Distance between the propylbenzylcholine mustard attachment site and carbohydrates and thiol groups in muscarinic acetylcholine receptor protein from rat cerebral cortex*

Daniel M. ALPERIN, Cecilia B. BOUZAT and Francisco J. BARRANTES†

Instituto de Investigaciones Bioquímicas, Universidad Nacional del Sur/Consejo Nacional de Investigaciones Científicas y Técnicas, 8000 Bahía Blanca, Argentina

When rat cerebral-cortex membranes were labelled with [³H]propylbenzylcholine mustard ([³H]PrBCM), a single protein of *M_r* 68 000 was found to carry the atropine-sensitive covalent label. After trypsinolysis of the receptors solubilized in 0.075% SDS, the resulting fragments were submitted to size analysis in combination with wheat-germ agglutinin (WGA)–Sephacrose and organomercurial–agarose chromatography. Peptides of *M_r* 75 000, 50 000, 30 000, 18 000 and 8000 were specifically released from the receptor. All fragments above *M_r* 8000 were able to bind WGA–Sephacrose and therefore the peptide of *M_r* 18 000 was taken as the upper limit of the distance between the antagonist and the glycan moieties. The limit fragment of *M_r* 8000 carried chemical groups which were modified by *N*-ethylmaleimide and reacted with an immobilized organomercurial. About 65–80% of the labelled receptors were adsorbed on concanavalin A–Sephacrose with low affinity, generating two further components after sequential application to WGA–Sephacrose. About 50% of the receptors were susceptible to neuraminidase treatment, with a concomitant slight modification of the SDS/polyacrylamide-gel-electrophoretic pattern.

INTRODUCTION

In the central nervous system the most abundant cholinergic receptor (AChR) is of the muscarinic (m) type (Krnjevic, 1974). The mAChRs of brain and other tissues have been characterized as glycoproteins of the ‘N-Asp’ type (Gavish & Sokolovsky, 1982; Scott Herron & Schimerlick, 1983; Shirakawa *et al.*, 1983; Peterson *et al.*, 1986; Rauh *et al.*, 1986), and one role of the glycan moiety has been elucidated (Conrad Liles & Nathanson, 1986). Another important structural feature with functional consequences is the occurrence of thiol groups in the mAChR, their modification with thiol reagents and heavy metals, resulting in changes in the affinity state of the receptor (Aronstam *et al.*, 1978). The position of the carbohydrate moiety (Sharon, 1984) and thiol groups (Malbon *et al.*, 1987) along the polypeptide backbone is a rather constant feature in the various mAChRs whose primary structures are now known (Kubo *et al.*, 1986a,b; Peralta *et al.*, 1987), thus providing intramolecular landmarks which serve as reference points for other structural features. The present work deals with the following aspects of mAChR from rat cerebral cortex labelled with tritiated propylbenzylcholine mustard ([³H]PrBCM): (1) *M_r* of the glycopeptides carrying the [³H]PrBCM; (2) estimation of the distance between the irreversible antagonist and sugar moieties; (3) identification of thiol groups located close to the binding site; and (4) properties related to the glycan structure and heterogeneity of the glycoprotein. The data are compared with the theoretical expectations

for the primary sequence of the M1 subtype of the mAChR (Kubo *et al.*, 1986a), and the implications are discussed.

EXPERIMENTAL

Materials

[propyl-2,3-³H]Propylbenzylcholine mustard hydrochloride (43–58 Ci/mmol), Protosol and Aquasol-2 were purchased from New England Nuclear. The following compounds were obtained from Sigma: aprotinin, phenylmethanesulphonyl fluoride, bovine serum albumin, dithiothreitol, *N*-ethylmaleimide, concanavalin A–Sephacrose 4B, WGA–Sephacrose 6MB, neuraminidase from *Clostridium perfringens*, Tos-Phe-CH₂Cl-treated trypsin and all the proteins used as electrophoretic standards. Bio-Gel P-10, Affi-Gel 501, urea, acrylamide and bisacrylamide were purchased from Bio-Rad.

Preparation of membranes

Cerebral cortices were obtained from adult Wistar rats (250 g) and rapidly homogenized (9 vol./vol. of cortex) in cold 0.32 M-sucrose/10 mM-Tris/HCl buffer, pH 7.4, containing the following proteinase inhibitors: 1 mM-EDTA, aprotinin (8 µg/ml) and 0.1 mM freshly prepared phenylmethanesulphonyl fluoride. All subsequent steps were performed at 4 °C. The homogenate was centrifuged at 1090 *g* for 10 min. The resulting supernatant was centrifuged at 15000 *g* for 30 min (Hulme *et al.*, 1978). The pellet thus obtained was then resuspended, to a final

Abbreviations used: Con A, concanavalin A; mAChR, muscarinic acetylcholine receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-propane-1-sulphonic acid; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide-gel electrophoresis; PrBCM, propylbenzylcholine mustard; WGA, wheat-germ agglutinin; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane (‘TPCK’); DTT, dithiothreitol.

