77. *Characterization of Muscarinic Acetylcholine Receptors as Glycoproteins*

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(Communicated by Setsuro EBASHI, M. J. A., Nov. 12, 1983)

Muscarinic acetylcholine receptors (mAChRs) are now thought to consist of a group of different subclasses.¹⁾ The evidences for this have been provided mainly from functional or pharmacological studies. For example, the mAChRs can mediate either excitatory, inhibitory, or metabolic responses, and are pharmacologically distinguishable in the sense of affinity to agonists or antagonists.²⁾ Nevertheless, no direct evidence suggesting the structural heterogeneity of the mAChRs has been so far available.

As many of the membrane proteins do, some surface receptors for neurotransmitters or neurotoxins have been shown to possess glycosyl residues. All the polypeptide subunits of nicotinic acetylcholine receptors are known to glycosylated.³⁾ Purified GABA/benzodiazepine receptor molecules are glycoproteins, and bind to concanavalin A (Con A).⁴⁾ Saxitoxin receptors of the sodium channel bind to wheat germ agglutinin (WGA).⁵⁾ In the present study we investigated the properties of the mAChRs focusing on their nature of glycosyl residues. For this purpose, we carried out a screening of lectins to examine whether any of them could interact with the detergent-solubilized mAChRs from porcine brains. The results indicated that a major population of the mAChR molecules are glycoproteins which have strong affinity to WGA. Furthermore, the results also showed the existence of a minor population of the receptors which are not able to interact with the lectin, suggesting that there may exist structural heterogeneity of the receptors in terms of glycosyl residues.

Porcine brain tissue was obtained from a local slaughter house. Crude mitochondrial (P_2) fraction was prepared according to the method of Cotman⁶⁾ using 0.32 M sucrose in 10 mM Tris/HCl (pH 7.4) supplemented with 1 mM EDTA and 0.5 mM PMSF as homogenizing buffer. The fraction was then labeled with [³H]quinuclidinyl benzilate (QNB) for 45 min at 25°C in phosphate buffered saline (PBS). Under our conditions, at least 95% of labeling was specific,

i.e. displacable by 10 μ M atropine. The labeled receptors were washed, and solubilized with 1.0% Lubrol-PX in PBS for 60 min at 4°C. Supernatants resulting from centrifugation at 100,000 $\times$ g for 60 min were gel-filtered on Sephacryl S-300 to eliminate free ligands and used as labeled and solubilized receptors.

Eight kinds of lectins with different carbohydrate specificities, which are listed in the legend to Fig. 1, were examined for their abilities to interact with the solubilized receptors as follows. The lectins were coupled to CNBr-activated Sepharose 4B, and each of them was incubated with [3 H]QNB-labeled and Lubrol-PX-solubilized mAChRs in buffered saline in the presence or absence of inhibitory sugar. After the incubation for 4 h at 4°C with gentle agitation, the reaction mixture was centrifuged and the radioactivity of the supernatant was measured by a liquid scintillation counter. The amount of the receptors bound to the gels was determined by subtracting the radioactivity of the supernatant from that was initially added.

Among the lectins tested, WGA was found to bind the receptors (Fig. 1). The binding was completely inhibited by N-acetylglucosamine (GlcNAc), an inhibitory sugar for WGA. Other lectins except

Fig. 1. Interaction of solubilized mAChRs with immobilized lectins. [3 H]QNB-prelabeled and Lubrol-PX-solubilized membranes were added to each lectin-Sepharose 4B suspension in the absence (open column) or presence (shaded column) of the inhibitory sugar and incubated for 4 h at 4°C. The lectins examined are wheat germ agglutinin (WGA), jack bean lectin (Con A), lentil lectin (LcA), castor bean lectin (RCA), horse gram agglutinin (DBA), peanut agglutinin (PNA), soybean agglutinin (SBA), and whin seed agglutinin (UEA). The inhibitory sugars are N-acetyl-D-glucosamine (GlcNAc), α -methyl-D-mannoside (α -MM), D-galactose (Gal), N-acetyl-D-galactosamine (GalNAc) and L-fucose (Fuc). Results of two experiments were shown. Exp. 1 was carried out with gels whose lectin capacity was 7.5 mg/ml, and with 0.04% Lubrol-PX in Tris buffered saline (pH 7.4) supplemented with 0.4 mM CaCl_2 and 0.4 mM MnCl_2 . Exp. 2 was performed with gels whose lectin capacity was 1 mg/ml, and with 0.08% Lubrol-PX in phosphate buffered saline (pH 7.2). Each value represents the mean of three samples with standard deviation. Asterisk represents $P \leq 0.001$.

