Biochemical Study on Nicotinic Acetylcholine Receptor

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Abstract : Nicotinic acetylcholine receptor (AChR) is a multisubunit membrane glycoprotein which functions as a ligand-triggered cation channel. Receptors from electric tissues and skeletal muscle have a molecular weight of approximately 290,000 as a monomer and are composed of four types of polypeptide chains which assemble into a heterologous $\alpha_2\beta\gamma\delta$ pentamer.

We determined the carbohydrate structure of *Torpedo* AChR, which was the first determination for neurotransmitter receptors. About 70 % of the oligosaccharides of the AChR were of the high mannose-type, $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$. These two types of oligosaccharides were distributed in all the subunits. The remaining were various kinds of complex-type oligosaccharides, existing mainly in the γ and δ subunits. The α and β subunits had only one carbohydrate chain each, while the γ and δ subunits had two and three carbohydrate chains, respectively. These glycosylation sites were identified by sequencing glycopeptides obtained by lectin-affinity chromatography. The participation of oligosaccharides in ligand-binding of AChR was examined using a newly developed binding assay. The sialic acids and high mannose-type oligosaccharides on AChR were found to be unnecessary for its ligand binding.

Next we found that the β and δ subunits of *Torpedo* AChR were phosphorylated on their tyrosine residues. The level of the phosphorylation was enhanced by incubating the AChR-rich membrane fraction with cholinergic ligands. This suggests that cholinergic agonists physiologically regulate phosphorylation on tyrosine *in vivo*, which might be included in the desensitization mechanism of the receptor.

We also examined the spatial relation of proteins surrounding AChR using *Torpedo* AChR-rich membrane fraction. Bifunctional crosslinkers revealed an intimate relation among the AChR γ subunit, 43-kD protein, and dystrophin. Finally, we found neurotoxin-binding activities in the supernatant fraction obtained by ultracentrifugation of a homogenate of the electric organ, which usually does not contain AChR. This new activity was different in nature from AChR, and could function as a regulator or a modulator for AChR function.

Keyphrases : Nicotinic acetylcholine receptor, electric organ, *Torpedo californica*, carbohydrate structure, glycosylation sites, cholinergic ligands, tyrosine phosphorylation, α-bungarotoxin, 43k-protein

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The nervous system regulates all aspects of bodily function. Millions of specialized nerve cells sense changes in both the external and internal environments. Millions more nerve cells regulate the contraction of muscles and the secretion of endocrine or exocrine glands. Neurons make specific contacts with other cells at specialized sites called synapses, across which signals are passed. Synapses generally conduct signals in only one direction: an axon terminal from the presynaptic cell sends signals that are picked up by the postsynaptic cell. The two types of synapses, electrical and chemical, differ in both structure and function.

In chemical synapses, the axon terminal of the presynaptic cell contains vesicles filled with a particular neurotransmitter substance. When the action potential, or electric impulse, reaches the axon terminal, these vesicles are exocytosed, releasing their contents into the synaptic cleft, the narrow space between the cells. The transmitter diffuses across the synaptic cleft and, after a lag period of about 0.5 ms, binds to a receptor on the postsynaptic cells. Upon binding, it induces a change in the ionic permeability of the postsynaptic membrane that results in a disturbance of the electric potential at this point. This electrical disturbance induces an action potential, a muscle contraction, or a hormone release. When the transmitter is supplied continuously to receptors, the receptor molecules begin to lose their responsiveness. After initially opening the ion channel, they slowly take on a desensitized conformation over the course of seconds or minutes. They bind to transmitters but maintain a closed channel.

Neuron-neuron or neuron-muscle synapses using acetylcholine as a neurotransmitter are termed cholinergic. A family of acetylcholine receptors is distinguished from each other by postsynaptic cell responses to agonists of acetylcholine, such as nicotine and muscarine. Nicotine causes an excitory response lasting only milliseconds, which is far shorter than muscarinic responses. Nicotinic cholinergic synapses are widespread in the peripheral nervous system and form the neuromuscular junctions (endplates) of voluntary muscles (Fig.1). They are also present in the central nervous system.

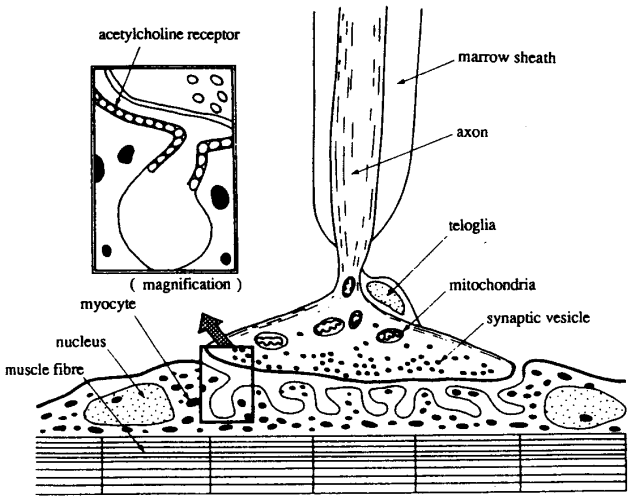

Fig.1. Diagram of neuromuscular junction.

Solubilization of the membrane-bound nicotinic acetylcholine receptor (AChR) with non-denaturing detergents and the advent of efficient affinity columns opened the door to characterization of the AChR, which is considered to be the prototype of a whole class of receptor molecules. Many studies on this receptor have been facilitated by snake venom toxins. Among these the most widely used is α-bungarotoxin (BGT), which binds specifically and irreversibly to the receptor and prevents its function. Recent tremendous progress in AChR research is also indebted to sting ray (*Torpedo*) and electric eel, whose electric organs are non-contractile, embryologic derivatives of skeletal muscle stem cells and have served as an excellent model, owing to its similarity to the vertebrate neuromuscular junction and to the relative ease of isolating its abundant postsynaptic membranes [1-3].

AChR in the electric organs and muscle is a 290-kD transmembrane glycoprotein, composed of four distinct types of subunits assembled into a heterologous $\alpha_2\beta\gamma\delta$ pentamer (Fig. 2) ; the apparent molecular weights of the α, β, γ, and δ subunits are 40,000, 50,000, 60,000, and 65,000, respectively (see Fig.4). The primary structures of all four of these subunits and those from some mammalian muscle AChR have been elucidated by cloning and sequencing cDNAs or genomic DNA encoding these polypeptides. The four subunits exhibit marked sequence homology and are similar in hydrophilicity profile and predicted secondary structure, and thus are oriented most probably in pseudosymmetric fashion across the membrane. The four subunits have four hydrophobic regions called M1 to M4 (from the *N*-terminal), which probably traverse lipid bilayer. M2 domains of the five subunits constitute the ion channel pore. Among the subunits, the α subunit bears an agonist/competitive antagonist binding site. Reconstitution with purified protein and mRNA expression experiments in frog oocytes have shown that the $\alpha_2\beta\gamma\delta$ oligomer contains all the structural elements required for the physiological response, i. e., the agonist/antagonist binding site, the ion channel, the mechanism that mediates fast coupling between them, and the desensitization mechanism [1-3].