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† To whom correspondence and reprint requests should be sent, at the following address: INIBIBB, C.C. 857, camino de la Carrindanga km 7, 8000 Bahía Blanca, Argentina.

protein concentration of 10 mg/ml, in 20 mM-Tris/HCl buffer, pH 7.4, containing 0.9% NaCl. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The membrane preparation was divided into small aliquots and frozen at -80°C until use.

AChR labelling

Precyclized [^3H]PrBCM (Burgen *et al.*, 1974) was added to the membrane suspension (1 mg of protein/ml) to give a final concentration of 10 nM (Dadi & Morris, 1984), either with or without 1 μM -atropine in a total volume of 1 ml of 20 mM-Tris/HCl buffer, pH 7.4, containing 0.9% NaCl. Incubation was carried out for 30 min at 30°C . At the end of the labelling period the suspension was centrifuged at 15000 *g* for 3 min (Hulme *et al.*, 1978), washed twice with the above buffer and submitted to the procedures indicated below.

Saturation of specific muscarinic binding was achieved at 1 nM of [^3H]quinuclidinyl benzilate, representing 0.82 ± 0.12 pmol/mg of protein (mean $\pm$ S.D. for seven different preparations). Labelling with [^3H]PrBCM under the conditions described above gave specific muscarinic binding of 0.28–0.32 pmol/mg of protein, thus resulting in a fractional occupancy of about 0.4 for different batches of the tracer. The specific/non-specific binding ratio for [^3H]PrBCM was about 1:1.2, as described by other authors (Dadi & Morris, 1984).

Solubilization of [^3H]PrBCM-labelled receptors

Membrane samples were heated at 100°C for 3 min with 0.1 ml of 2% SDS. Subsequently, the solution was adjusted to 0.075% SDS with 50 mM-Tris/HCl buffer, pH 7.4, containing 0.02% NaN_3 .

In some cases solubilized membranes were centrifuged at 100000 *g* for 1 h, and the resulting supernatant was filtered through 0.2 μm -pore-size membrane. Samples obtained before and after the application of the latter procedure were submitted to exclusion chromatography on Bio-Gel P-10 in order to separate the non-binding radioligand, as indicated below. It was found that 95% of the labelled receptors were solubilized from the membranes by SDS.

Reduction, alkylation and desalting of mAChRs

When indicated in the Results section, samples solubilized in 0.075% SDS/50 mM-Tris/HCl (pH 7.4)/0.02% NaN_3 were reduced with 20 mM-dithiothreitol at 100°C for 3 min. They were then rapidly cooled to room temperature and alkylated by incubation with 50 mM-NEM for 30 min at 30°C . Desalting was carried out at room temperature on a bed (15 cm $\times$ 2.5 cm) of Bio-Gel P-10 equilibrated in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.075% SDS and 0.02% NaN_3 . The atropine-sensitive radioactive material was eluted in the void volume, whereas salts and free [^3H]PrBCM were included in the gel. Similar procedures were followed with the peptides obtained by proteolytic degradation of labelled receptors, taking advantage of the fact that they were desalted in this type of gel.

Lectin affinity chromatography

The procedure followed was essentially as previously described (Alperin *et al.*, 1986). Briefly, Con A–Sephacrose and WGA–Sephacrose columns (3 cm $\times$ 0.8 cm) were used at room temperature. The buffer consisted of 50 mM-

Tris/HCl, pH 7.4, containing 0.075% SDS and 0.02% NaN_3 . Flow rates of 0.6 ml/min were used. The sugars employed for the specific elution of the columns are indicated in the Results section. In all cases the columns were regenerated by an alkaline–acid cycle. When peptides derived from labelled receptors were analysed for their WGA–Sephacrose-binding capacity, a 6 cm $\times$ 0.8 cm bed was used. The elution was carried out with 250 mM-GlcNAc (*N*-acetylglucosamine) after extensive washing with buffer alone and buffer containing 250 mM-NaCl until the background of radioactivity was reached. In a separate series of experiments it was determined that free [^3H]PrBCM, as well as the non-binding radioligand derived from labelled membranes, were not retained on WGA–Sephacrose.

Polyacrylamide-gel electrophoresis

Samples were electrophoresed in 1.5 mm-thick slab gels at 18°C by using one of the following procedures.

(a) The discontinuous buffer system of Laemmli (1970) at fixed acrylamide concentration either with or without the addition of 4 M-urea, as described elsewhere (Rauh *et al.*, 1986), was used. In the latter situation the sample buffer was made 8 M in urea. Gels were stained with Coomassie Brilliant Blue in methanol/acetic acid/water (45:9:46, by vol.), sliced and submitted to radioactivity measurement as described below.

