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AMINO TERMINAL AMINO ACID SEQUENCE OF THE MAJOR POLYPEPTIDE SUBUNIT OF TORPEDO CALIFORNICA ACETYLCHOLINE RECEPTOR

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SUMMARY The amino terminal sequence of the 40,000 dalton polypeptide subunit of Torpedo californica acetylcholine receptor has been determined for twenty-five cycles using automatic microsequencing procedures. The results demonstrate a unique polypeptide sequence for this receptor subunit and quantitation of amino acid recoveries shows that no significant amounts of polypeptides with blocked amino terminii are present.

INTRODUCTION

The AcChR from electroplax membranes has been purified from several species (1-8) of electric ray as well as from electroplax of electric eel and denervated muscle (see 9 for a review). Torpedo californica AcChR has been shown to be composed of four polypeptides both in membrane bound form (10) and in purified form obtained by detergent extraction of such membranes followed by affinity chromatography (10,6). The apparent molecular weights of the four polypeptides are 40, 50, 60 and 65 $\times 10^3$ daltons, with the 40,000 dalton species being the most abundant.

This polypeptide (Subunit I) has also been shown to contain the binding site for cholinergic antagonists (6,13) and agonists (7,14,15) using affinity labeling methods. In addition the neurotoxin α-BuTx has been shown to

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Abbreviations: AcChR, acetylcholine receptor; α-BuTx, α-Bungarotoxin; HPLC, high pressure liquid chromatography; Pth, phenylthiohydantoin; SDS, sodium dodecyl sulfate.

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interact with this same subunit (6,13). However, the number of α-toxin binding sitesis exactly two-fold higher than that for the affinity reagents (6,13-15). This raises the question as to whether Subunit I as observed on SDS polyacrylamide gel electrophoresis is actually composed of equal numbers of two polypeptides of equal or close to equal molecular weights.

In this communication we present evidence that Subunit I a unique polypeptide by virtue of determining the first 25 amino acids of its primary sequence from the amino terminal using previously published microsequencing procedures (21).

MATERIALS AND METHODS

Preparation of AcChR:

Membrane fragments enriched in AcChR were prepared from Torpedo californica electroplax by the method of Elliott et al (17). All proteins except for the AcChR were removed by base treatment, essentially as described previously (17,18). Membrane fragments were diluted to 1-2 mg/ml in H_2O and titrated to pH 11 with NaOH. After stirring for 1 hr at 4°C, the membranes were centrifuged at 45,000 xg for 45 min. The supernatant and the loose, soft pellet were removed and the small hard pellet resuspended in 5 mM Tris. After readjustment to pH 11, the centrifugation step was repeated and the hard pellet resuspended in a small volume of 10 mM Tris, pH 7.4, giving a preparation containing approximately 4 nmol α-BuTx sites per mg protein.

Purification of the 40,000 Dalton Subunit:

The base-treated membrane fragments were heated in gel sample buffer (5% β-mercaptoethanol, 3% SDS, 10% glycerol in 0.125 M Tris, pH 6.8) and electrophoresed according to Laemmli (19) on a preparative slab gel measuring 11 cm × 13.5 cm × 0.6 cm, with a 7.5% acrylamide, 0.2% methylene-bis-acrylamide separating gel and a 3% acrylamide, 0.08% bis-acrylamide stacking gel. Electrophoresis was at 20 mA for 18 hrs, after which the gel was stained in 0.25% Coomassie brilliant blue, 25% methanol, 10% acetic acid for 5-10 min. A narrow horizontal band at the position of the 40,000 dalton subunit was cut from the gel and the protein removed by electroelution into a dialysis bag in Tris-glycine buffer containing 0.1% SDS at 5 mA/tube for 18 hrs. The protein solution was dialyzed against several changes of 0.15 M NaCl, 0.02% SDS and then against 0.02% SDS in water for 48 hrs, after which it was lyophilized, and its purity confirmed by re-electrophoresis.

For amino acid analysis, samples were hydrolyzed in 6 N Hcl for 22 hrs at 110°C, dried under N_2, and analyzed on a Durrum D-500 amino acid analyzer.