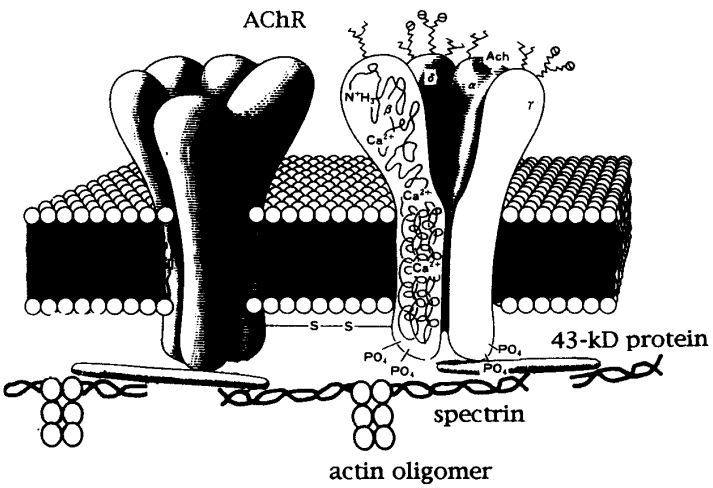

Fig.2. Schematic model of the structure of *Torpedo* AChR in membrane with the peripheral cytosolic proteins.

Furthermore, the fifth type of subunit, ε, was found in calf muscle, which had high homology to the γ subunit [4]. In cattle the AChR exists in the $\alpha_2\beta\gamma\delta$ form in muscle tissues at the fetal stage and changes into the $\alpha_2\beta\varepsilon\delta$ form with development of the body system [4]. It is also known that AChR from brain has a different subunit structure from the protein found in the peripheral nervous system [3].

AChR is surrounded by various proteins in the membrane. Such proteins include several cytoskeletal proteins or enzymes. Among them, four cytoplasmic peripheral membrane proteins have been well characterized; namely, 43-kD, 58-kD, and 87-kD proteins, and dystrophin [68]. Aggregates of AChR at the synapse are apparently stabilized and maintained by interaction with the underlying electron-dense cytoskeletal meshwork, which is composed of these peripheral membrane proteins [5].

Myasthenia gravis is a disease characterized by fatigue and weakness of skeletal muscles due to a reduction in the number of functional AChR at the neuromuscular junctions. Circulating antibodies (autoantibodies) against the receptors cause their degradation. Normally, only a fraction of the AChR in the neuromuscular junction are activated in response to a nerve impulse. However, because of the reduced numbers of the receptors in myasthenia gravis, the lower levels of acetylcholine released during repetitive firings are insufficient to depolarize the membrane to the threshold for triggering muscle action potentials [6].

In order to further understand the molecular mechanism of AChR, we have studied various biochemical aspects of AChR ; namely, the carbohydrate structure and its role, the role of tyrosine phosphorylation, spatial relationship with other proteins surrounding AChR, and a protein similar to AChR. *Torpedo* AChR and its membrane fraction provide good materials for such biochemical analyses.

1. Structures of oligosaccharides of *Torpedo* AChR [17, 35]

The carbohydrate moieties of glycoproteins are thought to serve many functions, including determination of protein conformation, stability, turnover, cellular recognition, and intracellular transport [9, 10]. Removal of the carbohydrate moieties from AChR by site directed mutagenesis [12] or tunicamycin treatment [13, 14] resulted in loss of surface expression of AChR. These results suggest that AChR cannot be assembled or is unstable in the absence of the carbohydrate moieties. It has also been reported that inhibition of glucosidase at the initial step of the oligosaccharide processing pathway changed the channel properties of AChR, reducing the rate of channel opening and of agonist dissociation [16]. The correct processing of oligosaccharides seems to define expression of functional AChR.

A lectin, concanavalin A (Con A), is known to bind to AChR with high affinity. In some instances, however, sera from patients with myasthenia gravis were found to inhibit the binding of

Con A to AChR [8]. This suggests that carbohydrate moiety of AChR could be included in an antigenic site in this autoimmune disease, and might have some relation to the onset of the disease.

It had been reported that AChR of *Torpedo californica* contained a significant amount of glucose [22, 23], which was not expected from the processing pathway of glycoprotein oligosaccharides. So, to start with, we analyzed sugar composition. AChR was isolated from the electric organ using a cobrotoxin-Sepharose column [20]. The carbohydrate composition of one molecule of *Torpedo* AChR is 28, 53, 2.0, 11, 4.9 residues of glucosamine, mannose, fucose, galactose, and *N*-acetylneuraminic acid, respectively. No glucose was detected. Since galactosamine was not detected in the AChR molecule, it is quite unlikely that *O*-glycosidic-type oligosaccharides exists in *Torpedo* AChR.

Oligosaccharides were released by sequential digestion with pepsin and *N*-oligosaccharide glycopeptidase. The oligosaccharides were fractionated according to negative charge by HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection). After derivatization with 2-aminopyridine, the neutral fraction and the desialylated fractions obtained from each mono-, di-, tri-, and tetrasialylated fraction was analyzed by reverse-phase HPLC. The structures of the pyridylamino-oligosaccharides were analyzed by two-dimensional sugar mapping with combination of exoglycosidase digestion [21]. Some oligosaccharides were analyzed further with ^{1}H-NMR spectroscopy. The deduced structures are shown in Table 1 with number of sialic acids and relative composition. The oligosaccharides of *Torpedo* AChR are very heterogeneous, while two high mannose compounds, A ($Man_9GlcNAc_2$) and B ($Man_8GlcNAc_2$), comprise about 70 % of the total sugar chains.

