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ACTIVE ACETYLCHOLINE RECEPTOR FRAGMENT OBTAINED BY TRYPTIC DIGESTION OF ACETYLCHOLINE RECEPTOR FROM TORPEDO CALIFORNICA

Daniel Bartfeld and Sara Fuchs

Department of Chemical Immunology
The Weizmann Institute of Science, Rehovot, Israel

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SUMMARY

Tryptic digestion of acetylcholine receptor (AChR) from Torpedo californica did not change the pharmacological specificity and the pathological myasthenic activity of the receptor molecule. The product obtained after tryptic digestion was repurified by affinity chromatography on a toxin-Sepharose resin and was designated T-AChR. T-AChR has a sedimentation coefficient of 8.0S and in SDS acrylamide gel electrophoresis shows one major band with a molecular weight of 27,000. Immunological studies reveal that T-AChR binds to anti-AChR antibodies directed only against conformational antigenic determinants.

The nicotinic acetylcholine receptor (AChR) is an integral protein of the postsynaptic membrane. The receptor can be isolated and purified from electric organs of electric fish by extraction with non-ionic detergents and affinity chromatography on a cobra toxin-Sepharose resin, and was shown to be a multisubunit high molecular weight glycoprotein (1-4). Immunization of animals with purified AChR results in a muscular weakness and additional signs similar to those observed in the human disease myasthenia gravis (4-6). Enzymic degradations of AChR provide a useful approach for attempting the isolation and analysis of fragments of the receptor molecule which retain the specific cholinergic binding site and/or the pathologic myasthenic activity. In the following we describe the biochemical and immunochemical characterization of an active AChR derivative obtained following tryptic digestion of AChR from Torpedo californica. This AChR derivative which contains as its principal component a polypeptide of 27,000 molecular weight, retains the pharmacological and myasthenic activity of the intact receptor molecule.

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TABLE 1. Effect of tryptic digestion of AChR on the specific activity

Sample	Digestion with trypsin (min)	Specific activity (nmole α-Bgt/mg)
AChR	-	10.6
AChR + Trypsin	0	8.6
AChR + Trypsin	5	12.0
AChR + Trypsin	20	7.5
AChR + Trypsin	60	10.8
AChR + Trypsin	240	12.0

MATERIALS AND METHODS

AChR was isolated from the electric organ of Torpedo californica (Pacific Bio-Marine, Venice, Ca.) and was purified by affinity chromatography on a Naja naja siamensis toxin-Sepharose column (4). Reduced carboxymethylated AChR (RCM-AChR) was prepared by reduction and carboxymethylation of AChR in 6M guanidine-HCl (7). α-Bungarotoxin (α-Bgt) and Naja naja siamensis neurotoxin were prepared according to Clark et al. (8) and Karlsson et al. (9), respectively. ^{125}I-AChR and ^{125}I-α-Bgt were prepared by the chloramine-T method (10). Trypsin (bovine, 2.0 U/mg) was obtained from Merck and trypsin inhibitor (from Lima bean, type II-L) from Sigma.

Nicotinic receptor sites in AChR and trypsinated AChR were measured by the ^{125}I-α-Bgt binding assay (4,11). Inhibition constants for cholinergic ligands were determined by measuring their effect on the initial rate of formation of ^{125}I-α-Bgt-AChR complex (4,12,13). Fractionation of anti-AChR antibodies was performed by chromatography of sera from rabbits immunized with AChR on a denatured AChR-Sepharose adsorbent (RCM-AChR-Sepharose resin) (14). The unadsorbed antibody fraction was designated as anti-native AChR (nAChR) antibodies. Antibodies adsorbed to and eluted from the column were designated anti-denatured AChR (dAChR) antibodies. Radioimmunoassay using ^{125}I-AChR as the radiolabeled antigen was performed as described elsewhere (4,15).

RESULTS AND DISCUSSION

AChR (1 mg/ml in receptor buffer, i.e., 0.01M Tris-buffer, pH 7.5, 0.1M NaCl, 10^{-3}M EDTA, 5 x 10^{-4}M NaN_3 and 0.1% Triton X-100) was digested at room temperature with trypsin (10:1 w/w) and the digestion was terminated by the addition of trypsin inhibitor (2:1, weight ratio of inhibitor to trypsin). As can be seen in Table 1 tryptic digestion did not result in a significant change in the binding capacity of ^{125}I-α-Bgt to the receptor. SDS acrylamide gel electrophoresis demonstrates that tryptic digestion resulted in a gradual disappearance of the various receptor polypeptide