(b) To estimate the size of proteolytic fragments, a wide-*M_r*-range electrophoretic system was prepared and polymerized as described by Falke *et al.* (1985). The stacking gel was composed of 7.5% (w/v) acrylamide, 0.19% (w/v) bisacrylamide, 24% (w/v) urea and 0.1% (w/v) SDS. The running gel was prepared with 16% acrylamide, 0.5% bisacrylamide, 21.6% urea, 13.3% (v/v) glycerol and 0.1% SDS. Since the eluate obtained from the WGA–Sephacrose step had to be analysed with the latter system, the following procedure was implemented. All samples were made of the same buffer composition in a final volume of 3 ml and then concentrated to 0.8 ml under a stream of N_2 at 37°C for about 3 h. The advantage of this procedure compared with freeze-drying is that samples containing a large amount of GlcNAc remain soluble and are reduced in volume within a shorter period. At the end of the concentration step the pH was verified to be 7.4 in all samples. Subsequently, the concentrated samples were adjusted to 8 M-urea, 5% (v/v) 2-mercaptoethanol and 0.005% Bromophenol Blue (as tracking dye). The final concentration of SDS in the samples was approx. 0.2%.

The entire procedure was also applied to the electrophoretic standards and non-proteolysed receptors. A similar pattern was found in the gels whether or not they were submitted to the concentration step. The samples were loaded in three to five wells of the slab gel, each one corresponding to a maximum of 200 μg of protein. Upon completion of a run the gels were immediately fixed in 10% trichloroacetic acid containing 3% 5-sulphosalicylic acid for 5 h, then stained and washed as described above.

Enzymic treatments

Non-reduced, non-alkylated, membranes solubilized in 0.075% SDS were treated with neuraminidase in the presence of 1 mM-EDTA at 37°C under the conditions indicated in the Results section. The reactions were stopped by heating at 100°C for 3 min, and the samples

were analysed by affinity chromatography on WGA-Sepharose or concentrated as described above and examined by SDS/urea/PAGE (Rauh *et al.*, 1986).

Non-reduced, non-alkylated, solubilized membranes (3 mg of protein in 9 ml) were supplemented with Tos-Phe-CH₂Cl-treated trypsin at an enzyme/protein (w/w) ratio of 1:20. Incubation was performed for 1–48 h in the presence of 0.02% NaN₃ under a toluene-saturated atmosphere. As a control, samples without proteinase were also incubated for the same period under similar conditions. At the end of the digestion the samples were heated at 100 °C for 3 min and adjusted to a final concentration of 0.1 mM-phenylmethanesulphonyl fluoride at room temperature. The sample was then reduced, alkylated and desalted in two 4.5 ml portions. The materials eluted in the void volume of the Bio-Gel P-10 column (4.7 ml; determined with Blue Dextran) were pooled and processed as follows: (a) 3 ml (about 1 mg of protein) were made to a final concentration of 250 mM with the addition of GlcNAc and submitted to the concentration procedure (control samples); and (b) 6 ml (about 2 mg of protein) were exposed to the largest WGA-Sepharose column. The first 3 ml desorbed from the latter column with 250 mM-GlcNAc were then processed as before for gel electrophoresis.

Covalent chromatography on organomercurial-agarose

Affi-Gel 501 (3 cm × 0.8 cm) was used for detection of thiol groups in peptides derived from proteolysed mAChR. The buffer consisted of 50 mM-Tris/HCl (pH 6.5)/0.075% SDS/8 mM-urea/10 mM-EDTA/0.1 M-NaCl. The samples were made of this buffer composition, then applied to the gel and allowed to react for 30 min. After this period the column was extensively washed with the same buffer until background levels of radioactivity were detected. Specific desorption was carried out with 20 mM-DTT after 30 min of reaction. All the procedures were carried out at room temperature. The resins were regenerated with 10 mM-HgCl₂/0.1 M-NaCl/50 mM-sodium acetate buffer, pH 5.0.

Radioactivity measurements

Radioactivity of solutions and membranes dissolved in Protosol was determined by liquid-scintillation counting in Aquasol-2. Polyacrylamide slices were incubated for 5 h with 1 ml of 1 M-NaOH, then neutralized with HCl and counted for radioactivity as described above. The alkaline treatment was found to be very effective in removing the label from fixed gels as established by comparison with unfixed ones. This effect is possibly related to the splitting off of the label from the protein as reported by other authors (Dadi & Morris, 1984).

RESULTS

When membranes of cerebral cortex were labelled with [³H]PrBCM, most of the radioactivity found upon SDS/10%-PAGE was in a peak migrating with an *M_r* of 68000 (Fig. 1). Virtually identical apparent *M_r* values were determined in samples previously reduced and alkylated and upon varying the polyacrylamide concentration between 7.5 and 20% (Leach *et al.*, 1980). In all cases only background radioactivity was observed with membrane samples labelled in the presence of 1 μM-atropine.

