirred for 20 hr at 24° in the dark. The mixture was desalted on a Sephadex G-25 column (3.5 × 60 cm) equilibrated with 0.05 M AcOH at 4° in the dark. The protein fraction was liophilized and chromatographed on a Bio-Rex 70 column. The protein fractions were freeze-dried.

Localization of modified amino acid residues in neurotoxins M_{10} and NT-II. Modification of M_{10} by 2,4-dinitro-5-fluoroaniline, as well as isolation of the respective monoderivatized neurotoxin (DNPAn–M_{10}), were carried out under the same conditions as those used for the DNPA–M_{10} preparation. The yield of DNPAn–M_{10} amounted to 18%. The carboxymethylation and tryptic hydrolysis of this compound were performed as in[5]. The tryptic digest was separated by the method of peptide maps using the Schleicher–Schüll cellulose plates as was earlier done for the M_{10} chymotryptic peptides[5]. Only one spot had a yellow color characteristic of the 2,4-dinitro-5-fluoroaniline modified products. This spot was extracted with 50% aqueous pyridine and, after removing the solvent, the material was subjected to the structural analysis. Thus only one peptide was identified which corresponds to the fragment 50–59 of the M_{10} sequence and, therefore, contains a modified Lys[5] residue.

For localizing the modified residues in the NT-II *p*-azidobenzoyl derivatives (Fig. 6), 0.4 mg (0.06 μmol) of each derivative was dissolved in 1 ml buffer (Tris · HCl, pH 7.5, 8 M urea, 1 mM EDTA) and incubated with 40 mg (260 μmol) of dithiothreitol for 48 hr in the dark. After carboxymethylation with 310 mg (1.68 mmol) of iodoacetic acid at pH 7.5, the mixture was desalted, lyophilized and subjected to trypsinolysis at pH 7.5. The hydrolysates were separated by Sephadex G-25 sf column chromatography (0.4 × 125 cm) in 0.1 M NH_4OAc, pH 4.5. The eluates were monitored by their absorbance at 206 and 280 nm. An increase in the A_{280}/A_{206} ratio, as compared to that for the tryptic hydrolysate of the NT-II, was an indication of a peptide bearing the *p*-aminobenzoyl group. Structural analysis of the labeled peptides provided identification of the modified residues in each derivative. For example, for one of them the labeled peptide **26–32** was isolated, the evidence of Lys 26 modification; with another derivative, the presence of peptide **1–25** in the hydrolysate showed that it was modification of Lys **15** which prevented the peptide chain cleavage at this residue by trypsin.

Influence of M_{10} on the binding of ^{125}I–DNPA–M_{10} to synaptosomes. The investigation of the competition of ^{125}I–DNPA–M_{10} with the native toxin for the receptors in the synaptosome membrane was carried out at 20° in an incubation buffer containing 130 mM choline chloride (Serva), 50 mM HEPES-tris, pH 7.4 (Pierce), 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl (all reagents of os.ch. ["very pure"] grade, Soyuzreaktiv, U.S.S.R.), 0.1% BSA, and 1 μm TTX (Calbiochem). A suspension of synaptosomes in 0.75-ml portions containing 0.625 mg of membrane protein was incubated in the dark with 2.5 nM ^{125}I–DNPA–M_{10} and toxin M_{10} (0–3.5 μM). After 30 min from the addition of the label, each sample was transferred to a QS-115 quarts cell (Gilford) and was irradiated for 10 min a I-18 instrument (LOMO, U.S.S.R.) with a SVD-120 Å mercury lamp, the light being passed through a BS-6 filter.