There were various kinds of complex-type oligosaccharides, composed of bi-, tri-, and tetraantennary oligosaccharides, each of which varied in the number of linking sialic acids. Among the complex-type oligosaccharides, fraction M predominated, comprising about 5 % of the total oligosaccharides. A notable feature of the complex-type oligosaccharides of AChR is the localized distribution of the fucose or bisecting *N*-acetylglucosamine residues ; fucose existed only in the biantennary oligosaccharides and bisecting *N*-acetylglucosamine existed only in the triantennary oligosaccharides. Moreover, the peculiar structures, Gal α 1-3Gal and Gal β 1-3GlcN Ac, existed only in the fucose-containing biantennary oligosaccharides and the triantennary oligosaccharides, respectively. In contrast to these complexities, the tetraantennary oligosaccharides were quite homogeneous. Fucose residues did not exist in the tri- or tetraantennary oligosaccharides.

We initially expected that there would be peculiar oligosaccharides in a neurotransmitter receptor such as AChR, but the deduced structures are those commonly found in a variety of glycoproteins. Poulter *et al.* [18, 19] studied structures for the major oligosaccharides of *Torpedo* AChR using liquid secondary ion mass spectrometry and confirmed the structures of the major

Table 1. Carbohydrate structure of *Torpedo* AChR

	Structure of Oligosaccharide	Number of Sialic acid	Compositions (%)
A	Manα2Manα6 Manα6 Manα3 Manβ4GlcNAcβ4GlcNAc Manα2Manα2Manα3	0	43
B	Manα2Manα6 Manα6 Manα2Manα3 Manβ4GlcNAcβ4GlcNAc Manα2Manα2Manα3	0	32
C	Manα6 Manα6 Manα3 Manβ4GlcNAcβ4GlcNAc Manα2Manα3	0	
D	Manα6 Manα6 Manα3 Manβ4GlcNAcβ4GlcNAc Manα3	0	
E	GlcNAcβ2Manα6 Manβ4GlcNAcβ4GlcNAc GlcNAcβ2Manα3	0	3.8
F	Galβ4GlcNAcβ2Manα6 Manβ4GlcNAcβ4GlcNAc GlcNAcβ2Manα3	0	
G	GlcNAcβ2Manα6 Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ2Manα3	0, 1	
H	Galβ4GlcNAcβ2Manα6 Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ2Manα3	0, 1, 2	
L	GlcNAcβ2Manα6 Fucα6 Manβ4GlcNAcβ4GlcNAc GlcNAcβ2Manα3	0	2.5
O	Galβ4GlcNAcβ2Manα6 Fucα6 Manβ4GlcNAcβ4GlcNAc GlcNAcβ2Manα3	0, 1	
P	GlcNAcβ2Manα6 Fucα6 Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ2Manα3	0	
Q	Galβ4GlcNAcβ2Manα6 Fucα6 Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ2Manα3	1	
R	Galα3Galβ4GlcNAcβ2Manα6 Fucα6 Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ2Manα3	1	1.3
S	Galα3Galβ4GlcNAcβ2Manα6 Fucα6 Manβ4GlcNAcβ4GlcNAc Galα3Galβ4GlcNAcβ2Manα3	0	

J	GlcNAcβ2Manα6 GlcNAcβ4 \ Manβ4GlcNAcβ4GlcNAc Manα3 Galβ4GlcNAcβ2	0, 1	7.5
K	Galβ4GlcNAcβ2Manα6 GlcNAcβ4 \ Manβ4GlcNAcβ4GlcNAc Manα3 Galβ4GlcNAcβ2	1, 2	
M	Galβ4GlcNAcβ2Manα6 Galβ4GlcNAcβ4 \ Manβ4GlcNAcβ4GlcNAc Manα3 Galβ4GlcNAcβ2	1, 2, 3	
N	Galβ4GlcNAcβ2Manα6 Galβ3GlcNAcβ4 \ Manβ4GlcNAcβ4GlcNAc Manα3 Galβ4GlcNAcβ2	3	
T	GlcNAcβ2Manα6 GlcNAcβ4 — Manβ4GlcNAcβ4GlcNAc GlcNAcβ4 Manα3 GlcNAcβ2	0	6.3
U	GlcNAcβ2Manα6 GlcNAcβ4 — Man β4GlcNAcβ4GlcNAc Galβ4GlcNAcβ4 Manα3 GlcNAcβ2	0, 1	
V	(Galβ4)2 { GlcNAcβ2Manα6 GlcNAcβ4 — Manβ4GlcNAcβ4GlcNAc GlcNAcβ4 Manα3 GlcNAcβ2	0, 1	
W	Galβ4GlcNAcβ2Manα6 GlcNAcβ4 — Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ4 Manα3 Galβ4GlcNAcβ2	0, 1, 3	
I	Galβ4GlcNAcβ6 Manα6 Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ4 Manα3 Galβ4GlcNAcβ2	3, 4	3.0

high mannose-type oligosaccharides which we had reported. They also detected molecular ions and fragmentation patterns for some complex-type oligosaccharides, whose structures were defined in detail by our study.

2. Distribution of each oligosaccharide among the AChR subunits [17]

The primary structures of *Torpedo* AChR subunits have revealed potential sites for *N*-glycosylation, Asn-X-Ser/Thr ; there are one, one, four, and three such sites in the α, β, γ, and δ subunits, respectively [1]. Two of the four sites in the γ subunit were supposed to exist in the lipid bilayer or cytoplasmic face, and are not likely to be glycosylated.