N-Terminal Amino Acid Sequence Analysis:

The spinning cup sequenator used for the Edman degradation was designed and built at Caltech and will be described in detail elsewhere (M. Hunkapiller and L. Hood, submitted to Science). It incorporates many of the design features described by Wittmann-Liebold (20) and M. Hunkapiller and L. Hood (21) with further refinements in the delivery head assembly and reagent/solvent reservoir system, and is equipped with a straight-edge cup from Beckman Instruments.

The sequenator program was similar to that described previously (21). It included a 25 min coupling step using 0.17 m Quadrol buffer, a single 5 min cleavage step, and automated conversion of the amilinothiazolinones to Pth amino acids by treatment with 25% aqueous trifluoroacetic acid for 40 min. The cup and conversion flask were maintained at 52°C.

Polybrene (Aldrich, 6 mg) and glycylglycine (100 nmol) dissolved in 0.5 ml of distilled water were loaded into the sequenator cup and subjected to 8 complete degradation cycles. The 40K subunit (1-10 nmol dissolved in 0.5 ml distilled water plus SDS) was then loaded and the sequencing program commenced with the coupling stage. Pth amino acids were identified by high performance liquid chromatography on a DuPont Zorbax CN column. Details on identification of Pth amino acids and standard chromatograms have been described elsewhere (22).

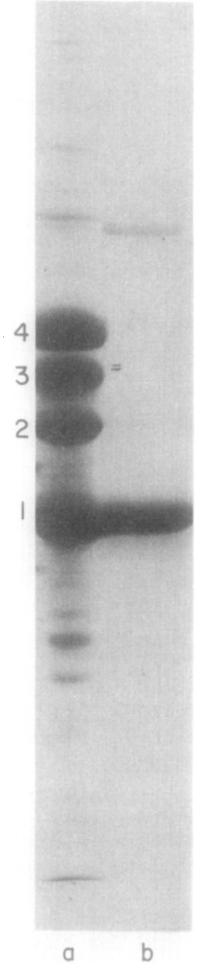

FIGURE 1: SDS polyacrylamide gels of (a) base-treated membrane fragments and (b) the 40,000 dalton polypeptide (Subunit I) purified by preparative SDS gel electrophoresis of material from the membrane fragments. The four AcChR subunits are marked on the gel.

RESULTS AND DISCUSSION

It has previously been demonstrated that the four subunits from Torpedo californica AcChR can be isolated by preparative SDS polyacrylamide gel electrophoresis in quantities sufficient for determination of amino acid composition (23) and for generation of subunit specific antibodies (24). In Figure 1 analytical gels of whole AcChR and of Subunit 1 purified as described in the Materials and Methods Section are shown. No major contamination of Subunit I by other receptor subunits or by extraneous proteins was observed. Since quantities of this purified subunit in the range of 10-20 nmoles could be readily prepared, it was feasible to subject it to amino terminal analysis using microsequencing procedures (21). This analysis revealed that Subunit I contains a single amino terminal amino acid, namely serine, and this result allowed application of the sequential Edman degradation method. Twenty-five successive cycles gave the following amino acid sequence:

$$\overset{1}{\text{Ser}}\text{-Glu-His-Glu-}\overset{5}{\text{Thr}}\text{-Arg-Leu-Val-Ala-}\overset{10}{\text{Asn}}\text{-Leu-Leu-Glu-Asn-}\overset{15}{\text{Tyr}}\text{-Asn-Lys-}$$
$$\text{Val-Ile-}\overset{20}{\text{Arg}}\text{-Pro-Val-Glu-His-}\overset{25}{\text{His}}\text{---}$$

In Figure 2 the HPLC chromatograms obtained for cycle numbers 1, 7 and 18 are shown to illustrate the quality of the data.

