A photoaffinity ligand of the acetylcholine-binding site predominantly labels the region 179–207 of the α-subunit on native acetylcholine receptor from *Torpedo marmorata*

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Regions of the *Torpedo marmorata* acetylcholine receptor (AChR) α-subunit involved in the binding of acetylcholine were probed with two different covalent ligands. The sulfhydryl-directed affinity reagent 4-(*N*-maleimido)phenyltrimethylammonium iodide labeled a single α-subunit cyanogen bromide fragment on the reduced AChR which was identified as α 179–207. The novel photoaffinity ligand *p*-(*N*,*N*-dimethylamino)-benzenediazonium fluoroborate, on the other hand, labeled three distinct α-chain cyanogen bromide fragments on the unmodified AChR in a carbamylcholine-protectable manner. The major radiolabeled species was purified and identified by sequence analysis as α 179–207. The acetylcholine-binding site on the native AChR may thus involve several distinct portions of the α-chain, with the region α 179–207 making a major contribution to the site.

Acetylcholine receptor *Photoaffinity labeling* *Protein structure* *Acetylcholine-binding site* *Aryldiazonium salt*

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) mediates the permeability response to acetylcholine (ACh) at the vertebrate neuromuscular junction and the electromotor synapse of *Torpedo* fish. The minimal functional unit is a heterologous pentamer of four different polypeptide chains ($\alpha_2\beta\gamma\delta$) which carries two binding sites for ACh located, at least in part, on the two α-chains, and contains the agonist-gated ion channel (review [1]).

The complete primary structures of the four AChR polypeptides in *Torpedo californica* [2,3], of the α-chain in *T. marmorata* [4] and of various chains in several other species (review [5]) have been elucidated by DNA cloning and sequencing. A major goal of current research on the AChR is to localize functional domains within the known amino acid sequence of the constituent polypeptide chains. One approach consists in the labeling of pharmacological sites using appropriate covalent ligands and the identification of the modified amino acids by protein chemical techniques (e.g. [6,7]).

Abbreviations: HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid

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In the case of the ACh-binding sites, the reduction of a disulfide bond in the vicinity of the tetraalkylammonium-binding site permits labeling of the α-subunit by cholinergic affinity ligands specific for sulfhydryl groups [8]. Employing one such ligand, 4-(*N*-maleimido)benzyltrimethylammonium iodide (MBTA), Kao et al. [6] were able to demonstrate that the residues Cys 192, and possibly Cys 193, represent the sites of incorporation of this class of affinity ligands on the reduced AChR. Owing to the limited reactivity of such reagents, however, it is unclear to what extent the region surrounding Cys 192 and 193 participates in the binding of ACh in the native AChR. Furthermore, reduction of the disulfide bond which links these consecutive Cys residues [9] produces marked alterations in the affinity and selectivity of the AChR for cholinergic ligands [8,10].

In order to probe in greater detail the structure of the ACh-binding site on the native AChR, we employed a recently developed photoaffinity ligand *p*-(*N,N*-dimethylamino)benzenediazonium fluoroborate (DDF) [11], a stable derivative of a previously described cholinergic affinity reagent [12,13]. Photolabeling experiments using [^{3}H]DDF recently permitted the identification of a peptide fragment localized in the tetraalkylammonium-binding site on *Electrophorus electricus* acetylcholinesterase [14]. As described in a separate report (submitted), DDF can also be used to label the ACh-binding sites on the AChR. In the dark, DDF behaves as a reversible competitive antagonist of the AChR but irreversibly blocks the binding of cholinergic ligands following irradiation at 295 nm. Under appropriate conditions, labeling of the native AChR by [^{3}H]DDF is associated predominantly with the α-subunit and is protected by cholinergic agonists and antagonists.

Here, we have analyzed the regions of the α-subunit labeled by [^{3}H]DDF via the ACh-binding sites on native AChR. The resulting pattern of labeling was compared with that obtained for the sulfhydryl-specific affinity reagent 4-(*N*-maleimido)phenyltrimethylammonium iodide ([^{3}H]-MPTA) on reduced AChR. Our findings indicate that [^{3}H]DDF is incorporated at multiple sites on the α-subunit, but that the majority of the photolabeling is associated with a fragment containing the residues Cys 192 and 193 labeled by [^{3}H]MPTA.