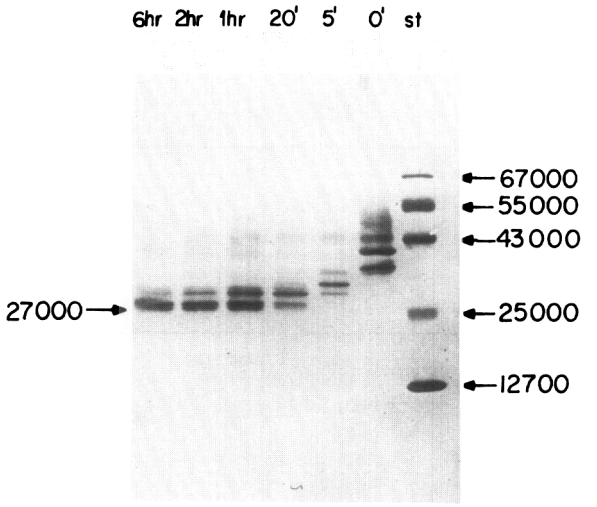

Fig. 1. SDS-Poly acrylamide gel electrophoresis (PAGE) of AChR following tryptic digestion. Aliquots of AChR (0.1 mg) were digested with trypsin (0.01 mg). The digestion was stopped at different times by the addition of trypsin inhibitor and a sample of each aliquot was layered on a gradient gel (7.5% - 15%). In the control (0') the trypsin inhibitor was added to the trypsin before the receptor.

chains and an appearance of bands with lower molecular weight, concentrating in the region of an apparent molecular weight of 27,000, following prolonged digestion (Fig. 1). Immunologically, the binding capacity to antibodies against conformational determinants in AChR (anti-nAChR) was also not affected by tryptic digestion (Fig. 2A). In contrast, the ability to bind to antibodies against sequential determinants (anti-dAChR) was gradually decreased (Fig. 2B), suggesting that such non-structural sequences in the receptor molecule were digested by trypsin.

Since tryptic digestion of AChR did not result in a significant change in the binding properties to α-Bgt, the product obtained after 2.5 hours of digestion was repurified by affinity chromatography on a Naja naja siamensis toxin-Sepharose resin. The material adsorbed to the resin was eluted with carbamylcholine (0.7 M (4)), dialysed against receptor buffer and was designated T-AChR. T-AChR displayed a specific activity of 11.9 nmole toxin-binding sites per mg protein. On SDS-acrylamide gel electrophoresis

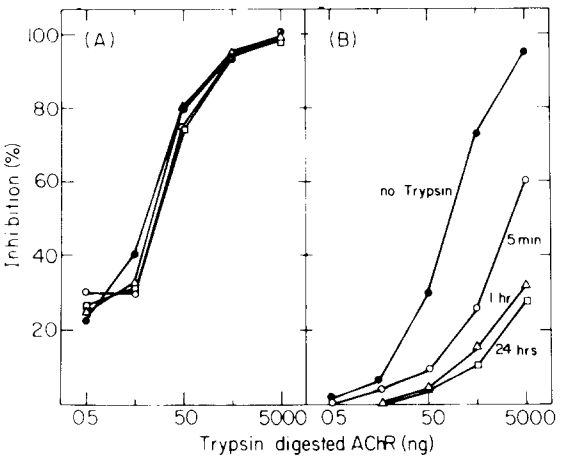

Fig. 2. Effect of tryptic digestion on the immunolgoical specificity of AChR. The curves represent the inhibition of the binding of ^{125}I-AChR to anti-nAChR (A) and to anti-dAChR (B) by AChR (●) and by AChR following digestion with trypsin for 5 min (o), 1 hr (Δ) and 24 hr (□).

T-AChR shows one major band with a molecular weight of 27,000 whereas intact AChR generally shows four bands, the major one with a molecular weight of 40,000 and the other three with a higher molecular weight (Fig. 3,(1,2)). The band observed in the AChR gel in the range of molecular weight of ca. 37,000 (Fig. 3) may have resulted from some degradation during purification of the receptor, as has been reported earlier (1,2). Low molecular weight peptides which were present in the tryptic digest of the receptor did not adsorb to the toxin-Sepharose resin. The sedimentation coefficient of T-AChR was determined by sucrose gradient centrifugation (16) and by analytical ultracentrifugation and was compared with that of the intact AChR. By sucrose gradient centrifugation AChR and T-AChR displayed sedimentation coefficients of 9.3S and 8.0S, respectively, and by analytical ultra-centrifugation 9.4S and 8.1S, respectively.

