Muscarinic cholinergic receptor of rat cerebral cortex

Location and characterization of ligand binding site-carrying peptides in synaptosomal membranes and isolated neuronal perikarya

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

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

A careful examination of the location and biochemical properties of the tryptic peptides identified by site-specific labelling of the muscarinic cholinergic receptor (mAChR) of rat cerebral cortex has been carried out. In brain synaptosomal membranes and isolated neuronal perikarya, mAChR labelled with [³H]propylbenzilylcholine mustard (PrBCM) was tryptically cleaved to peptides of *M*_r 50000, 30000, 18000 and a limiting fragment of *M*_r 8000. All of these binding site-carrying fragments, characterized in terms of their content of carbohydrates and thiol groups, were quantitatively recovered as membrane-bound peptides. The delipidated [³H]PrBCM-labelled tryptic limiting fragment was found to be highly hydrophobic and insoluble in aqueous media. Experiments performed with proteinase on the tryptic limiting fragment suggest the existence of an ester linkage between the ligand and the peptide. The results strongly support the hydropathicity profile which predicts the location of the muscarinic receptor protein with respect to the membrane bilayer.

INTRODUCTION

According to studies based on cDNA techniques (Bonner *et al.*, 1987, 1988), muscarinic acetylcholine receptors (mAChRs) occur in rat cerebral cortex as several distinct glycoprotein species. Considerable similarity between the sequences of the mAChR, the adrenoceptor and the opsins has been reported; similarly, their hydropathicity profiles suggest the existence of seven common membrane-bound segments (Kubo *et al.*, 1986; Dohlman *et al.*, 1987a).

Studies performed on native mAChR labelled with tritiated propylbenzilylcholine mustard (PrBCM) demonstrated that the tracer can be recovered covalently attached to a tryptic limiting fragment devoid of carbohydrates and containing thiol groups (Alperin *et al.*, 1987). We have also suggested that the latter fragment represented the amino acid stretch 58–123 identified by Kubo *et al.* (1986). Wheatley *et al.* (1988) came to a similar conclusion using purified mAChR; their assignment of the [³H]PrBCM binding site corresponded to the theoretical transmembrane regions II–III, which match the aforementioned stretch. The two sets of data are in agreement with the proposed ligand-binding site for the very similar β -adrenergic receptor, deduced either by site-specific labelling (amino acids 83–96; Dohlman *et al.*, 1988) or by directed mutagenesis (amino acid 113; Strader *et al.*, 1988).

Despite the differences in the amino acid compositions of each cloned mAChR, their degree of similarity in the region described is very high (about 80% amino acid identity, calculated from Bonner *et al.*, 1988). Thus,

information on the properties of this limited region is relevant to all mAChR species in this receptor family.

If the models postulated for the location of the receptor with respect to the membrane are correct, the tryptic-limiting peptide described above will remain attached to the membrane, and will be a highly hydrophobic fragment. However, experimental confirmation of such a theoretical model is still absent for the mAChR.

In the present work, a careful examination of the location of the mAChR with respect to the membrane, and of the biochemical properties of the tryptic peptides identified by site-specific labelling of mAChRs with [³H]PrBCM, has been carried out. Fragments of [³H]PrBCM-labelled mAChR have been proteolytically generated both in brain synaptosomal membranes and in neuronal perikarya isolated from rat cerebral cortex. Their association with the bilayer, solubility and hydrophobic behaviour and the nature of the [³H]PrBCM linkage to the mAChR have been determined. The data strongly support the current view concerning the membrane location of the ligand-binding region of the muscarinic receptor protein.

MATERIALS AND METHODS

Materials

[³H]PrBCM (48 Ci/mmol), [³H]quinuclidinyl benzilate (QNB) (35 Ci/mmol) and Aquasol-2 were purchased from New England Nuclear. Tos-Phe-CH₂Cl-treated trypsin, proteinase (protease type XXI from *Streptomyces griseus*), electrophoresis *M*_r standards, wheat-

Abbreviations used: mAChR, muscarinic cholinergic receptor; PrBCM, propylbenzilylcholine mustard; QNB, quinuclidinyl benzilate; WGA, wheat-germ agglutinin; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; TCA, trichloroacetic acid.

* 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.