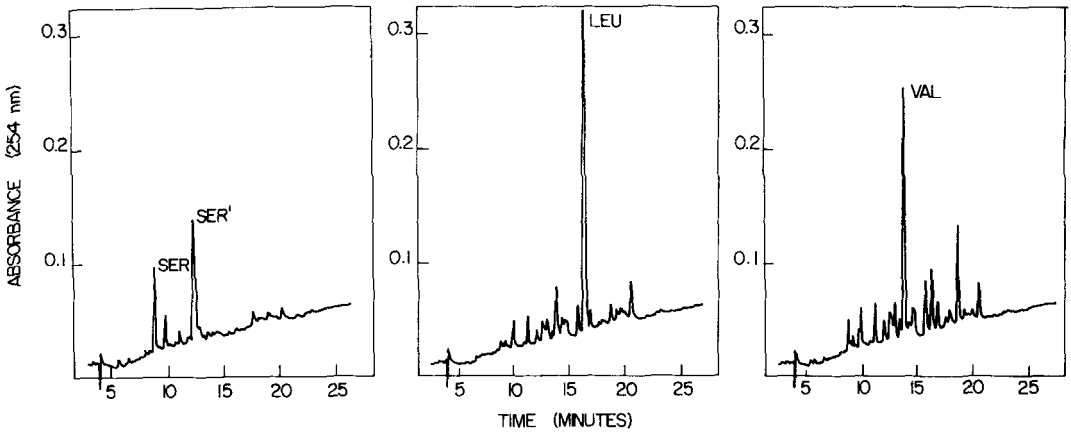

FIGURE 2: HPLC chromatograms of the Pth amino acids from cycles from cycles 1, 7 and 18 in a sequenator analysis of 9.6 nmol of Subunit I. 10% aliquots from each cycle were injected into the HPLC system. The Pth-Ser' derivative marked in cycle 1 is a serine product formed in the sequenator (21) and does not represent serine modified in native Subunit I.

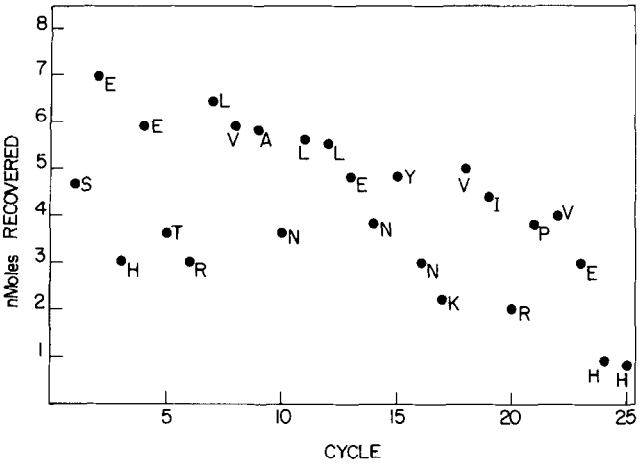

FIGURE 3: Yields of Pth amino acids identified in the amino terminal sequenator analysis of 9.6 nmol of Subunit I. The yields were calculated by comparison of HPLC peak heights with those of a standard Pth amino acid mixture. They are not corrected for the different recoveries of the various Pth amino acids.

Figure 3 shows the yields of Pth amino acids for each cycle from a sequenator analysis of 9.6 nmol (determined by amino acid analysis of a separate aliquot of protein) of Subunit I. A successive cycle efficiency of 96% for this run can be calculated from the yields of the Pth aliphatic amino acids.

It was possible to calculate the percentage of protein giving the unique sequence shown above relative to the total protein applied to the sequenator. This was done by assuming 95% recovery of the Pth aliphatic amino acids (21) and extrapolating the yields of these Pth amino acids given in Figure 3 back to cycle 0. This high initial yield of the unique sequence, 86 ± 5% (8.2 nmol), eliminates the possibility that a second polypeptide (even with a blocked amino terminus) could be present in amounts equimolar with Subunit I. Thus the two-fold differences in values obtained for the binding of α-neurotoxins such as α-BuTx compared with those obtained for a variety of cholinergic affinity reagents (6,7,13-15) are not the result of heterogeneous peptides of similar or identical molecular weight being present.

Note added in proof: The first twenty amino-terminal residues of the sequence agree exactly with a recent report by Devillers-Thiery et al. (25) for the corresponding residues of the 40,000 dalton subunit from the related species Torpedo marmorata.

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