The detergent-solubilized mAChR was used first to

Fig. 1. Radioactivity profile of [³H]PrBCM-labelled membranes subjected to SDS/10%-PAGE

Each point corresponds to a 2.5 mm slice. The amount of protein applied was 200 μg. The migration of the protein standards (1, myosin, *M_r* 205000; 2, β-galactosidase, *M_r* 116000; 3, phosphorylase b, *M_r* 97400; 4, bovine serum albumin, *M_r* 66000; 5, ovalbumin, *M_r* 45000; 6, carbonic anhydrase, *M_r* 29000 and the dye (7, Bromophenol Blue) is indicated.

Fig. 2. Affinity chromatography on WGA-Sepharose of [³H]-PrBCM-labelled membranes solubilized in 0.075% SDS

The membranes (1 mg of protein) were labelled either in the presence (○) or absence (●) of 1 μM-atropine. The chromatography was performed at a flow rate of 0.6 ml/min.

examine the behaviour of its glycan moieties on immobilized WGA. As shown in Fig. 2, two components were specifically desorbed from the WGA-Sepharose column by sequential elution with 10 mM- and 250 mM-GlcNAc, each one representing about 50% of the bound

Fig. 3. Radioactivity profile of [^3H]PrBCM-labelled receptors subjected to SDS/4 M-urea/10%-PAGE

Labelled membranes dissolved in 0.075% SDS were incubated either with (●) or without (○) neuraminidase before electrophoresis. Each slice represents a 2.5 mm section of the gel. Each sample of 400 μg of membrane protein was applied to two wells. The migration of the same M_r standards as those in Fig. 1 are shown.

radioactivity. The radioactivity associated with both peaks decreased when membranes were labelled in the presence of 1 μM -atropine (Fig. 2). By this criterion, most of the label retained by the affinity gel was specifically associated with muscarinic-receptor protein. The recovery of applied mACHR from immobilized WGA was $60 \pm 7\%$ (mean $\pm$ S.E.M., $n = 5$). Re-application of the flow-through material to another column resulted in an additional 25% recovery with a similar affinity pattern, thus reflecting that the capacity of the column was exceeded in the first case.

Potential sources of the heterogeneity revealed by WGA-Sepharose chromatography were subsequently investigated. Membranes labelled with [^3H]PrBCM were solubilized in 0.075% SDS and then reduced, alkylated and desalted on Bio-Gel P-10. The material thus obtained was submitted to lectin affinity chromatography as detailed above without noticeable changes in the affinity pattern and recovery of applied receptors (56 and 61% in two separate determinations). It is thus apparent that thiol-mediated interactions are unlikely to be responsible for the heterogeneous behaviour of mACHR found with immobilized WGA. In another series of experiments, membranes of cerebral-cortex homogenates were prepared at 0–4 $^{\circ}\text{C}$ with the 'anti-proteinase cocktail' and labelled at 0 $^{\circ}\text{C}$ for 1 h, then dissolved and affinity-chromatographed on WGA-Sepharose. A similar pattern to that shown in Fig. 2 was found, and an M_r of 68000 was also determined, being similar to that shown in Fig. 1. This result suggests that endogenous glycosidases were not involved in the observed heterogeneity. Samples containing various amounts of protein (175 μg –3 mg), and also the Con A-Sepharose eluate (105 μg of protein), rendered a similar pattern on WGA-Sepharose as described below. It therefore appears that the heterogeneity found is not a consequence of exceeding the capacity of the lectin column (Adair & Kornfeld, 1974).

Fig. 4. Affinity chromatography on Con A-Sepharose of [^3H]PrBCM-labelled membranes solubilized in 0.075% SDS

The sample contained 1 mg of labelled membrane protein. Conditions were as in Fig. 2. Abbreviation: MeMan, α -methyl D-mannoside.

The contribution of *N*-acetylneuraminate residues to the receptor binding to immobilized WGA was estimated by neuraminidase treatment. In order to render the assay more sensitive, 2 mg of labelled membrane protein were solubilized in 0.075% SDS and incubated either with 0.5 unit of enzyme or without additions (control) for 3 h at 37 $^{\circ}\text{C}$ and analysed on WGA-Sepharose. A decrease in the binding to the lectin occurred, accounting for 53–57% of that of the control in two separate experiments. In addition, a pattern similar to that shown in Fig. 2 was obtained, thus indicating that both of the retained components were equally affected by the neuraminidase treatment.