2. EXPERIMENTAL

Methyl[^{3}H]DDF (1 Ci/mmol) was synthesized and purified as in [15]. Methyl[^{3}H]MPTA (10 Ci/mmol) was obtained from the Commissariat à l'Energie Atomique (Saclay) and phencyclidine was generously provided by A. Jaganathen (Université Louis Pasteur, Strasbourg). Native and ^{125}I-labeled α-bungarotoxin were from Boehringer/Mannheim and Amersham, respectively, carbamylcholine from Sigma and CNBr from Kodak. Live *T. marmorata* were provided by the Biological Station of Arcachon (France).

AChR-rich membrane fragments were purified and alkali-treated as described [16,17]. For affinity alkylation with [^{3}H]MPTA, the membranes (1 μM α-toxin sites) were preincubated for 45 min under nitrogen at 22°C with 0.3 mM dithiothreitol in 15 mM Tris-HCl, 150 mM NaCl, 1.5 mM EDTA, pH 8.0. After reduction, the pH was adjusted to 7 by addition of 0.5 M sodium phosphate buffer, pH 6.7, and isotopically diluted [^{3}H]MPTA (0.5 Ci/mmol) added to a final concentration of 5 μM; a control aliquot of this mixture had been preincubated with 7 μM α-bungarotoxin. Following 10 min at room temperature, the reaction was quenched with β-mercaptoethanol and the membranes washed by centrifugation.

Photolabeling of AChR-rich membranes with [^{3}H]DDF was performed as described in [15] with the following modifications. Membranes (1 μM α-toxin sites in 10 mM sodium phosphate buffer, pH 7.0) were preincubated for 30 min at 10°C with 100 μM phencyclidine to block the high-affinity binding site for noncompetitive blockers [15]. [^{3}H]DDF was then added to a final concentration of 0.2 mM; control incubations involved a 30 min preincubation with 100 μM carbamylcholine. The suspensions (0.5 ml) were irradiated with monochromatic light (295 nm) for 30 min at 10°C with mixing and then the membranes washed by centrifugation. Based on the number of toxin-binding sites measured [18] before and after labeling, [^{3}H]MPTA ad [^{3}H]DDF were incorporated into approx. 60 and 50%, respectively, of the available ACh-binding sites.

The labeled membranes were solubilized in SDS sample buffer [19], aliquots were run on analytical slab gels (10% polyacrylamide/0.13% bisacryl-

amide) and the incorporation of radioactivity into the AChR subunits quantified as in [20]. The remainder was subjected to preparative SDS-polyacrylamide gel electrophoresis [21] and the α-subunit cut out and eluted from the gels [7]. Following dialysis, lyophilization, carboxymethylation and acetone precipitation [7], the purified α-chain was dissolved in 70% formic acid and cleaved with CNBr [22].

The HPLC system used for peptide fractionations was from Waters and was equipped with an Anacomp controller/data module (Kontron) and a Valco injector. Gel-permeation HPLC was performed on two TSK 125 columns (Bio-Rad) connected in series and run with 8 M urea/10% acetic acid. Reversed-phase HPLC was done on a Waters μbondapak C_{18} column (3.9 × 300 mm) eluted with gradients of 90% 1-propanol in either 0.1% TFA or 0.13% HFBA.

Purified CNBr fragments were subjected to automated Edman degradation on a gas-phase sequenator (Applied Biosystems) [23]. Aliquots of each cycle were used for PTH-amino acid identification by HPLC [24] or for radioactivity measurement.

3. RESULTS

Batches of AChR-rich membrane fragments (approx. 100–200 nmol α-toxin-binding sites) were either photolabeled with [^{3}H]DDF, or labeled with [^{3}H]MPTA after dithiothreitol treatment. The AChR α-chain, which in both cases incorporated the radioactivity predominantly, was then purified by preparative polyacrylamide gel electrophoresis. Addition of specific ligands for the ACh-binding site markedly reduced the labeling of this particular chain; the incorporation of [^{3}H]DDF was inhibited by 75% in the presence of 100 μM carbamylcholine, while that of [^{3}H]MPTA was decreased 90% by 7 μM α-bungarotoxin.