for Con A did not bind to the receptors at all. The fact that Con A showed a slight ($p < 0.005$) interaction with the receptors suggests that mannose residues may also be present in carbohydrate moieties of the receptors. Since glycosyl residues of high mannose type have strong affinities for Con A, glycosyl residues of mAChRs may not belong to this type. Presumably, glycosyl residues of mAChRs to which WGA binds consist of complex type carbohydrate chains containing di-N-acetyl chitobiose structure linked to aspartic residues of the protein moiety.

We tried to absorb most of [^3H]QNB-labeled and Lubrol-PX-solubilized mAChRs by addition of an excess amount of WGA-Sepharose. A portion of the receptors, however, was left unbound to the immobilized lectin. The binding isotherm indicated that only about 70% of the receptors were adsorbed even when WGA was added at a saturation level. In order to investigate whether there existed any muscarinic receptor populations which were incapable of binding to WGA, we passed the [^3H]QNB-labeled and solubilized preparation through a column of excess amount of WGA-Sepharose, and fractionated into two fractions, one which binds to the column and the other which flows through. Most of the radioactivity which was bound to the WGA-Sepharose column was recovered by eluting out with 0.2 M GlcNAc. When this fraction (GlcNAc-eluate) was re-analysed for its binding to WGA-Sepharose, significantly larger proportion of the radioactivity was found to bind compared to unfractionated control preparations (Fig. 2). On the other hand, the flow-through fraction of the WGA-column showed no interaction at all with WGA-Sepharose when re-analysed in the same way. Since [^3H]QNB binds reversibly to and is gradually dissociated from the receptors, increase of free [^3H]QNB leads to decrease in apparent maximal binding of the radioactivity to WGA-Sepharose (this may explain at least partly why the maximal binding of the radioactivity in the eluate was less than 100%). Gel filtration analysis of the flow-through fraction showed that at least 30% of the tritium radioactivity was associated with proteins. If these labeled proteins were bound to WGA to the same extent as receptors in the control fraction, at least about 20% of the radioactivity should have been bound to WGA-Sepharose gels. However, the results (Fig. 2A, open squares) indicated that this was not the case.

These results strongly suggest the existence of at least two populations of mAChRs, a major one which binds to WGA and a minor one which cannot bind to the lectin. In other words, there may exist a structural heterogeneity of the receptors in terms of glycosyl residues, although it is not clear at present whether this

Fig. 2. Interaction of the fractionated mAChR populations with WGA-Sepharose. $[^3\text{H}]\text{QNB}$ -labeled and solubilized mAChRs were fractionated by passing through a WGA-Sepharose column. The flow-through fraction ($\square$), the fraction which bound to and was eluted from the column with 0.2 M GlcNAc (GlcNAc-eluate, $\blacksquare$), or unfractionated control mAChRs ($\circ$) was examined for the binding to WGA-Sepharose as in the legend to Fig. 1. A: Binding curves. B: Double reciprocal plots of the binding curves. Each value represents the mean of triplicate samples.

heterogeneity is physiologically significant or not. The relationship between these "structural" subclasses and so-far reported "functional" or pharmacological subclasses remains to be established.

The finding that the majority of mAChRs can bind to WGA provides a simple and convenient method of affinity chromatography to purify, at least partly, this class of the mAChRs. Preliminary results showed that 12-fold purification of mAChRs with 13% recovery was attainable with the WGA-Sepharose column, indicating its potential usefulness for the mAChR preparation especially in a large scale.

We thank Drs. T. Osawa of Univ. of Tokyo and T. Ozaki of N.I.P.S. for their kind help to this work.

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