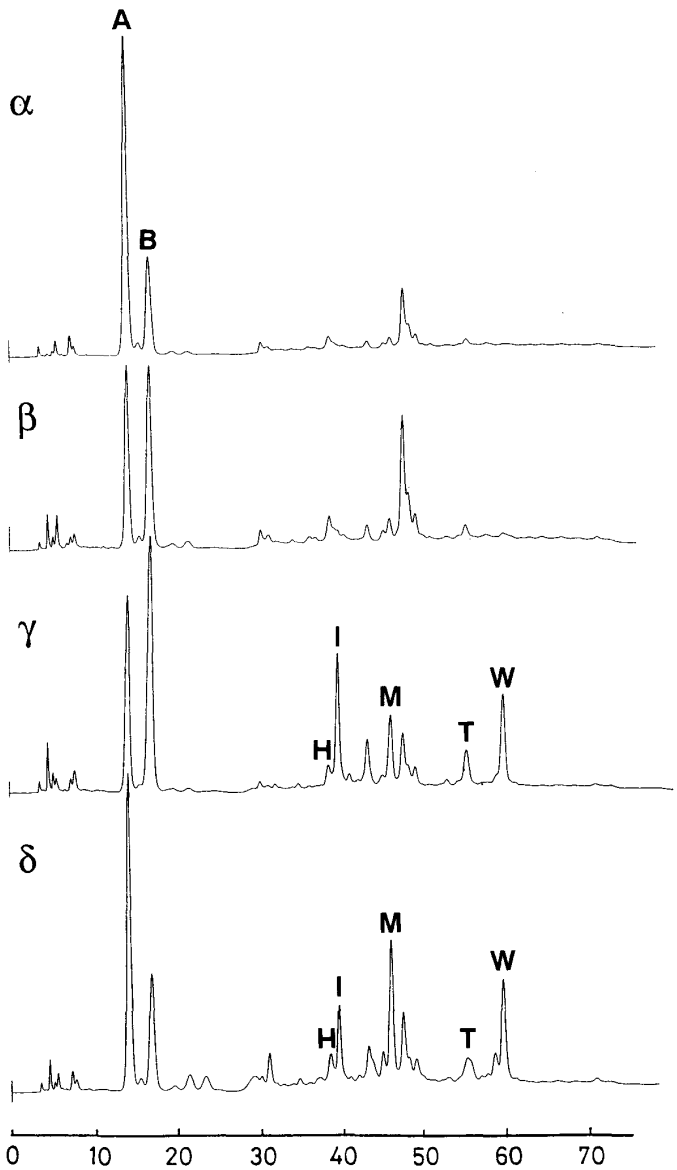

Fig.3. HPLC profiles of pyridylamino-oligosaccharides from AChR subunits. Alphabets in the figure correspond to those in Table 2.

AChR subunits separated from each other by SDS-PAGE were digested sequentially with pepsin, sialidase, and *N*-oligosaccharide glycopeptidase. Oligosaccharides derived from each subunit were subjected to pyridylamination and then to HPLC analysis (Fig.3). Each subunit contained high mannose-type oligosaccharides, A and B, as the main components, while in the γ and δ subunits a variety of complex-type oligosaccharides become more abundant.

Total amounts of the oligosaccharides in each subunit were compared ; the ratio of the sum of the peak area for each subunit is nearly $\alpha : \beta : \gamma : \delta = 2 : 1 : 2 : 3$. This result means that α, β, γ, and δ subunits have one, one, two, and three oligosaccharides, respectively. Please note that the AChR monomer has two α subunits. Thus, the γ subunit is glycosylated only at two sites out of four.

The oligosaccharides in each subunit were further characterized by the susceptibility to glycosidases and by binding to lectins. All the subunits were susceptible to endo-β-*N*-acetylglucosaminidase H (endo-H), indicating that high mannose-type oligosaccharides were present in all the subunits. The molecular weights of the γ and β subunits decreased after sialidase digestion, which was consistent with the fact that complex-type oligosaccharides are abundant in these subunits.

Affinity of each subunit to lectins was examined on a nitrocellulose membrane onto which AChR subunits had been transferred after SDS-PAGE (Fig.4). Con A, which is specific to mannose, bound all subunits, whereas leucoagglutinating phytohemagglutinin (L-PHA), which has high affinity to tri- and tetraantennary oligosaccharides, bound only to the γ and δ subunits. These results were quite consistent with the results of HPLC and glycosidase digestion experiments. On the other hand, wheat germ agglutinin (WGA), which is specific to *N*-acetylglucosamine and *N*-acetylneuraminic acid, bound specifically to the δ subunit in spite of the similar oligosaccharide patterns on HPLC for the γ and δ subunits. Since the lectin bound

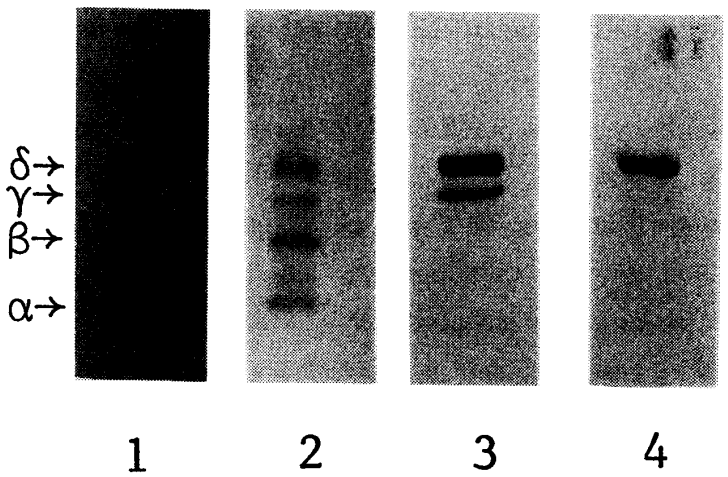

Fig.4. Binding of AChR subunits to lectins.
AChR was electrophoresed, blotted onto nitrocellulose paper, and reacted with Con A (lane 2), L-PHA (lane 3), or WGA (lane 4). Lane 1 was stained amido black for all proteins.

with the δ subunit even after sialidase digestion, *N*-acetylneuraminic acid is not included in this binding. Although the reason for this specific binding is now unknown, Lindstrom *et al.* [24] utilized this specificity of WGA for analyzing the spatial relation of the AChR subunits.

3. Identification of glycosylation sites in *Torpedo* AChR

In general, it is difficult to define glycosylation sites of glycoproteins by chemical methods ; they are usually estimated from specific amino acid sequence, Asn-X-Ser/Thr. The glycosylation sites of AChR were estimated only from the amino acid sequences. Here, we have tried to determine the glycosylation sites of AChR with a combination of lectin column chromatography and protein sequencing. This protein is an interesting example, having different types of oligosaccharides in the same polypeptides.

AChR was digested with pepsin and successively applied to columns of Con A- and L-PHA-Sepharose. The bound glycopeptides were further separated from each other with HPLC. Analysis of each peak with a protein sequencer revealed glycopeptide sequences as shown in Table 2. As mentioned above, there are one, one, two, and three glycosylation sites for α, β, γ, and δ subunits, respectively, so the putative glycosylation sites of γ and δ subunits are termed $\gamma 1$ and $\gamma 2$ or $\delta 1$, $\delta 2$, and $\delta 3$ from the *N*-terminal. As shown in the table, we obtained the glycopeptides corresponding to α, β, $\gamma 1$, $\gamma 2$ and $\delta 1$. The glycopeptides α, β, and $\gamma 2$ were retained by the Con A column, suggesting that they have high mannose-type carbohydrates. On the other hand, the glycopeptides $\gamma 1$ and $\delta 1$ were retained by the L-PHA column, which indicates that these glycopeptides have complex-type carbohydrates. The asparagine residues to which carbohydrates were supposed to link were not detected as PTH-Asn because the linkage between carbohydrate and asparagine was not cleaved in the sequencing process. We obtained no glycopeptide which was retained by the both lectin columns. The glycopeptides $\delta 2$ and $\delta 3$ were not obtained, perhaps due to the low efficiency of pepsin cleavage or the low yield of the peptides.