Bacterial neuraminidases may contain contaminating Ca^{2+} -independent proteinases capable of splitting the polypeptide regions of the receptor containing the oligosaccharides responsible for the binding to WGA-Sepharose. For this reason, incubation with the enzyme was performed as described above, and a portion of the sample was analysed by SDS/urea/PAGE (Rauh *et al.*, 1986). As shown in Fig. 3, although no detectable change occurred in the apparent M_r of the peak corresponding to monomeric mACHR, a displacement to the lower- M_r region of the gel can be observed around the half-way point in the neuraminidase-treated samples. Lower- M_r components could not be observed, thus indicating that proteolysis was prevented under our incubation conditions. When 0.5 unit of the enzyme was added again after 1.5 h of incubation and the reactions continued for 3 and 12 h, the binding of atropine-sensitive components to WGA-Sepharose was not diminished more than 42% with respect to samples incubated without the enzyme. In control experiments even after 12 h incubation the amount of radioactivity recovered from WGA—

Fig. 5. Time course of the trypsinolysis of [^3H]PrBCM-labelled receptors obtained before and after WGA-Sepharose chromatography and examined by means of wide- M_r -range PAGE

Labelled receptors were solubilized in 0.075% SDS and incubated either without trypsin (a) or with the addition of trypsin for 1.5, 3, 6 and 8 h (b, c, d and e respectively). At the end of the incubation the samples were electrophoresed before (●) and after (○) chromatography on WGA-Sepharose. Each slice represents a 5 mm section. The amounts of radioactivity recovered for WGA-Sepharose-desorbed samples from about 2 mg of protein as starting material were: 7300, 6400, 6300, 4300 and 2600 c.p.m. for (a), (b), (c), (d) and (e) respectively. Radioactivities recovered before application of the affinity chromatography on a basis of 500 μg /three wells were: 4700, 5100, 6800, 6200 and 5800 c.p.m. for (a), (b), (c), (d) and (e) respectively. (f) Represents radioactivity profiles obtained after chromatography on WGA-Sepharose either from the same sample shown in (e) (○) or from the same amount of protein labelled in the presence of atropine and submitted to a similar procedure (●). The migrations of protein M_r standards (1–7 as described for Fig. 1, plus the following are shown: 8, trypsin inhibitor, M_r 21 500; 9, myoglobin, M_r 16 950; 10, myoglobin fragments I+II, M_r 14 400; 11, lysozyme, M_r 14 400; 12, myoglobin fragment I, M_r 8 160; 13, myoglobin fragment II, M_r 6 210; and 14, myoglobin fragment III, M_r 2 510).

Sepharose was found not to differ from that of non-incubated samples.

The binding of mAChR to Con A-Sepharose was examined as follows. Solubilized, labelled mAChRs were allowed to react with the affinity gel for 30 min at room temperature. The column was then washed and sequentially eluted with 10 mM- and 250 mM- α -methyl -D-mannoside. As shown in Fig. 4, a large amount of the label retained on Con A-Sepharose was desorbed by the lower concentration of sugar, the high-affinity component varying between 20 and 35% in three separate

experiments. The recovery of applied receptors from this column was poor (30%). However, re-application of the flow-through material to another column permitted the recovery of an additional 30% of [^3H]PrBCM-labelled material. This result makes evident the limited capacity of this gel, as described above for WGA-Sepharose. Similar observations were reported for the chromatography of mAChR solubilized in CHAPS (Shirakawa *et al.*, 1983). In order to determine the WGA-binding ability of those receptors retained by Con A-Sepharose, sequential lectin affinity chromatography was attempted.

Solubilized labelled membranes (2 mg of protein) were exposed to Con A-Sepharose under the conditions indicated above, and the column was then washed until background radioactivity levels were reached. Specific elution of the gel was subsequently performed with 10 mM sugar, and the eluate was recycled on WGA-Sepharose at a flow rate of 1 ml/min for 1 h at room temperature. After washing and specific elution of the WGA column, an affinity pattern similar to that shown in Fig. 2 was obtained, with high recovery of the applied receptors (75–82% in two separate experiments).

To estimate the proximity of the covalently bound [^3H]PrBCM to the glycan moieties responsible for WGA-Sepharose binding, the following strategy was employed. Labelled membranes dissolved in SDS were treated with trypsin and subsequently reduced, alkylated and desalted. The resulting material was exposed to WGA-Sepharose and the fraction specifically desorbed from the latter gel was examined on a wide- M_r -range SDS/urea/PAGE system. The molecular mass of the smallest radioactive fragment found upon electrophoresis was taken as an upper limit of the distance between the site of covalent radioligand attachment and the carbohydrate moiety. As shown in Fig. 5(c), a close correlation was found between the radioactivity profiles derived from samples previously submitted, or not, to affinity chromatography. The radioactivity of the detectable peptides migrated with M_r values of 75000, 50000, 30000, 18000, 8000 and 2500 (Fig. 5). Receptors incubated under similar conditions without trypsin resulted in only one peak of radioactivity of M_r 75000 in this gel system (Fig. 5a). It was verified that the same sample generated an homogeneous single peak of M_r 68000 when analysed in the SDS/urea/PAGE system described in Fig. 3.

When the tryptic peptides (corresponding to about 1 mg of protein) obtained as described in Figs. 5(c) and 5(e) were chromatographed on WGA-Sepharose as described for Fig. 2, the same pattern as with non-degraded receptors was found, with a recovery of 53 and 21% of the applied receptors respectively. The latter values are in agreement with the recovery of radioactivity after SDS/PAGE described in the legend to Fig. 5.

