TRANSMEMBRANE NATURE OF ACETYLCHOLINE RECEPTOR AS EVIDENCED BY PROTEASE SENSITIVITY

Leaf HUANG
Department of Biochemistry, University of Tennessee, Knoxville, TN 37916, USA

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1. Introduction

The nicotinic acetylcholine receptor is an integral protein in the postsynaptic membranes of some nerve and muscle tissues. Electrophysiological measurements have shown that it mediates cation permeation across the endplate membrane when the receptor is stimulated with cholinergic agents [1]. From the rate of cation movement across the membrane, it was inferred that the AchR is an ion channel rather than a mobile carrier, and hence the transmembrane nature of the receptor was postulated [2–5]. We describe here some experimental evidence which supports this hypothesis.

2. Materials and methods

2.1. *Membrane vesicle preparation*

Frozen electroplax of *Torpedo californica* (Pacific Biomarine, Venice, CA) was thawed and homogenized with an Omni mixer (Sorvall) in 0.2 M NaCl, 5 mM EDTA, 0.02% NaN_3 and 10 mM Tris (pH 7.8). The homogenate was sieved through a nylon screen to remove connective tissues and was then centrifuged at 100 000 × *g* for 30 min. The pellet was washed once in the same buffer except that NaCl was raised to 1 M. Crude membrane vesicles were resuspended in NENT buffer (100 mM NaCl, 5 mM EDTA, 0.02% NaN_3 and 10 mM Tris (pH 7.8) and used for subsequent experiments. This preparation contained ~0.6 nmol toxin binding sites/mg protein. Protein was assayed by the Lowry method [5].

Abbreviations: AchR, nicotinic acetylcholine receptor; EDTA, ethylene dinitrilo-tetraacetic acid; α-BGT, α-bungarotoxin; TPCK, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone

2.2. *Purification and iodination of α-bungarotoxin (α-BGT)*

α-BGT was purified by ion-exchange column chromatography from the lyopholysed venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, FL) as in [7]. ^{125}I-Labeled α-BGT was prepared by using chloramine-T [8] to spec. act. ~40 μCi/nmol. Radioactivity was counted in a Beckman Biogamma II counter.

2.3. *Assay of α-BGT binding*

Crude membrane vesicles (0.1 mg protein in 400 μl NENT buffer) were solubilized with 1% Triton X-100 (30 min, 25°C). ^{125}I-Labeled α-BGT (0.2–0.3 nmol) was added and incubated 1 h at 37°C. The mixture was briefly centrifuged in a desk top centrifuge and an aliquot (100 μl) of the clear supernatant was applied to a Bio-gel P-60 column (0.6 × 18 cm) equilibrated with 10 mM Tris, 1% Triton X-100, (pH 7.8). The column was eluted with the same buffer and fractions of ~ 0.4 ml were collected. The ^{125}I cpm of the toxin–receptor complexes eluted at the void volume were counted. The recovery from the column was > 95%. The specific toxin binding activity of the membrane vesicles was calculated from the specific radioactivity of the ^{125}I-labeled α-BGT.

2.4. *α-Chymotrypsin digestion of AchR*

Crude membrane vesicles (0.1 mg in 200 μl) were incubated with either ^{125}I-labeled α-BGT (0.2 nmol)

or unlabeled α-BGT (25 nmol) for 1 h at 37°C, followed by washing twice to remove excess toxin They were incubated with α-chymotrypsin (Worthington, 200 μg/ml) for 1 h at 37°C Reaction was terminated by adding 2 7 mM L-1-tosylamide-2-phenyl-ethyl-chloromethyl ketone (TPCK, Sigma) Toxin–receptor complexes were solubilized with 1% Triton X-100 (30 min, 25°C) The supernatants after a brief centrifugation were fractionated on a Bio-gel P-60 column as described above. The void volume eluents were pooled and further analysed by sucrose density gradient centrifugation To digest the Triton X-100 solubilized AchR, an identical protocol was followed, except that no further solubilization after enzyme digestion was done.

2 5 *Sucrose density gradient centrifugation*

Samples (100–200 μl) were loaded on a 5 ml linear gradient of sucrose (5–20%, w/w) in NENT buffer containing 1% Triton X-100 The tubes were spun at 48 000 rev /min for 7 h in a SW-50 1 rotor Fractions of the gradient were collected from the bottom of the tube and counted for ^{125}I Markers used for the sedimentation were catalase (11 S), lactate dehydrogenase (7 S) and α-BGT (2 S)

2 6 *Electron microscopy*

Crude membrane vesicles or α-chymotrypsin in digested vesicles were centrifuged at 100 000 × *g* for 30 min The pellet was fixed in glutaraldehyde and post-fixed with OsO_4 [9] Samples were dehydrated in a graded series of water–acetone mixtures and embedded in Epon [9] Thin sections were viewed in a JEM 6c electron microscope operating at 80 kV after staining with uranium acetate and lead citrate Pictures were taken at 25 000 × magnification and further enlarged photographically