It is interesting that glycopeptides α, β, and $\gamma 2$ have the same type of oligosaccharides, the high mannose-type. Those sites and $\delta 2$ are in homologous regions in their amino acid sequences. Further, $\gamma 1$ and $\delta 1$ have complex-type oligosaccharides and they are also in homologous regions. Although we can not say that each site is glycosylated exclusively by one type of oligosaccharides, high mannose- or complex-type, it seems probable that homologous sequences have the same type of oligosaccharides. It is probably because their environments around the glycosylation sites, including the secondary and tertiary structures, are similar. The mechanism and significance of the carbohydrate processing in AChR molecule should be resolved in near future.

Table 2. Amino acid sequences of glycopeptides obtained with lectin columns and HPLC aligned with corresponding sequences of each subunit.

Sequences following names of each subunit are those deduced from cDNA cloning. Sequences beneath them are those of glycopeptides isolated by successive lectin columns and HPLC, and determined by aequencer. N shows putative glycosylation site (NXS/T). X shoes a residue which was not determined.

Glycopeptides obtained by Con A column	
α	KSYCEIIVTHFPFDQQNCTMKLGI
	IIVTXFPFDQQXXTMKL
	KSYXEIIVTXFPFDQQXXTMKLGI
β	RSSCTIKVMYFPFDWQNCTMV
	FPFDWQXXTMV
γ2	RSTCPIAVTYFPFDWQNCSLVFR
	XSTXPXAVTYFXFDWQX
	XXTXPIAVTYFPFDXQXXS
Glycopeptides obtained by L-PHA column	
γ1	YRLSWNTSEYE
	YXLXWXTXE
δ1	WYDHRLTWNASEYS
	WYDXXLTXXASE
	WYDXXLTXXAXE
Undetected peptides	
δ2	RSSCPINVLYFPFDWQNCSLVFR
δ3	KKNIYPDKFPNGTNY

4. Solid-phase neurotoxin binding assay for AChR and changes in the binding ability of the receptor with various treatments [42]

The activity of AChR is usually assessed by binding capacity for postsynaptic long neurotoxins such as BGT, which are generally radio-labeled. These neurotoxins bind to AChR irreversibly with high affinity [25]. However, these methods are rather troublesome because of the use of isotopes. Previously we reported an enzyme immunoassay (EIA) for anti-AChR antibodies, where we used horseradish peroxidase (HRP)-labeled BGT for specific labeling of the AChR [48]. A good correlation was observed between titers of anti-AChR antibodies measured with our EIA and conventional RIA. Since this method was practical and easy to perform, we applied this enzyme-labeled conjugate to detect AChR activity.

Our binding assay for AChR is based on the capacity of the receptor to bind enzyme-labeled BGT. The procedures are as follows; purified AChR was adsorbed to the well walls of a microtiter plate and was incubated with HRP-BGT conjugate. Then, HRP activity left in the wells after washing was assayed. BGT and HRP were conjugated according to the periodate method [48]. This system is useful because it avoids use of radioactive isotopes such as ^{125}I and no special equipment is necessary.

With this binding assay, AChR could be detected at concentrations as low as 100 ng/ml in the coating solution and the HRP activity increased depending on the concentration of AChR up to 2 mg/ml. Specific inhibition of HRP-BGT binding by BGT and by carbamylcholine, an agonist of AChR, was observed. Half-maximal inhibition by BGT was observed at almost the same concentration of BGT-HRP. The concentration of carbamylcholine for half-maximal inhibition was greater than that of BGT in accordance with the difference in their binding affinity for AChR [26].

Using our assay, we examined changes in the binding activity of AChR treated with various agents which are known to denature or reduce proteins. Decreased binding ability was observed with AChR treated with 2.5 M urea or 0.6 M NaSCN. The binding ability of AChR treated with 2 % SDS was completely lost. In contrast, the binding ability of AChR treated with 0.6 M NaCl or 3.5 mM dithiothreitol did not change from that of the control AChR. These results show that denaturing reagents such as SDS, urea, or caotropic ion destroy the conformation of AChR, especially of the ligand-binding region. The reduction of the S-S linkage in the AChR does not seem to affect its binding ability, though an S-S linkage between adjacent half-cystinyl residues is known to be included in the acetylcholine and BGT binding site [27, 28]. On the other hand, introduction of the bulky carboxymethyl group to cysteine destroyed the binding ability of AChR. This was shown using reduced carboxymethylated (RCM)-AChR instead of native AChR to coat the solid-phase in our assay. The coating efficiencies of treated AChR were almost the same irrespective of the treatments, which was examined by EIA with RCM-AChR antiserum.

Next, we examined inhibition of BGT-HRP binding to AChR by various antisera. The antisera are against native AChR, asialo-AChR, and RCM-AChR. The anti-RCM-AChR antiserum showed higher reactivity than other antisera, but inhibited the BGT-HRP binding with AChR most weakly. The anti-native AChR and the anti-asialo-AChR sera behaved similarly. These results also indicate that the binding site of RCM-AChR is destroyed and the antiserum contained no antibody targeted to the binding site.

Although our binding assay is simple and useful, a weak point is its requirement of purified AChR for efficient coating of the solid-phase. To overcome this disadvantage, we developed other methods to detect binding activity of AChR. These are based on the sandwiching of the AChR between BGT-HRP and BGT or an antibody against AChR, where BGT or an antibody was used to coat the solid-phase. The sandwiching with BGT and BGT-HRP is based on the fact that AChR contain two ligand-binding sites in one pentamer. As an antibody immobilized on the wells we chose anti-RCM-AChR IgG since it inhibited BGT-HRP binding to AChR most weakly among antisera we tested. With the sandwich assay using BGT and BGT-HRP, we could detect AChR at concentrations as low as 500 ng/ml, and with the assay using anti-RCM-AChR IgG and BGT-HRP the detection limit was about 10 ng/ml, indicating the latter to be far more sensitive than the former. However, these methods have their own merits and can be used effectively. These sandwich assay methods are very useful since with these assays we can detect and quantitate AChR that has not been purified.

5. Participation of carbohydrates in ligand-binding of AChR [29]

Most receptors in the plasma membrane for various ligands are glycoproteins, and the carbohydrate moieties are thought to be involved in their functions. For example, enzymatic removal of the carbohydrate from human placental insulin receptor increases both the affinity for insulin and the tyrosine kinase activity of the receptor [36]. The maximal binding capacity and phosphorylation activity of the epidermal growth factor receptor of A-431 cells are reduced by the removal of its carbohydrates [37]. It was also found that enzymatic deglycosylation of the P_2 receptor from porcine temporal cortex membrane decreases the affinity for prostaglandin D_2 and E_2 [38]. On the other hand, it has been reported that carbohydrates do not contribute significantly to the pharmacological ligand binding properties of α-adrenergic [39], β-adrenergic [40], and neuronal D_2 dopamine receptors [41].