Increasing the incubation period for a fixed enzyme/protein ratio (1:20, w/w) resulted in a progressive shift of the radioactive pattern to the lower- M_r regions of the gels. This is illustrated in Figs. 5(a)–5(e). The latter corresponds to 8 h of incubation with two additions of the enzyme. Although the M_r values for the fragments are similar to that shown in Fig. 5(c), the correlation between the radioactive profiles obtained before and after WGA-Sepharose chromatography differs in the following respect: there is a large increase in the peak migrating with M_r 8000 that is not accompanied by a concomitant increase of the M_r -8000 peak after the WGA-Sepharose step (Fig. 5e). A more direct approach involved treatment with 1 unit of neuraminidase for 3 h at 37 °C of samples previously trypsin-treated for 12 h with four additions of the enzyme at regular intervals. The major component of M_r 8000 thus produced represented 90% of the total radioactivity recovered, and its mobility was not affected by the neuraminidase treatment (results not shown).

In order to investigate the nature of the peptides retained by WGA-Sepharose as shown in Fig. 5(e), a similar amount of membranes labelled in the presence of atropine was submitted to an identical procedure.

Fig. 6. Covalent chromatography on Affi-Gel 501 of the [^3H]PrBCM-labelled fragment of M_r 8000

Solubilized labelled membranes (1.5 mg of protein) were exhaustively digested with trypsin, reduced, alkylated when appropriate and desalted. The material was then analysed on organomercurial-agarose. The specific elution was carried out with 20 mM-DTT (arrow). Symbols: ●, samples previously reduced; △, samples reduced and alkylated with NEM; ○, membranes labelled in the presence of atropine and submitted to the entire procedure without alkylation.

Fig. 5(f) illustrates the non-specific nature of two small components retained by the WGA column migrating with M_r values of 2500 and 8000. In no case were peptides smaller than the above-mentioned M_r -2500 component observed. This also applies to samples corresponding to WGA-Sepharose eluates obtained from the gels immediately after electrophoresis, slicing and counting for radioactivity without fixation, thus making loss of small glycopeptides during fixation unlikely. The possibility that free radioligand accounted for some of the components was also considered and discarded, because it was shown not to be retained on WGA-Sepharose, and furthermore it was found to be totally released from the gels during fixation and staining.

The following experiments were performed in order to investigate the presence of thiol groups in the limit fragment of M_r 8000. Cerebral-cortex membranes (1.5 mg of protein) were labelled with [^3H]PrBCM and solubilized as described in the Experimental section. The samples were then proteolysed for 48 h at 37 °C under a toluene atmosphere with six regular additions of trypsin at an enzyme/protein ratio of 1:20 (w/w). The completeness of the digestion was verified by wide- M_r SDS/PAGE; almost all of the label was found to migrate as an M_r -8000 fragment when analysed in this system. The digested samples were then reduced with 20 mM-DTT at 100 °C for 3 min and subsequently desalted on Bio-Gel P-10. The material eluted in the void volume of the column had the same composition as the buffer used for Affi-Gel 501 (see the Experimental section). The sample was then analysed on organomercurial-agarose. As shown in Fig. 6, radioactive material was specifically

desorbed by DTT. As a control, receptors labelled in the presence of 1 μ M-atropine were submitted to the entire procedure. After elution with DTT, a loss of radioactivity was found, thus making evident that the larger peak originated in the labelled receptor (Fig. 6). In a subsequent experiment the reduced peptide was alkylated with 50 mM-NEM for 30 min at 37 °C, then desalted on Bio-Gel P-10 and analysed on Affi-Gel 501. An amount of radioactivity similar to that corresponding to non-specific labelling was recovered after desorption with DTT (Fig. 6). The recovery of applied receptors from the column was from 75–92 % in four separate experiments.

DISCUSSION

Low concentrations of SDS are reported here to result in 95 % solubilization of [3 H]PrBCM-labelled mAChR from rat cerebral-cortex membranes, as demonstrated by sedimentation analysis and filtration through small-pore membranes. An apparent M_r of 68 000 was determined for [3 H]PrBCM-labelled mAChR under our experimental conditions, in good agreement with values reported by other laboratories (Dadi *et al.*, 1986; Rauh *et al.*, 1986). In some instances a relatively minor amount of radioactivity was also found in the high- M_r regions; its occurrence, described as an artefact by some authors (André *et al.*, 1983; Dadi & Morris, 1984; Rauh *et al.*, 1986), was prevented neither by extensive alkylation of reduced samples with NEM nor by the inclusion of 4 M-urea in the SDS/PAGE (Fig. 3). Fragments with an M_r lower than 68 000 were not found; proteolysis of the mAChR thus seems to be avoided (Ventner, 1983; Dadi & Morris, 1984).