The distribution of these two compounds within the α-chain was compared on radioactive peptide maps. Analysis of small aliquots of the total CNBr digests of purified labeled α-chains by reversed-phase HPLC yielded the radioactivity profiles shown in fig.1. A major radioactive peak eluting between 35 and 39 min represented 54% of the eluted [^{3}H]DDF-labeled material (II in fig.1A), the remainder being distributed mainly in two peaks

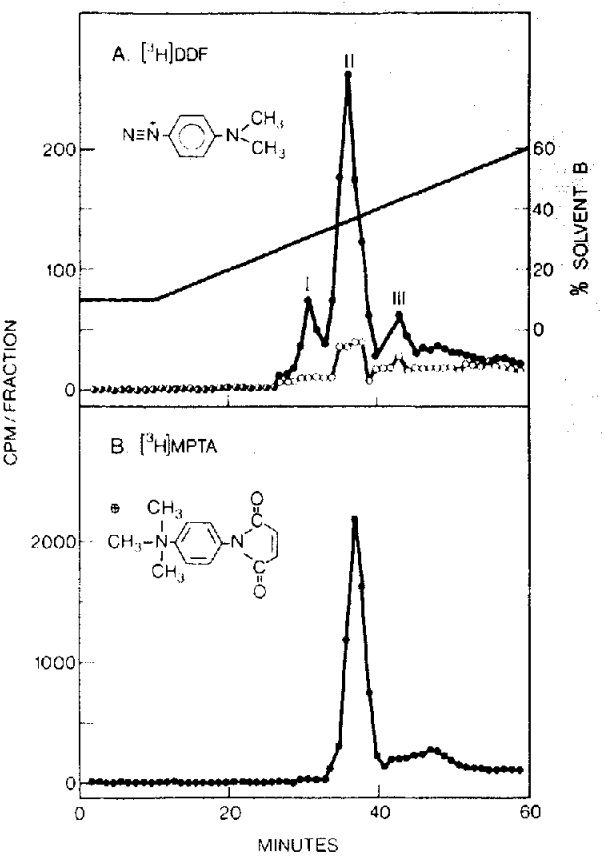

Fig.1. Analytical peptide maps of [^{3}H]MPTA- and [^{3}H]DDF-labeled α-chain CNBr digests. Aliquots of the total CNBr digests of labeled α-chain in 70% formic acid were diluted with 0.1% TFA/10% 1-propanol and subjected to reversed-phase HPLC using the gradient of solvent B (90% 1-propanol/0.1% TFA) shown in A at a flow rate of 0.7 ml/min. Radioactivity in 0.7 ml fractions was measured by liquid scintillation counting. The formulae of the covalent ligands employed are shown as insets. (A) Radioactivity profiles observed with CNBr digests of equivalent amounts of α-chain labeled by [^{3}H]DDF in the absence (●) and presence (○) of 100 μM carbamylcholine. I–III denote peaks of specific labeling by [^{3}H]DDF. In both cases, the recovery of injected radioactivity was 66%. (B) Radioactivity profile observed with CNBr digest of [^{3}H]MPTA-labeled α-chain. The recovery of injected radioactivity was 75%.

eluting between 29 and 32 min (I) and 41–45 min (III). The incorporation of [^{3}H]DDF into peaks I–III was markedly reduced (by 65–80%) when photolabeling was performed in the presence of carbamylcholine (open circles in fig.1A); [^{3}H]DDF