† Present address: The Weizmann Institute of Sciences, Rehovot, Israel.

germ agglutinin (WGA)–Sephacrose 6MB, dithiothreitol, *N*-ethylmaleimide, aprotinin and phenylmethanesulphonyl fluoride (PMSF) were obtained from Sigma. Bio-Gel P-10, Affi-Gel 501 and the reagents for polyacrylamide-gel electrophoresis (PAGE) were purchased from Bio-Rad. Dowex 50W-X4 resin and a wide pore C₁₈ reverse-phase column (RP 7104-0) were from Baker. Sartorius SM 16534 membrane filters of 0.2 µm pore size were used throughout.

Preparation and labelling of synaptosomal membranes

Cerebral cortices from adult Wistar rats were homogenized at 4 °C in 0.32 M-sucrose/20 mM-Tris/HCl, pH 7.4, containing the following protease inhibitors: 0.5 mM-PMSF, 1 mM-EDTA and 8 µg of aprotinin/ml. Membranes were obtained by centrifugation of the post-nuclear fraction (1090 *g* for 10 min) at 15000 *g* for 30 min and resuspension of the pellet in 20 mM-Tris/HCl buffer, pH 7.4, containing 155 mM-NaCl. Protein was determined by the method of Lowry *et al.* (1951).

[³H]PrBCM was cyclized for 45 min in 10 mM-phosphate buffer, pH 7.4. The covalent binding was carried out on 1 mg of protein with 10 nM precyclized ligand at 30 °C for 30 min in 1 ml of 20 mM-Tris/HCl buffer, pH 7.4, containing 155 mM-NaCl. The incubation was stopped by centrifugation at 12000 *g* for 3 min. Reversible binding with [³H]QNB was performed under similar conditions, using up to 350 µg of protein/ml. Saturation binding was found at 1 nM of the ligand, accounting for 1 ± 0.098 pmol of mAChR/mg of protein (mean $\pm$ S.D., *n* = 5). Non-specific binding was determined in all cases by preincubation for 15 min and labelling in the presence of 10 µM-atropine. Since [³H]PrBCM is an irreversible ligand, site occupancy by preincubation with atropine reduces the specific attachment of the tracer when non-specific binding is determined.

Isolation and labelling of neuronal perikarya

Neuronal bodies were isolated from 30-day-old rat cerebral cortex according to the method of Sellinger *et al.* (1971) with minor modifications. The advantage of this procedure is that proteolytic enzymes were not required to dissociate the cells. Briefly, cerebral cortices from three rats were minced and transferred into 120 ml of an ice-cold solution of 50 mM-Tris/HCl buffer, (pH 7.4)/7.5% (w/v) poly(vinylpyrrolidin-2-one)-40/0.22 M-sucrose/0.5% (w/v) bovine serum albumin/10 mM-CaCl₂/0.5 mM-PMSF (freshly prepared)/8 µg of aprotinin/ml. The minced tissue was then eased into a truncated disposable syringe and the plunger was used to push the tissue through a 330 µm pore size mesh. The same procedure was repeated with meshes of 130 and 73 µm pore size. The last filtrate was divided into six portions, each of which was layered on to a 4-step gradient of (from top to bottom) 3 ml of 1.0 M-sucrose, 4 ml of 1.25 M-sucrose, 4 ml of 1.4 M-sucrose and 5 ml of 1.65 M-sucrose, all prepared in 50 mM-Tris/HCl buffer, pH 7.4, containing 1% (w/v) bovine serum albumin. The tubes were centrifuged in a Beckman SW-28 rotor at 40000 *g* for 30 min. Pure neuronal perikarya were recovered in the pellet, which was washed once by resuspension in 20 mM-Hepes buffer, pH 7.4, containing 110 mM-NaCl, 1.8 mM-CaCl₂, 1 mM-MgCl₂, 25 mM-glucose and 70 mM-sucrose, and centrifugation at 500 *g* for 5 min. The latter medium, in which neurons remain

metabolically active (Yanagihara, 1979), was used for radioactive labelling and all other studies performed with neuronal bodies.

Neuronal bodies (about 1 mg of protein) were incubated in 1 ml of the above medium at 30 °C with the addition of 20 nM precyclized [³H]PrBCM. After 30 min the cells were centrifuged at 500 *g* for 5 min. Binding with [³H]QNB was carried out by incubation of the neurons (up to 0.5 mg of protein) in the presence of 1 ml of 2 nM-[³H]QNB for 30 min at 30 °C. The reaction was stopped by centrifugation and the pellet was washed three times. Non-specific binding was determined by preincubation for 15 min and labelling in the presence of 10 µM-atropine, and was found to account for less than 10% of the total binding.