3 Results and discussion

The crude membrane vesicles were not clumped but were quite heterogeneous in size, as revealed in electron microscopy (fig 1a) Most of the vesicles were bound by a single membrane The binding of ^{125}I-labeled α-BGT to the AchR in these vesicles was studied (table 1) When saturation amounts of ^{125}I-labeled α-BGT were incubated with Triton-solubilized receptors, all binding sites were occupied, as assayed by a gel filtration method However, when the same amount of ^{125}I-labeled α-BGT was incubated with intact membrane vesicles only ~50% of the total sites were bound The ~50% latency of the toxin binding activity suggests that about half of the membrane vesicles in this crude preparation were right-side-out and the other half were inside-out

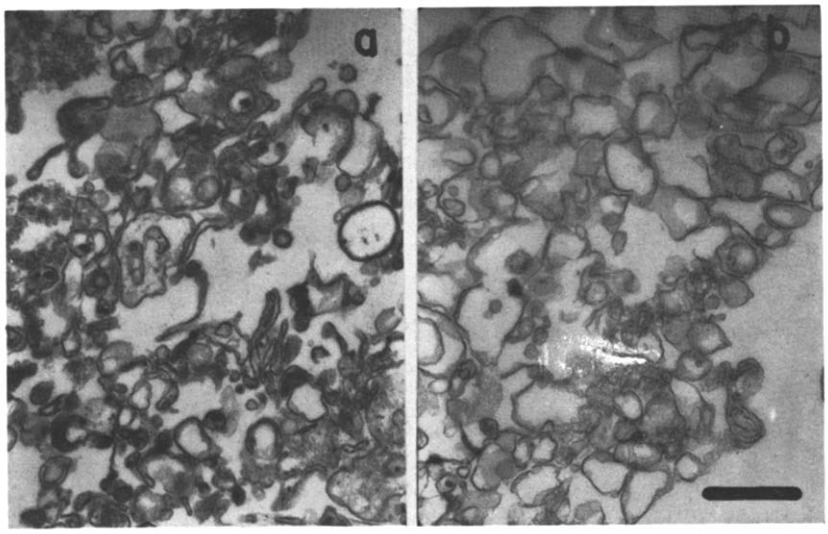

Fig 1 Thin-section electron micrographs of the crude *Torpedo* membrane vesicles with (b) or without (a) α-chymotrypsin digestion Bar is 0 5 μm

Table 1
Latency of ^{125}I-labeled α-BGT binding to *Torpedo* membrane vesicles

Treatment	^{125}I-labeled α-BGT added (nmol)	^{125}I-labeled α-BGT bound (nmol)	% bound
^{125}I-labeled α-BGT → Triton[a]	0.2	0.062 ± 0.01[b]	31 ± 5
Triton → ^{125}I-labeled α-BGT[a]	0.2	0.126 ± 0.014	63 ± 7

[a] After incubation with ^{125}I-labeled α-BGT, 25 nmol unlabeled α-BGT was added to dilute the specific radioactivity of the labeled α-BGT. The exchange of the bound toxin with free toxin during Triton solubilization was < 12%
[b] Mean ± standard deviation from 3 separate experiments

We then reasoned that if the receptor molecules spanned the membrane, the portion of the molecule exposed on either side of the membrane may have different sensitivity to protease digestion. α-Chymotrypsin was found to be the most effective enzyme for this purpose. After the exposed toxin-binding sites were occupied with ^{125}I-labeled α-BGT, the membrane vesicles were incubated with α-chymotrypsin (100 or 200 μg/ml gave similar results). The enzymic reaction was stopped by addition of TPCK. Vesicles were then solubilized with Triton X-100 and fractionated on a Biogel P-60 column. The radioactive toxin–receptor complexes eluted at the void volume were pooled and analyzed by sedimentation in a linear sucrose density gradient. The toxin–receptor complex obtained in this manner sedimented as a high molecular weight substance (~13 S) which was indistinguishable from the toxin–receptor complexes without protease treatment (compare fig.2a,d). Therefore α-chymotrypsin was without effect on those toxin–receptor complexes exposed on the right-side-out vesicles. In a parallel experiment, membrane vesicles were first incubated with a large amount of unlabeled α-BGT to block all sites on the right-side-out vesicles, then digested by α-chymotrypsin. The latent toxin-binding sites were then revealed by Triton solubilization and tagged with ^{125}I-labeled α-BGT. The resulting radioactive toxin–receptor complexes cosedimented with the lactate dehydrogenase marker as smaller molecular weight substances of ~7 S (fig.2b). These results suggested that the acetylcholine receptor molecule was accessable to protease digestion in the inside-out vesicles, but the toxin-