To separate the unbound toxin, a 300-μl aliquot of the suspension was filtered through glass filters of the GF/3F type (Whatman) in a FH 224v instrument (HSI) at a pressure of 10–15 mm Hg. The filters had previously been incubated overnight at 4° in a solution containing 164 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, and 5 mM HEPES-tris, pH 7.4, and 1% of BSA to decrease the sorption of the label. Before the deposition of the membranes, the filters were washed with 3 ml of buffer containing 161.9 mM choline chloride, 5 mM HEPES-tris (pH 7.4), 1.8 mM $CaCl_2$, 0.8 M $MgSO_4$, 0.55 mM glucose, 0.54 mM KCl, and 0.1% of BSA cooled to 4°. The synaptosomes were washed on the filter with 3 ml of the same buffer three times. The radioactivities of the filters were measured in an Ultragamma gamma-counter (LKB in the ^{125}I channel for 1 min.

Each measurement was performed 2–3 times. The results obtained were calculated in the form of the dependence of the displacement of the bound ^{125}I–DNPA–M_{10} (in %) on the concentration of native toxin M_{10} (Fig. 3).

Analysis of the specifically modified components of the synaptosomes. The covalent modification of the synaptosomes was carried out by the scheme described above in the presence of 6.6×10^{-8} M ^{125}I–DNPA–M_{10}. After irradiation, the suspension was diluted with 7 ml of 5 mM tris-HCl, pH 7.4, containing 5 mM $CaCl_2$. The synaptosomes were lysed by freezing the suspension at −20° with rapid thawing at 35° in a thermostat. The membranes were precipitated by centrifuging at 18,000 rpm, 4°, for 30 min in a JA-21 rotor on a JA-21B centrifuge (Beckman). To eliminate the Ca^{2+} ions the precipitate was

resuspended in 8 ml of 0.32 M sucrose containing 1 mM Na_2EDTA and 5 mM tris-HCl, pH 7.4, and was reprecipitated. A further increase in the number of successive washings did not lead to an appreciable fall in the radioactivity bound to the membrane. On the other hand, when the precipitate was extracted with acetone or with a mixture of acetone and methanol (2:1), less than 0.2% of the radioactivity passed into the solution.

A control experiment was run in parallel using a 50-fold excess of toxin M_{10} to suppress the specific binding of the ^{125}I-DNPA-M_{10}.

The membrane precipitates obtained were resuspended in 0.32 M sucrose containing 5 mM tris-HCl, pH 7.4 so that the concentration of membrane protein amounted to 4 mg/ml. To the sample so obtained was added an equal volume of 125 mM tris-HCl, ph 6.8, containing 20% of glycerol, 4 mM Na_2EDTA, 4% sodium dodecyl sulfate, 10% β-mercaptoethanol (Bio-Rad) and 0.002% of bromophenol Blue (Serva). To dissolve the membranes, the samples were heated in a boiling water bath for 5 min.

The electrophoresis of the solutions obtained was carried out in a Bio-Rad 220 instrument in Laemmli's system[27] in polyacrylamide gel blocks 1.5 mm thick. A 10% separating gel 9 cm high contained 0.375 M tris-HCl, pH 8.8 and 5% concentrating gel 1.5 cm high contained 0.125 M tris-HCl, pH 6.8. Both gels also contained 0.1% sodium dodecyl sulfate, 2 mM Na_2EDTA, and 5% glycerol. In each of the cells of the gel we placed 150–200 μg of membrane protein. Electrophoresis was carried out at 30 mA (60–70 V) for 1.5 hr and then at 50 mA (120–150 V) for 3.5–4 hr. The gel was fixed overnight in 10% AcOH, 10% isopropanol, and it was then stained for 2–4 hr in the same soln containing 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad). The decoloration of the gels was carried out in the fixing soln for 4–6 hr.

To determine mol wts we used the following mixture of marker proteins (mol wts in parentheses): γ-crystallin (20,000, Protein Institute of the Academy of Sciences of the U.S.S.R.), chymotrypsinogen A (25,000), aldolase (40,000), ovalbumin (45,000), catalase (60,000), BSA (68,000), all from Pierce, phosphorylase A (94,000, Sigma) and β'-subunit of *E. coli* RNA-polymerase (165,000, M. M. Shemyakin Institute of Bioorganic Chemistry of the Academy of Sciences of the U.S.S.R.).