Previous attempts to solubilize AChR from chemically excitable membranes relied on the use of mild non-ionic detergents (Gavish & Sokolovsky, 1982; Scott Herron & Schimerlick, 1983; Shirakawa *et al.*, 1983; Yagisawa *et al.*, 1983; Rauh *et al.*, 1986) in which equilibrium ligand binding can be assessed. Instead, we have taken advantage of the more extensive solubilizing properties of SDS. The binding of mAChR to immobilized Con A in SDS-containing media is similar to that of bovine cerebral-cortex mAChR in CHAPS (Shirakawa *et al.*, 1983) and of porcine brain mAChR in Lubrol PX (Yagisawa *et al.*, 1983). In contrast, brain mAChRs solubilized in digitonin or Triton X-100 (Rauh *et al.*, 1986), and cardiac mAChR solubilized in digitonin/cholate (Scott Herron & Schimerlick, 1983), failed to bind immobilized Con A. These results suggest that the detergent may affect not only the solubilization (Rueß & Löffler, 1979), but also the availability of sugar moieties for appropriate interaction with the lectin. This is illustrated by the absolute requirement for SDS and not other detergents to promote the release of carbohydrates from several glycoproteins by glycosidases (Trimble & Maley, 1984), the detrimental effect of Triton X-100 on the desorption of glycoproteins from Con-A-Sepharose (Schmidt-Ullrich *et al.*, 1975) and the interference of digitonin in the binding of glycoproteins to immobilized WGA (Lotan *et al.*, 1977; Meier *et al.*, 1984) and Con A (George *et al.*, 1986).

Among several lectins that have been previously used to evaluate the glycoprotein nature of the mAChR, immobilized WGA was the most effective, followed in some cases by Con A-Sepharose (Gavish & Sokolovsky, 1982; Scott Herron & Schimerlick, 1983; Shirakawa *et al.*,

1983; Yagisawa *et al.*, 1983; Rauh *et al.*, 1986). Accordingly, the mAChR was investigated here with these two lectins. The results obtained upon chromatography on WGA-Sepharose suggest the existence of similar amounts of two mAChR subpopulations which are resolved on the basis of their differential affinity for the immobilized lectin (Fig. 2). This heterogeneity appears not to be related to thiol-group-mediated interactions, endogenous glycosidases or a loss of selectivity of WGA-Sepharose. The central role of NeuAc (acetylneuraminate) and GlcNAc in the interaction of WGA-Sepharose with glycoconjugates is well established (Bhavanandan & Katlic, 1979). However, the structural features of the glycoproteins responsible for binding to immobilized WGA are complex and not fully understood (Furukawa *et al.*, 1986). The behaviour of the mAChR on the latter gel provides evidence of the presence of terminal sialic acid residues, since cleavage with neuraminidase results in a reduction of the binding to WGA-Sepharose. Even after extensive treatment with the enzyme, a subpopulation of the mAChR retained its ability to bind the lectin. This may be a consequence of the unavailability of sialic acid residues to the enzyme or of the binding to the lectin through GlcNAc residues. Sialic acid appears not to be involved with the heterogeneity detected in the WGA-Sepharose experiments, because no shift in the distribution of the affinity components was found after cleavage of NeuAc. The displacement found in the migration on SDS/PAGE of monomeric brain mAChR after neuraminidase treatment (Fig. 3) indicates a small contribution of NeuAc residues to the M_r estimates.

A large proportion of the mAChR retained on Con A-Sepharose was eluted as a low-affinity component (Fig. 4), although high-affinity binding to this lectin is preserved in the presence of SDS (Alperin *et al.*, 1986). Sequential affinity chromatography on Con A-Sepharose, followed by WGA-Sepharose, clearly demonstrates that the former gel retained a subset of receptors equivalent to that bound to the latter lectin (Fig. 2). These results are consistent with the behaviour of biantennary complex or hybrid-type oligosaccharides (Narasimhan *et al.*, 1978; Cummings & Kornfeld, 1982). Assuming two glycosylated sites per receptor molecule (Kubo *et al.*, 1986a), our results are more consistent with the carbohydrate molecular mass of 4000 Da determined for brain receptors (Rauh *et al.*, 1986) than with that of 18 550 Da found for mAChR of atrial origin (Peterson *et al.*, 1986). In the latter case, however, double the number of potential sites for glycosylation should be taken into account (Peralta *et al.*, 1987).

After trypsinolysis of the labelled mAChR and electrophoresis, a discrete peptide pattern was obtained (Fig. 5). Although all radioactive peptides appeared to bind WGA-Sepharose (Fig. 5c), the most extensive proteolysis results in the accumulation of a fragment of M_r 8000 that does not bind the lectin (Fig. 5e). Neuraminidase treatment does not result in further modification of the M_r -8000 component. These results therefore suggest that the latter peptide is devoid of sugar residues. A fragment of M_r 18 000 binds proportionally to the lectin (Fig. 5). The amount of this component is small, as described elsewhere (Venter, 1983), and probably represents a degradation intermediate. A fragment migrating with an M_r of 2500 is also consistently observed (Fig. 5); it binds proportionately to the lectin, but does not increase upon extensive proteolysis (Fig. 5e). The

origin of this and another component migrating with M_r 8000 (Fig. 5e; after chromatography on the WGA column) cannot be attributed to the receptor, because they were also recovered when a sample labelled in the presence of atropine was similarly analysed (Fig. 5f). For this reason the M_r -18000 peptide must be taken as the most reliable value for the smallest tryptic fragment containing the oligosaccharides and the [^3H]PrBCM-binding site.