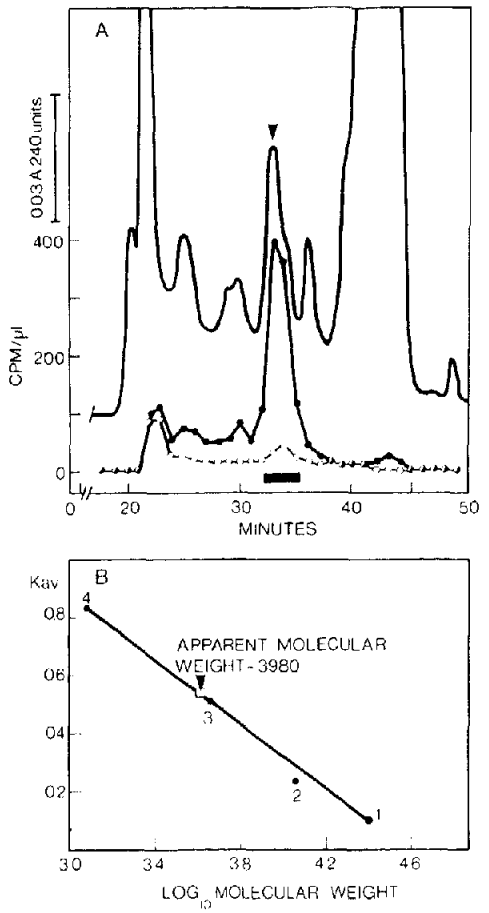

Fig.2. Preparative gel permeation HPLC of the CNBr digest of [^{3}H]DDF-labeled α-chain. (A) The dried CNBr digest was solubilized in 8 M urea/10% acetic acid (approx. 1 mg protein/ml) and 200 μl aliquots subjected to gel permeation HPLC at a flow rate of 0.5 ml/min as described in section 2. The absorbance of column eluates was monitored on line at 240 nm (solid line). Radioactivity in the fractions (0.5 ml) was measured in aliquots (2 μl) by liquid scintillation counting (•). Radioactivity in eluate fractions for the carbamylcholine-protected batch analyzed in parallel (○) have been normalized to equivalent amounts of protein. The bar denotes fractions pooled for further purification. (B) Calibration curve for the gel permeation HPLC system. The K_{av} was calculated as $(V_t - V_e)/(V_t - V_0)$, where V_t, V_0 and V_e represent elution times for the total volume (dinitrophenyllysine), void volume (bovine serum albumin) and sample, respectively. K_{av} is plotted vs $\log_{10}$ M_r for the standard proteins: (1) chymotrypsinogen A (M_r 25000); (2) ribonuclease A (M_r 14000); (3) insulin (M_r 5700); and (4) gramicidin S (M_r 1100). The arrow denotes the K_{av} observed for the major [^{3}H]DDF-labeled peak.

thus appeared to label specifically various α-chain CNBr fragments. With [^{3}H]MPTA-labeled material, on the other hand, a single peak of radioactivity was observed (fig.1B) which eluted in the same region of the chromatogram as the predominant [^{3}H]DDF-labeled component (II in fig.1A). To establish whether these radioactive species corresponded to the same peptide, they were purified to homogeneity and sequenced.

Preparative purification of the major [^{3}H]DDF-labeled species (II in fig.1A) was achieved by a combination of gel permeation and reversed-phase HPLC. Size fractionation of the α-chain CNBr digest solubilized in urea/acetic acid resolved numerous UV-absorbing species (fig.2A). The majority of the [^{3}H]DDF-labeled material eluted with an apparent molecular mass of 4 kDa (fig.2B). The carbamylcholine-protected sample showed a 75–80% reduction in the extent of [^{3}H]DDF labeling of this peak. The corresponding fractions were pooled and subjected to reversed-phase HPLC in 0.1% TFA (fig.3A). Two [^{3}H]DDF-labeled components, corresponding to peaks I and II in the analytical peptide maps (cf. fig.1), were resolved; labeling of both was reduced by carbamylcholine. The fractions containing the principal radioactive peak (II) were pooled and rechromatographed on the same column using 0.13% HFBA as counterion. In this last system, a symmetrical peak of carbamylcholine-protectable radioactivity corresponding to a well resolved peak in the absorbance profile was observed (fig.3B). The radioactivity associated with this purified [^{3}H]DDF-labeled species represented approx. 5% of that present in the uncut α-chain used. This value reflects the recoveries of radioactivity for the purification procedures (approx. 75% for each HPLC system) as well as the proportion of the α-chain labeling associated with this major radioactive component (approx. 50%).