Analysis of electrophoretograms of the membranes. The concentrating gels were removed and the separating gels were scanned at 570 nm in a Gilford spectrophotometer and were then cut into 2-mm pieces with a razor-blade slicer (model 120, Bio-Rad). The radioactivity of each piece was measured 4–6 times in a γ-counter for 1 min. From the average values of the radioactivity of each piece of gel found we deducted individually the previously measured background radioactivities of the inserts. The radioactivity profile obtained was drawn in correspondence with the scanning curve and then the radioactivity profile of the control sample was deducted. The radioactivity peaks that, together with the confidence interval, exceeded the background values by a factor of more than 3 were taken as the zone of the membrane components specifically binding the labeled toxin.

Binding of modified neurotoxins to the acetylcholine receptor. To determine acetylcholine receptor activity, to 0.4 ml of the solubilized AchR (concentration 0.1 mg/ml) was added 2–2.5 mg of bovine serum albumin and then 0.05 ml of the radioactive toxin solution (concentration 0.3 mg/ml, specific activity 5300 cpm/μg; the reaction mixture was incubated for 1 hr at room temp, then applied on a Sephadex G-75 column (1.5 × 40 cm), equilibrated with the same buffer, and the toxin-receptor complex was separated from a toxin excess. In order to determine radioactivity of the fractions containing the toxin-receptor complex, aliquotes of 0.4 ml each were taken and measurements were carried out in a Bray scintillator using a radioactivity counter "Intertechnique" SL-30.

To determine the extent of covalent binding, each of two quartz cuvettes was filled with 0.4 ml of solubilized AchR (concentration 0.1 mg/ml). One cuvette was filled with 0.05 ml of a radioactive toxin solution and the other with 0.03 ml of NT-II photosensitive derivative in 2-fold excess with respect to AchR active sites. Both cuvettes were incubated for 1 hr in the dark and then irradiated with a halogen lamp (250 Wt). After irradiation AchR from the reference cuvette was separated from unbound toxin on a Sephadex G-75 column. In the sample cuvette which contained photosensitive derivative, 0.05 ml of radioactive toxin was added, incubated for 1 hr and separated from unbound toxin on a Sephadex G-75 column. Covalent binding extent was found from the decrease in binding of radioactive neurotoxin as compared with the control tests.

Displacement of covalently unbound photosensitive derivatives of NT-II was assessed as follows: to 0.4 ml of an AchR solution (0.1 mg/ml) was added 0.03 ml of a soln containing 9.3 μg of photosensitive neurotoxin deriv, the mixture was incubated in the dark for 1 hr, then 0.05 ml of radioactive toxin (1.5-fold excess relative to the photosensitive deriv) was added, incubated for 1 hr in the dark and separated from the unbound toxin by gel-filtration. Specific activity of the AchR preparations was determined in parallel runs.