In case of AChR molecule, it was demonstrated that inhibition of glycosylation of AChR by tunicamycin in cultured muscle cells results in a significant decrease in AChR biosynthesis [13, 14]. Further, removal of the carbohydrate moiety from the receptor molecule disrupts the assembly and thereby abolishes surface expression of AChR on oocytes [12]. Additionally, inhibition of carbohydrate processing by 1-deoxynojirimycin or castanospermine results in less receptor

expression in BC3H-1 cells, which was interpreted to be a consequence of a more rapid degradation of the receptor [15, 16]. In the AChR with the incomplete oligosaccharides, the channel opening rate or the rate of agonist dissociation was reduced [16]. An inhibitor of a late processing step (swainsonin) did not produce such alteration [16]. These results suggest that AChR subunits cannot be assembled well or become unstable in the absence of their carbohydrate moiety and, moreover, that correct oligosaccharide processing seems to be required for usual expression of the receptor. Therefore the carbohydrate chains of AChR are considered to be indispensable for the assembly and transport of AChR subunits. It is unclear at present, however, whether or not the carbohydrate chains have some role in the expressed AChR in the plasma membrane.

In order to study whether the carbohydrate chains have some role in the expressed AChR, especially in its ligand-binding ability, we applied our binding assay just mentioned. We examined possible changes in the binding activity of the AChR in the presence of various carbohydrates or after glycosidase digestion. We could proceed with the present study, based on the oligosaccharide structures determined for *Torpedo* AChR [17, 35].

The inhibition of toxin-binding to AChR by various carbohydrates or glycoproteins was examined first. No inhibition was observed with *N*-acetylglucosamine, *N*-acetylgalactosamine, α-methyl mannoside, galactose, or fucose. Fetuin, a glycoprotein with triantennary complex-type oligosaccharides, was not inhibitory, either. On the other hand, the binding was inhibited by the presence of sialic acid, ganglioside, or ovalbumin, which contains high mannose-type oligosaccharides. However, in all cases a relatively high concentration of the inhibitor was needed for the inhibition.

Next, we analyzed the ligand-binding ability of AChR treated with sialidase or endoglycosidases. Sialic acid has a negative charge that could provide a possible negative subsite to interact with a positive charge of the ligand [7]. *Torpedo* AChR is abundant in high mannose-type oligosaccharides, which are susceptible to endo-H [17], and endo-β-N-acetylglucosaminidase F (endo-F) is known to have wider specificity and cleave both complex- and high mannose-type oligosaccharides [43]. Sialic acids of AChR were successfully removed [17], while endo-H or endo-F could remove half of the oligosaccharide chains at most. We could not see any significant difference in ligand-binding ability between intact and digested AChR. In order to assess any possible change in affinity for an agonist, we performed a competition experiment with carbamylcholine. No difference could be observed in the mode of inhibition by the ligand between intact and digested AChR.

Finally, the inhibition of toxin-binding to AChR by $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$, the main oligosaccharides of AChR, or by bi-, tri-, and tetraantennary oligosaccharides, which also exist in AChR, was examined. These oligosaccharides at a concentration up to 1 μM did not inhibit the binding of HRP-BGT to AChR.

The present result seems to contrast with that of Zeghloul *et al.* [50]. They showed that removal of asparagine-linked oligosaccharide chains from *Torpedo marmorata* electric organ membrane by endo-F or endo-H partially inhibited the binding of α-neurotoxin I from *Naja mossambica mossambica* snake venom to the membrane under conditions where about 70 % of concanavalin A binding to the membrane was lost. The discrepancy between their result and ours might be caused by the difference in type of neurotoxin used as a probe (BGT *vs.* α-neurotoxin I) or by the difference in the form of AChR used (purified AChR *vs.* electric organ membrane). The latter seems especially plausible since AChR may interact with many other proteins and lipids in the membrane. The interaction of the receptor with other proteins or lipids would have distinct effects on its function. Also, another glycoprotein in the vicinity of AChR, whose oligosaccharides was removed by endo-H or endo-F, might be important for α-neurotoxin I to bind to the AChR.

Recently it was reported that functional AChR expressed in oocytes was glycosylated but the oligosaccharides were exclusively of the high mannose type, which is different from the native AChR isolated from the electric organ [11]. This result appears to suggest that the fine structures of the carbohydrate moieties do not play an important role in AChR function.

Desialylated (asialo)-AChR seems to be similar to native AChR because BGT-HRP bound equally with the both AChR and asialo-AChR, and anti-asialo-AChR and anti-native AChR sera behaved in the same way in binding to AChR and in inhibition of BGT-HRP binding to AChR. However, AChR and asialo-AChR were different from each other in the following antigenic properties. When we injected rabbits with native AChR, muscle weakness was observed in the rabbits and they soon died after the second injection which was conducted two weeks after the first injection. In contrast, in rabbits injected with asialo-AChR such symptoms were observed only after the fourth injection with the injection interval of two weeks. These results suggest the possibility that sialic acids in AChR or conformatione perturbed by the removal of sialic acids are responsible for the occurrence of experimental myasthenia gravis, although further study is required.

6. Effects of cholinergic ligands on tyrosine phosphorylation of AChR [30]

Protein phosphorylation and dephosphorylation have been widely accepted as one of the principal regulatory mechanisms in the control of biological processes. They are also involved in the modulation of synaptic transmission and have been shown to regulate the function of ion channels and receptors, including the AChR [51]. The postsynaptic membrane fraction enriched in AChR prepared from *Torpedo californica* is reported to contain at least four different protein kinases: cAMP-dependent protein kinase [52-54], protein kinase C [55, 56], tyrosine-specific protein kinase [57], and calcium/calmodulin kinase [58]. Three of these kinases have been shown to phosphorylate AChR subunits *in vitro* : the cAMP-dependent protein kinase phosphorylates the

γ and δ subunits; the protein kinase C, the α and β subunits; and the tyrosine-specific protein kinase, the β, γ, and δ subunits.