Preparative purification of the [^{3}H]MPTA-labeled peptide was performed in parallel following exactly the same procedure. In all three HPLC steps, the [^{3}H]MPTA-labeled material yielded absorbance profiles identical with those in figs 2 and 3, but a single radioactive component which coeluted with the major [^{3}H]DDF-labeled species (not shown). The purified fragment contained approx. 10% of the initial [^{3}H]MPTA radioactivity present in the α-chain.

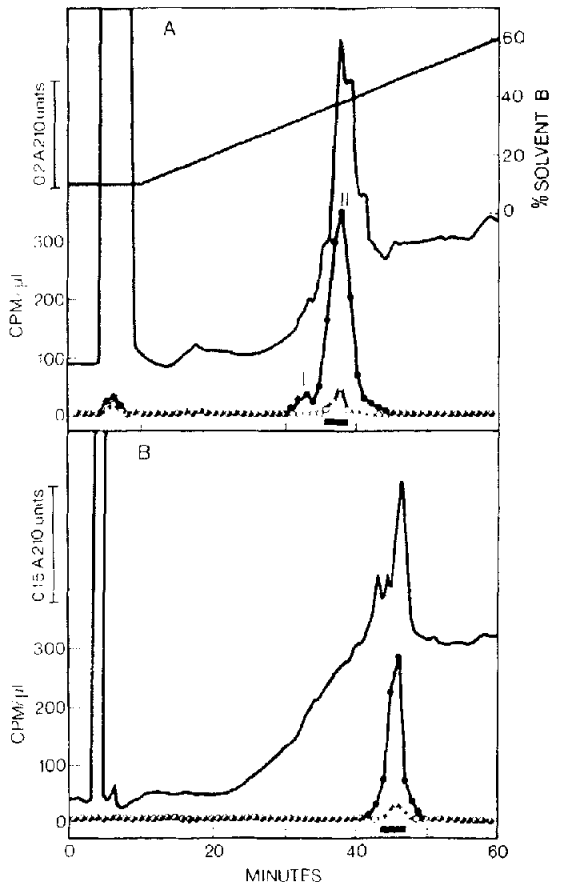

Fig.3. Purification of the major [^{3}H]DDF-labeled CNBr fragment by reversed-phase HPLC. (A) Fractions containing the major [^{3}H]DDF-labeled peak in fig.2 were subjected directly to reversed-phase HPLC in 0.1% TFA and eluted with the indicated gradient of solvent B (90% 1-propanol/0.1% TFA) at a flow rate of 0.7 ml/min. Absorbance of column eluates was monitored on line at 210 nm (solid line) and radioactivity measured in 2 μl aliquots of 1 min fractions (•). Radioactivity in eluate fractions for the carbamylcholine-protected batch (○) has been normalized to equivalent amounts of protein. The bar indicates fractions pooled for repurification. (B) Peak fractions from A were pooled, diluted with an equal volume of 0.13% HFBA and subjected to reversed-phase HPLC using 90% 1-propanol/0.13% HFBA as solvent B and the gradient indicated in the upper panel. Details are as described in A.

Following a final HPLC run in the 0.1% TFA HPLC system, the purified [^{3}H]MPTA- and [^{3}H]DDF-labeled components were subjected separately to sequence analysis (fig.4). In the case

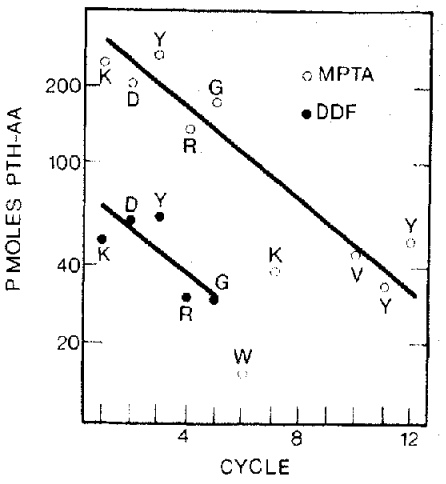

Fig.4. Sequence analysis of [^{3}H]MPTA- and [^{3}H]DDF-labeled CNBr fragments. The major [^{3}H]DDF- and [^{3}H]MPTA-labeled CNBr fragments purified by HPLC were dissolved in 50% formic acid and then loaded onto glass-fiber filters which had been pretreated with polybrene and precycled 5 times before sequencing on the gas-phase sequenator. Yields of PTH-amino acids observed upon sequence analysis of the [^{3}H]MPTA- (○) or of the [^{3}H]DDF- (•) labeled fragments are plotted against cycle number. Repetitive yields of approx. 80% were calculated by linear regression analyses of the data (solid lines).