REFERENCES

[1]T. Narahashi, *Physiol. Rev.* **54**, 813 (1974).
[2]C. C. Chang, *Handbook of Experimental Pharmacology* (Edited by C.-Y. Lee), Vol. 52, p. 309. Springer-Verlag, New York (1979).
[3]H. Rochat, P. Bernard and F. Courand, *Advances in Cytopharmacology* (Edited by B. Ceccarelli and F. Clementi), Vol. 3, p. 325. Raven Press, New York (1979).
[4]E. Karlsson, *Handbook of Experimental Pharmacology* (Edited by C.-Y. Lee), Vol. 52, p. 159. Springer-Verlag, New York (1979).
[5]E. V. Grishin, L. N. Soldatova, M. I. Shakhparonov and V. K. Kazakov, *Bioorg. Khim. U.S.S.R.* **6**, 714 (1980).
[6]E. V. Grishin, A. P. Sukhikh, N. N. Lukyanchuk, L. N. Slobodyan, V. M. Lipkin, Yu. A. Ovchinnikov and V. M. Sorokin, *FEBS Letters* **36**, 77 (1973).
[7]E. V. Grishin, N. M. Soldatov, Yu. A. Ovchinnikov, G. N. Mozhayeva, A. P. Naumov, A. N. Zubov and B. Ch. Nisman, *Bioorg. Khim. U.S.S.R.* **6**, 724 (1980).
[8]J. C. Fontecilla-Camps, R. J. Almassy, F. L. Sudath, D. D. Watt and C. E. Bugg, *Proc. Natl Acad. Sci. USA* **77**, 6496 (1980).
[9]E. V. Grishin, N. M. Soldatov and Yu. A. Ovchinnikov, *Bioorg. Khim. U.S.S.R.* **6**, 914 (1980).
[10]W. A. Catterall, R. P. Hartshorne and D. A. Beneski, *Toxicon* **20**, 27 (1982).
[11]J. I. Gillespie and H. Meves, *J. Physiol. London* **308**, 479 (1980).
[12]E. Carbone, E. Wanke, G. Prestipino, L. D. Possani and A. Maelicke, *Nature* **90**, 296 (1982).
[13]W. J. Culp and D. T. McKenzie, *Proc. Natl Acad. Sci. U.S.A.* **78**, 7171 (1981).
[14]M. A. Raftery, M. W. Hunkapiller, C. D. Strader and L. E. Hood, *Science* **208**, 1454 (1981).
[15]J. Kistler, R. M. Stroud, M. W. Klymkowsky, R. A. Lalancette and R. H. Fairclough, *Biophys. J.* **37**, 371 (1982).
[16]V. I. Tsetlin, E. Karlsson, A. S. Arseniev, Yu. N. Utkin, A. M. Surin, V. S. Pashkov, K. A. Pluzhnikov, V. T. Ivanov, V. F. Bystrov and Yu. A. Ovchinnikov, *FEBS Letters* **106**, 47 (1979).
[17]V. I. Tsetlin, E. Karlsson, Yu. N. Utkin, K. A. Pluzhnikov, A. S. Arseniev, A. M. Surin, V. I. Kondakov, V. F. Bystrov, V. T. Ivanov and Yu. A. Ovchinnikov, *Toxicon* **20**, 83 (1982).

[18]K. A. Pluzhnikov, A. A. Karelin, Yu. N. Utkin, V. I. Tsetlin and V. T. Ivanov, *Bioorg. Khim.-U.S.S.R.* **8**, 905 (1982).
[19]M. R. Kimball, A. Sato, J. S. Richardson, L. S. Rosen and B. W. Low, *Biochem. Biophys. Res. Commun.* **88**, 950 (1979).
[20]V. Witzeman and M. A. Raftery, *Ibid.* **85**, 623 (1978).
[21]F. Hucho, *FEBS Letters* **103**, 27 (1979).
[22]N. Nathanson and Z. Hall, *J. Biol. Chem.* **255**, 1698 (1980).
[23]R. E. Oswald and J.-P. Changeux, *FEBS Letters* **139**, 225 (1982).
[24]J.-P. Abita, R. Chicheportiche, H. Schweit and M. Lazdunski, *Biochemistry* **16**, 1838 (1977).
[25]D. E. Wilson, Y. Miyata, M. Erecinska and J. M. Vanderkoci, *Arch. Biochem. Biophys.* **171**, 104 (1975).
[26]R. Millon, M. Olomuski, G.-Y. Le Gall, B. Golinska, G.-P. Ebel and B. Ehresmann, *Eur. J. Biochem.* **110**, 485 (1980).
[27]U. K. Laemmli and M. Favre, *J. Mol. Biol.* **80**, 575 (1973).