The functional significance of phosphorylation of AChR has been examined with purified and reconstituted AChR [59]. Phosphorylation of the γ and δ subunits by cAMP-dependent protein kinase increased the rate of desensitization of the receptor, the process by which the receptor is inactivated in the presence of a cholinergic agonist [60]. The increase in desensitization rate was also observed when AChR was phosphorylated by protein kinase C [61] or by tyrosine-specific protein kinase [62]. Although the identity of the first messengers that regulate the phosphorylation of AChR has been ambiguous, it was recently suggested that calcitonin gene-related peptide (CGRP), a neuropeptide that coexists with acetylcholine in the motor nerve endings, regulates AChR desensitization by a mechanism that involves cAMP-dependent phosphorylation of the AChR [63, 64]. No explanation has been made concerning how the activities of tyrosine kinase or kinase C are regulated. Recently, a protein, agrin, which is an extracellular matrix protein, has been reported to increase tyrosine phosphorylation of the β subunit of the AChR prior to clustering in chick myotube cultures [31].

AChR-rich membrane, prepared from the electric organ [65], and purified AChR were applied to SDS-PAGE, electroblotted onto a nitrocellulose membrane, and reacted with anti-phosphotyrosine monoclonal antibody or polyclonal antiserum. The β and δ subunits reacted with these antibodies. The specificity of the immunoreaction was confirmed based on the specific inhibition by 1 mM phosphotyrosine, on the resistance of the reaction to 1 N NaOH at 37 ℃ for 30 min, under which conditions phosphoserine is unstable, and on its lability to alkaline phosphatase digestion. Both the β and δ subunits retained the reactivity to the antibodies in their peptides obtained by digestion with V8 protease. The present results demonstrate that the β and δ subunits of the *Torpedo* AChR is phosphorylated on its tyrosine residues *in vivo*, which is different from the *in vitro* results [57]. Overexposure revealed a faint band corresponding to the γ subunit, which means that a minor fraction of the γ subunit is also phosphorylated *in vivo*.

Next, we examined the possibility that cholinergic ligands might affect the tyrosine phosphorylation level of AChR subunits in the AChR-rich membrane fraction. Incubation with neurotoxins such as BGT or cobrotoxin or with an agonist such as carbamylcholine for 30 min at 37 ℃ enhanced the intensities of the bands of the β and δ subunits. The β subunit was more highly phosphorylated than the δ subunit by both neurotoxins ; maximal enhancements were approximately 5-fold for the β subunit and 2-fold for the δ subunit with 14 pM of toxin. On the other hand, carbamylcholine enhanced phosphorylation of both subunits almost equally ; the maximal enhancement was about 3-fold for both subunits, though a higher concentration (-90 mM) was necessary than those of the toxins. Phosphopeptide analysis by V8 protease digestion revealed that the phosphorylation sites of the β and δ subunits induced by the cholinergic ligands were the same as those of the native AChR. Presence of 5 mM ATP in the reaction

mixture only slightly increased the intensities of the bands, indicating that the AChR-rich membrane contains ATP or energy-rich source for the phosphorylation. This enhancement of tyrosine-phosphorylation was completely lost in the presence of 0.1 % NP-40 in the reaction mixture. This indicates that the enhanced intensities of bands certainly reflect the enhancement of tyrosine-phosphorylation, since NP-40 is reported to block tyrosine phosphorylation on AChR [57].

It is interesting that the mode of phosphorylation is quite different between the agonist and the antagonists, while it is almost the same for the neurotoxins regardless whether it is a long neurotoxin (BGT) or a short neurotoxin (cobrotoxin). The time dependencies of phosphorylation by the ligands were almost the same for both subunits. Among the ligands, carbamylcholine enhanced phosphorylation most rapidly ; 3 min of incubation enhanced tyrosine-phosphorylation to 50-60 % of maximum value and the reaction reached maximum in 10-15 min.

The mechanism of enhancement of tyrosine phosphorylation by cholinergic ligands is presently unknown. One possible mechanism is that some ligand-induced conformational change in the AChR makes the receptor more susceptible to tyrosine kinase. Another possibility is that the cholinergic ligands directly activate the tyrosine kinase. Anyway, the above results suggest the possibility that a cholinergic agonist physiologically regulates phosphorylation of the AChR *in vivo*. Since it is known that the presence of an agonist induces a desensitized state of AChR [60], this ligand-induced phosphorylation is likely linked to receptor desensitization.

There are fast (millisecond to second time scale) and slow (second to minute time scale) types of inactivation of the AChR channel by the agonists [60]. The enhancement of tyrosine phosphorylation by carbamylcholine seems to correspond to the slow inactivation. Since it is reported that phosphorylation of *Torpedo* AChR by an endogenous protein tyrosine kinase increased the rate of AChR desensitization [62], the following cascade may be envisioned : the agonist induces the fast desensitization through activation of cAMP-dependent protein kinase [59], which then induces tyrosine phosphorylation of the receptors, and the phosphorylated receptors tend to be desensitized. If this is the case, tyrosine phosphorylation of the AChR may function to maintain the desensitized state. Further research is obviously needed to explain the mechanism of receptor desensitization and its relation to phosphorylation of the receptor.

7. Crosslinking of proteins in AChR-rich membrane [32]

Several peripheral membrane proteins associated with the cytoplasmic face of the postsynaptic membrane have been identified [68]. The most extensively studied of the postsynaptic peripheral membrane proteins is 43-kD protein that exists at an equal stoichiometry with AChR in the postsynaptic membrane [69]. Accumulating evidence suggests a direct role for the 43-kD protein in the clustering of the AChR [44-47]. Much less is known about relatively minor postsynaptic proteins with a molecular masses of 87-kD and 58-kD, which are also concentrated at AChR-rich

sites in the electric organ and in mammalian muscle [70]. Dystrophin, the gene product of the Duchenne muscular dystrophy locus, also localizes to the postsynaptic membranes of the electric organ and over the entire inner surface of the sarcolemma in skeletal muscle [34]. Its deduced amino acid sequence predicts a 427-kD protein belonging to the spectrin superfamily and its C-terminal domain is homologous to 87-kD protein [34]. Those proteins are proposed to form a complex and participate in the immobilization of the AChR.

In order to study the interaction of the peripheral proteins with AChR, we utilized bifunctional crosslinkers to view the spatial relationships of these molecules. Since biotin-labeled maleimide reacted mainly with 43-kD protein in the AChR-rich membrane, free sulfhydryls were found to exist mainly in the 43-kD protein. Therefore, we used bifunctional crosslinkers which bind both free sulfhydryls and amines to find which subunit of the AChR or other proteins exist in the neighborhood of the 43-kD protein. It is known that all AChR subunits are rich in lysine within their predicted cytoplasmic domains [1]. The crosslinkers were 4-(*p*-maleimidophenyl)butyrate (SMPB, 14.5 Å span between reactive groups), 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, 11.6 Å span), and *m*-maleimidobenzoyl *N*-hydroxysulfosuccinimide ester (MBS, 9.9 Å span). There was no significant difference in the following results among the linkers.