T-AChR was characterized pharmacologically and immunologically. Table 2 shows the I_{50} values for inhibition of the binding of ^{125}I-α-Bgt to AChR and to T-AChR by several agonists and antagonists. The pharmacological specificity of T-AChR appears to be similar to that of AChR. Immuno-

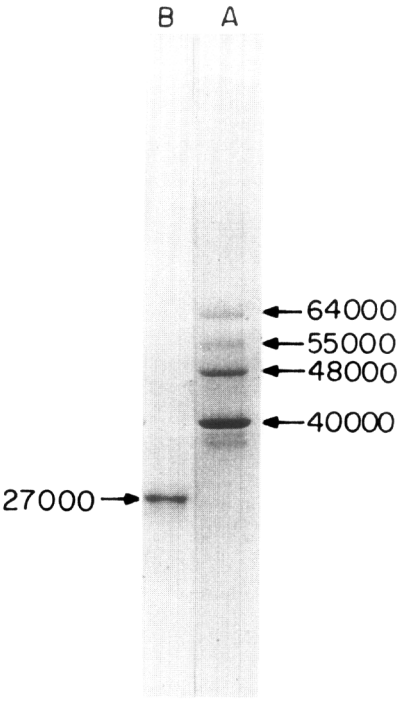

Fig. 3. SDS-Polyacrylamide gel electrophoresis of A, AChR and B, T-AChR. Gel conditions as in Fig. 1.

TABLE 2. I_{50} values for AChR and T-AChR

Ligand	I_{50} (M) AChR	T-AChR
Acetylcholine	3.6×10^{-6}	9.2×10^{-6}
Carbamylcholine	4.7×10^{-6}	3.3×10^{-5}
Decamethonium	5.0×10^{-5}	6.8×10^{-5}
Hexamethonium	1.6×10^{-3}	3.0×10^{-3}
Nicotine	2.0×10^{-4}	5.2×10^{-4}
D-Tubocurarine	9.0×10^{-6}	1.5×10^{-5}
Atropine	1.2×10^{-2}	5.0×10^{-2}
α-Bungarotoxin	1.5×10^{-10}	4.0×10^{-11}

logically, it was demonstrated that T-AChR binds to anti-nAChR whereas it binds very weakly to anti-dAChR (Fig. 4). This is in contrast to the denatured receptor (RCM-AChR) which does not bind to anti-nAChR and binds

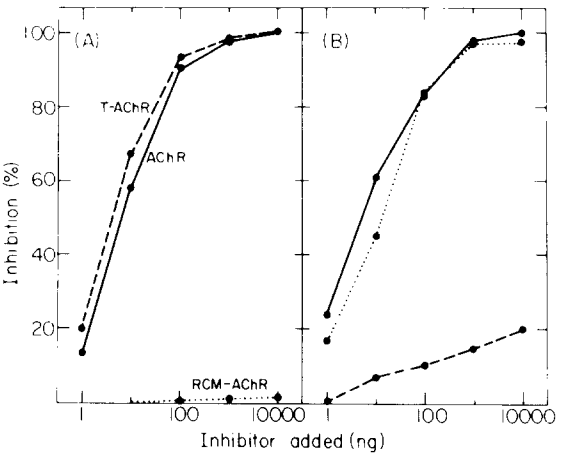

Fig. 4. Inhibition of the binding of ^{125}I-AChR to anti-nAChR (A), and to anti-dAChR (B) by AChR (——), T-AChR (– – –) and RCM-AChR (·····).

to anti-dAChR (Fig. 4, (14)). It thus appears that T-AChR binds to anti-AChR antibodies directed only against conformational determinants and does not bind to antibodies against sequential determinants. The exposed unfolded regions of AChR were probably digested by the trypsin, retaining only the folded regions which are resistant to proteolytic activity. The cholinergic binding sites seem to reside within this structured region. The myasthenic sites of AChR were also not affected by tryptic digestion as immunization of rabbits with T-AChR (one or two injections of 80 µg protein in complete Freund's adjuvant resulted in a muscular weakness, paralysis and death identical to those observed in experimental autoimmune myasthenia gravis obtained following immunization with AChR (4-6).

T-AChR represents an active receptor molecule with a lower structural complexity than that of the intact detergent purified receptor. The subunit structure of AChR is still under investigation. A general agreement exists about the 40,000 band which is dominant in almost all AChR preparations and is the only one labeled by affinity labeling reagents (1,17). No functional activity has yet been assigned to the higher molecular weight subunits present in highly purified AChR preparations.

These subunits seem, however, to have a different chemical composition from that of the 40,000 band (21,18), they do not share antigenic determinants with it (19) and therefore the 40,000 band is probably not derived from the heavier species.

On the basis of our results T-AChR provides a suitable preparation for further analysis of the structure and function of the cholinergic binding site as well as the myasthenic site. It was already reported that tryptic digestion of AChR-rich membrane fragments (16) and of purified AChR (20) results in preparations containing lower molecular weight components. We do not have yet direct evidence that the 27,000 polypeptide chain present in T-AChR results only from the 40,000 polypeptide, and that the other polypeptides with higher molecular weight have been digested to smaller peptides which do not bind any more to the toxin column. However, this seems very likely in view of the fact that the different subunits are not derived from each other. If this is indeed the case, it may be concluded that the myasthenic activity of AChR resides also within the 40,000 subunit of the receptor.

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