The combined NEM treatment and organomercurial-agarose chromatography experiments clearly demonstrate the presence of thiol groups in the 8000 Da peptide (Fig. 6).

The availability of the amino acid sequence of the mAChR from porcine brain, deduced from the cDNA sequence (Kubo *et al.*, 1986a), prompts comparison with the present experimental data. The first apparent congruence between the two sets of data lies in the correlation of predicted/observed peptide fragments generated by trypsinolysis. All possible trypsin-cleavage sites were calculated on the basis of (i) the published amino acid sequence (Kubo *et al.*, 1986a) and (ii) the known K (lysine) and R (arginine) residues on which trypsin acts. This yielded a total of 57 possible (theoretical) tryptic fragments. The relative abundance of cleavage sites was then calculated for stretches of the mAChR molecule; for an arbitrary 40-amino-acid-long stretch, for instance:

$$\frac{\text{Amount of trypsin attack sites} = \frac{\text{No. of K + R sites per 40-amino-acid stretch}}{\text{Total no. of sites in mAChR}} \times 100$$

It became apparent that the largest concentrations of trypsin-attack sites were in three discrete regions of the mAChR, namely between residues 120 and 160, 320 and 360, and 420 and 460 (12.5, 26.8 and 23% respectively; totalling 62.3% of all possible sites). Thus, under conditions of limited trypsinolysis, the SDS-unfolded receptors should be preferentially cleaved at the latter sites. Assuming that the assignation of the glycosylation sites to residues 2 and 12 (Kubo *et al.*, 1986a; Dohlman *et al.*, 1987) is correct, *all* fragments generated from these discrete regions and retained by the WGA-Sephacryl column should also be recognized by their radioactivity (the PrBCM label) if the *N*-terminal region is contained within these fragments. The first peptide detected under conditions of limited proteolysis has an M_r of 30000 (Fig. 5b). After an increased period of incubation, labelled peptides of M_r 18000, 30000 and 50000 were obtained, all carrying glycan moieties (Fig. 5c). More extensive proteolysis results in accumulation of a peptide, of M_r 8000, devoid of carbohydrates (Figs. 5d and 5e). This may correspond to the only possible peptide (M_r 7354) released from the amino-acid region corresponding to positions 58–123, of the sequence, which in accordance with the transmembrane assignation (Kubo *et al.*, 1986a) ought also to lack sugars and which contains cysteine residues at positions 69 and 98.

Out of a total of 57 possible tryptic-limiting fragments, eight cysteine-carrying peptides can theoretically be generated, and only one can have an M_r of 7354 (Kubo *et al.*, 1986a).

A major discrepancy remains between the two sets of data, namely the M_r of the intact mAChR. Experimentally determined M_r values oscillate around 75000

(Venter, 1983; Dadi *et al.*, 1986; Rauh *et al.*, 1986), whereas the value predicted from the cloned receptor is 51416 (Kubo *et al.*, 1986a). The present M_r estimates, obtained by SDS/PAGE of desialylated mAChR, and those for deglycosylated brain mAChR (Rauh *et al.*, 1986), do not support the idea of a large contribution of the carbohydrates to the total M_r of the receptor. Radiation-inactivation of rat brain mAChR also yielded a value of M_r 82000 (Venter, 1983), thus suggesting that an anomalous migration of the glycoprotein is not the only possible cause of this phenomenon. Finally, the assignation of transmembrane portions of the mAChR (Kubo *et al.*, 1986a) does not appear to correlate with experimental data on proteolytic cleavage of the receptor in membranes (Venter, 1983). The conclusion that about 50% of the mAChR mass protrudes from the membrane stems from the latter data. Residues Asn-110 and Asn-410 are supposedly non-glycosylated, because of their transmembrane assignation (Kubo *et al.*, 1986a). If this turns out not to be the case, these amino acids ought to be taken into account as potentially glycosylated sites. However, if the peptide of M_r 8000 described here does represent the amino-acid region corresponding to positions 58–123 of the sequence, then Asn-110 should not be glycosylated.

In summary, the major findings of the present work are: (i) the description of the different tryptic glycopeptides carrying the covalently attached labelled antagonist; (ii) the identification of the smallest fragment having [^3H]PrBCM and sugars; and (iii) the determination of the size of a limit [^3H]PrBCM-labelled peptide which is devoid of sugars and carries thiol groups.

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