AChR-rich membrane was incubated for 30 min at 22 ℃ in the presence of one of the bifunctional crosslinkers and analyzed by SDS-PAGE. With increasing concentration of crosslinker, crosslinked proteins accumulated at the top of the stacking gels and every protein band became faint, but among the AChR subunits the γ subunit diminished the most noticeably. The γ subunit band became faint almost at the same rate as the 43-kD protein. In these experiments *Torpedo* dystrophin was also found to diminish as effectively as the 43-kD protein and the γ subunit.

The crosslinked products were examined by immunoblot analysis. The crosslinked products were first found in the 110-kD region, which was recognized by both the anti-43-kD protein peptide antibody and by anti-RCM-AChR antibody. Then the 110-kD crosslinked products diminished, and the products moved to the top of the stacking gels, which was recognized by anti-43-kD, anti-dystrophin, and anti-RCM-AChR antibodies.

The 43-kD protein appears to be located in close proximity to dystrophin and the γ subunit, but not the β subunit of AChR. Burden *et al.* [69] reported that the crosslinked product composed of the 43-kD protein and the β subunit of the AChR appeared at the 110-kD region on SDS-PAGE in spite of the same reaction conditions as ours. In our experiment we did not observe any difference among the behaviors of the α, β, and δ subunits. The reason for this discrepancy is unknown. The nature of the arrangement of AChR, 43-kD protein, and *Torpedo* dystrophin is important for defining their possible function.

8. A neurotoxin-binding protein in the electric organ of *Torpedo californica* [33]

When we prepare AChR from the electric organ, we first ultracentrifuge the homogenate of the organ to obtain the membrane fraction as a pellet and then solubilize this pellet with a detergent. Then, the solubilized solution is subjected to a second ultracentrifugation, and the supernatant is applied to an neurotoxin-affinity column. This protocol is followed because AChR is a transmembrane protein, and detergent treatment is indispensable to solubilize the receptor.

We applied our solid-phase binding assay [42] to measure neurotoxin-binding activity in each fraction obtained in the course of preparing AChR. The supernatant of the first ultracentrifugation was found to have a significant amount of activity, so we tried to characterize the activity.

A portion of the supernatant was incubated with ^{125}I-BGT and applied to gel chromatography using a Sepharose CL-6B column. The radioactivity separated into five peaks. The first (largest in size) and the second eluting peaks diminished in the presence of cold BGT in the preincubation mixture, indicating that their binding with the toxin is specific. On the other hand, the radioactivity of the fourth peak increased in the presence of cold BGT, which means this peak corresponds to free ^{125}I-BGT. The radioactivity of the third and fifth peaks did not change by cold BGT, indicating these fractions bound non-specifically with the radioactivity. These peaks were not studied further. The second peak was assigned to AChR since it eluted at the same position as the activities in AChR-rich membrane and AChR itself, which were preincubated with 125I-BGT. The second peak also bound to anti-AChR antiserum and Con A in the same fashion as AChR. The AChR molecule may reside in well-dispersed microparticles and not sediment by the ultracentrifugation.

The AChR-rich membrane did not have the first peak observed in the supernatant. This large toxin-binding component in the supernatant were also detected when the supernatant was directly applied to the gel chromatography without Triton X-100 pretreatment or preincubated in 3% Triton X-100 for an extended period (3 days) before the chromatography.

Each fraction resulting from Sepharose CL-6B chromatography was examined with respect to its binding capacity with anti-RCM-AChR antiserum and Con A. AChR binds well to the antiserum and Con A. The anti-RCM-AChR antiserum bound with the earliest eluting peak in addition to the AChR-like peak in the supernatant. However, the extent of the binding with these peaks was less than that with the AChR peak. This indicates that these peaks are immunologically related to AChR. In contrast, only a small fraction of the supernatant first peak bound to Con A. When we applied the whole supernatant fraction to an anion exchange column at pH 7.4, about 80% of the toxin-binding activity was recovered in the pass-through fraction, while AChR was completely adsorbed by the column in the same condition.

The binding activity in the supernatant was calculated with the liquid-phase assay using anti-

RCM-AChR antiserum, and was about 50 pmol BGT/100 g of electric organ, which corresponds to one 400th of that of AChR in an equivalent amount of the organ, though we have to consider the low cross reactivity of the supernatant fraction.

The newly found toxin-binding activity in the supernatant is immunologically related to AChR, but is different from AChR: larger apparent molecular weight, less reactivity to Con A, and different isoelectric point. There are many possibilities for the existence of such a molecule: one is that this large component is composed of the same peptides as those of AChR (the α subunit must be included) but the processing or assembly mode following protein synthesis is different; the component might be an imperfect version of the AChR or it might be a complex composed of AChR subunit(s) and other proteins; another possibility is that the gene of the high molecular weight component is different from those of AChR, though they are related.

It has been suggested that acetylcholine may have a presynaptic effect on neuromuscular transmission, which is supposedly mediated through a presynaptic AChR [67]. The presynaptic AChR is presumed to serve a negative feedback role in controlling transmitter release : residual acetylcholine inhibits transmitter release and blockade of presynaptic AChR by *d*-tubocurarine enhances transmitter release [67]. It is also reported that BGT doubles the acetylcholine output from the phrenic motor nerve terminals [49] and that myasthenic serum or a monoclonal anti-AChR antibody, which blocks binding of BGT to AChR, facilitates quantal acetylcholine release [66]. These observations show that the presynaptic AChR includes binding site(s) for cholinergic ligands and immunological similarity to postsynaptic AChR. Thus it is interesting to consider the possibility that the supernatant components constitute such a presynaptic AChR.

Conclusion

Recent progress in biotechnology, especially recombinant DNA methodology, has enabled researchers to characterize AChR in detail. We have learned that AChR is structurally related to and form a superfamily with receptors responsive to GABA, glycine, and serotonin. The mechanism of AChR function is now well understood, but there still remain some problems to be resolved: for example, molecular mechanism to convert acetylcholine-binding to channel-opening, interaction between AChR and surrounding proteins and lipids, etc. The carbohydrate structure of AChR unveiled by the present study would be useful for studying the fine regulatory mechanism of AChR function and its interaction with other molecules. The enhancement of tyrosine phosphorylation of AChR subunits by cholinergic ligands, the interaction between AChR and the 43-kD protein, and newly found neurotoxin-binding activity are helpful for understanding molecular mechanisms underlying AChR function.

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