of the [^{3}H]MPTA-labeled fragment, PTH-amino acids could be identified up to cycle 12. A unique amino-terminal sequence was observed which, by comparison with the known sequence of the α-subunit from *T. marmorata* [4], corresponded unambiguously to a CNBr fragment extending from Lys 179 (cleavage after Met 178). Measurement of the radioactivity released on each cycle up to residue 19 revealed a clear release of tritium at cycles 14 and 15 (not shown), corresponding to Cys 192 and Cys 193 of the α-subunit; these residues are thus labeled by [^{3}H]MPTA. This result confirms the original observation of Kao et al. [6] which demonstrated that α Cys 192, and possibly Cys 193, of *T. californica* were labeled by the closely related [^{3}H]MBTA.

The purified [^{3}H]DDF-labeled fragment exhibited the same unique amino-terminal sequence beginning at Lys 179 (fig.4); no release of radioactivity was observed for the five sequence cycles yielding identifiable PTH-amino acids. Based on its apparent molecular mass of 4 kDa (fig.2) this

radioactive peptide most probably extends to the next possible CNBr cleavage site at Met 207 (calculated molecular mass = 3687 Da). Photolabeling by [^{3}H]DDF of the ACh-binding site on the native receptor thus occurs predominantly in the region α 179–207, which contains the residues Cys 192–Cys 193 labeled by [^{3}H]MBTA and [^{3}H]MPTA on the reduced receptor.

4. DISCUSSION

The regions of the AChR α-subunit involved in the binding of cholinergic ligands were probed in the present study using the novel photoaffinity ligand [^{3}H]DDF. This reagent offers significant advantages over the sulfhydryl-directed affinity markers in that: (i) it covalently labels the ACh-binding site in the native AChR without prior reduction, a modification known to produce marked changes in the conformation of this site [8,10]; (ii) the photogenerated aryl cation can react with any functional groups located in the immediate vicinity [14]; and (iii) the photolabile diazonium moiety may interact directly with the residue(s) forming the tetraalkylammonium-binding site [25].

Comparative peptide mapping of the [^{3}H]MPTA- and [^{3}H]DDF-labeled α-subunit indicated differences in the distribution of the two markers within this chain. Whereas the incorporated [^{3}H]MPTA was associated with a single α-chain CNBr fragment, [^{3}H]DDF appeared to label three distinct fragments in a carbamylcholine-sensitive manner.

Purification and amino-terminal sequence analysis of the predominant [^{3}H]DDF-labeled fragment permitted its identification as the segment α 179–207. This fragment contains the Cys residues 192 and 193 labeled by [^{3}H]MBTA [6] and [^{3}H]MPTA (this study) on the reduced AChR. It has also been reported that synthetic peptides corresponding to this region of the α-chain can bind α-toxins [26–28], albeit with low affinities and in an agonist-insensitive manner. The present finding that the majority of the incorporated [^{3}H]DDF was associated with the fragment α 179–207 shows that residues located within this region are actually involved in the binding of cholinergic ligands in the native AChR.

The identities of the two additional [^{3}H]DDF-labeled components were not determined in this study. Preliminary characterization of one of them (peak I), however, indicates that it is not a subfragment of the major radiolabeled species. The specific incorporation of [^{3}H]DDF into these two additional species suggests that regions of the α-chain distinct from that containing Cys 192 and 193 may participate in the binding of cholinergic ligands. Evidence from α-toxin-binding experiments supports this idea [29] though the findings are controversial [30].

Further characterization of the [^{3}H]DDF-labeled fragments, in particular the identification of the precise amino acids modified by this reagent, is currently in progress. These experiments should provide a more detailed view of the structure of the ACh-binding site in its native conformation.

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