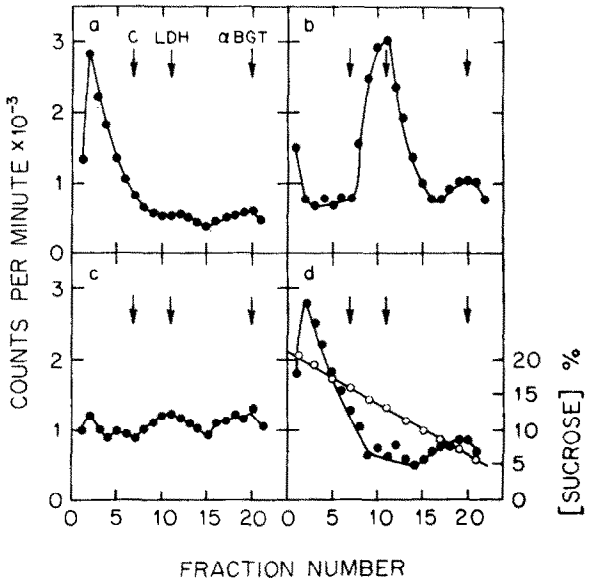

Fig.2. Sedimentation profiles of ^{125}I-labeled α-BGT–AchR complexes in sucrose density gradient. In (a) vesicles were labeled with ^{125}I-labeled α-BGT, washed twice to remove unbound toxin, then digested with α-chymotrypsin. The radioactive toxin–receptor complexes were Triton-solubilized, collected by gel filtration on Bio-gel P-60, and analysed by sucrose density gradient centrifugation. In (b) vesicles were first incubated with unlabeled α-BGT, washed twice, and then digested with α-chymotrypsin. In (c) vesicles were treated as in (a) except the radioactive toxin-receptor complexes were digested with α-chymotrypsin after Triton solubilization. In (d) vesicles were treated as in (a) except that the α-chymotrypsin digestion and TPCK steps were omitted. Closed circles are ^{125}I cpm and open circles are sucrose concentrations. Markers used were catalase (C), lactate dehydrogenase (LDH) and α-BGT as indicated by arrows.

binding site of the receptor was protected from digestion because it is located within these vesicles If the radioactive toxin–receptor complex obtained in right-side-out vesicles was solubilized by Triton X-100 before the chymotrypsin digestion, the 7 S species was also reproducibly seen in the gradient (fig 2c) In this case, the portion of the receptor molecule opposite to the toxin-binding site became accessible to protease when the membrane integrity was destroyed by detergent The toxin binding sites of receptor on the right-side-out vesicles were apparently protected from digestion by α-chymotrypsin by the binding of α-BGT, since little binding activity was found if the vesicles were treated with the enzyme before the incubation with toxin (data not shown) The α-chymotrypsin digestion of intact vesicles was sufficiently mild that the treated vesicles still had similar size distribution and appeared to be sealed vesicles, as were the original ones, when examined by electron microscopy (fig 1b)

The observed change in sedimentation behavior of acetylcholine receptor was unlikely due to a shift in the state of aggregation of the molecule involving intermolecular disulfide bonds, since the reported small oligomer of AchR from *Torpedo californica* has a sedimentation coefficient of ~9 S [10,11] which is considerably larger than the degraded species we found It is understandable that only the large oligomer (13 S) of intact AchR was observed in these experiments, since EDTA was included in our buffer systems The presence of Ca^{2+} facilitates the conversion of the 13 S into the 9 S species [10]

Figure 3 shows a schematic interpretation of the results described above In the random homogenization of cells in electroplax, both right-side-out and inside-out membrane vesicles are formed The toxin-binding sites, when occupied by α-BGT, of the receptor molecules in the right-side-out vesicles are resistant to the digestion by α-chymotrypsin, and therefore sediment as high molecular weight substances (13 S) The receptors in the inside-out vesicles, however, are accessible to digestion and therefore sediment as lower molecular weight species (7 S). These conclusions therefore indicate that the acetylcholine receptor molecule is a transmembrane protein This work agrees with the X-ray diffraction studies [12] where AchR was suggested to span the endplate membrane Our conclusion is also supported by the

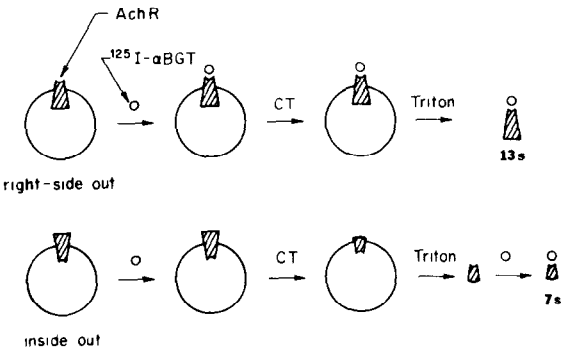

Fig 3 Schematic interpretation of chymotrypsin digestion of AchR in *Torpedo* membrane vesicles CT represents α-chymotrypsin

report [13] that the antibodies to the purified receptor bind to both sides of *T californica* microsacs

Acknowledgements

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