Treatment with trichloroacetic acid (TCA)

When indicated, labelled membranes were treated with 10% TCA, then washed once with 5% TCA and finally with ethanol/ether (2:1, v/v) until only background radioactivity was present in the supernatants (usually five washes). Sonication was used in all steps. This procedure was carried out to eliminate free residual radioligand (Dadi & Morris, 1984) and to delipidate the sample when analysis of small labelled fragments was required, as will be described in the Results section. Neither the protein pattern revealed by the staining with Coomassie Brilliant Blue of labelled membranes in SDS/PAGE (10% gels) nor the migration of the labelled mAChR were affected by the TCA treatment. A band ahead of the tracking dye and co-migrating with free ligand was observed in non-stained gels. This band, together with the tracer, was extracted during the ethanol/ether washings of the TCA pellet.

Centrifugation of the SDS-solubilized fragments, obtained after trypsinolysis of labelled membranes treated with TCA, was carried out at 165000 *g* for 1 h using a Beckman TI 80 rotor at 12 °C.

Solubilization of membranes

Labelled membranes treated or not with TCA were solubilized in 2% SDS/50 mM-Tris/HCl buffer, pH 7.4, at 100 °C for 3 min, and then diluted to 0.075% SDS with the same buffer containing 0.02% NaN₃. The initial detergent/protein ratio was about 1.8 in all cases. The latter buffer composition was used in all subsequent procedures.

Enzymic treatments

Tos-Phe-CH₂Cl-treated trypsin was added to synaptosomal membranes, neuronal bodies or SDS-solubilized receptors labelled with [³H]PBCM in their respective incubation media at 37 °C. Incubations of membranes and SDS-solubilized mAChRs were carried out under a toluene-saturated atmosphere. At the end of the digestion periods, membranes and cells were centrifuged under the conditions given in the Results section. The pellet and supernatant were then heated at 100 °C for 3 min and PMSF and EDTA were added to give final concentrations of 0.1 mM and 1 mM respectively. In the case of SDS-solubilized receptors, the extensive trypsinolysis was stopped only by heating as before. Further proteolytic and alkaline treatments were performed by the addition of proteinase or alkali to the latter samples under the conditions given in the Results

section. Centrifugation at 192000 *g* for 10 min was carried out in a Beckman SW 50.1 rotor at 12 °C.

Reduction and alkylation

In all cases the samples were reduced after proteolysis with 10 mM-dithiothreitol for 30 min at 37 °C followed by alkylation with 50 mM-*N*-ethylmaleimide under similar conditions.

Polyacrylamide-gel electrophoresis

To estimate the size of proteolytic fragments, a wide- M_r SDS/PAGE system in the presence of urea was used as described previously (Alperin *et al.*, 1987).

Gel exclusion and ion-exchange chromatography

Bio-Gel P-10 columns (50 cm × 1.2 cm) were used at room temperature and eluted at a flow rate of 30 ml/h. The gels were equilibrated and run in 50 mM-Tris/HCl buffer (pH 7.4)/0.2% NaN_3 , with or without 0.075% SDS as indicated in the Results section.

For cation-exchange chromatography a Dowex 50W-X4 resin (Na^+ form) column (5 cm × 0.6 cm) was used at room temperature, equilibrated and eluted with a gradient of NaCl in 5 mM-borate buffer, pH 9, at a flow rate of 0.5 ml/min. In all cases the samples were reduced and alkylated before chromatography.

Radioactivity measurements

The radioactivity of solutions and membranes was determined by liquid scintillation counting in Aquasol-2. In the case of polyacrylamide gels, slices were incubated for 5 h with 1 M-NaOH to split the label and then neutralized with HCl, and radioactivity was counted.

RESULTS

Limited trypsinolysis of mAChR in synaptosomal membranes and solubility of the products

The topology of the membrane-bound receptor was studied first by trypsin digestion of [^3H]PrBCM-labelled synaptosomal membranes. After labelling with this irreversible antagonist, the membranes (0.8 mg of protein in 300 μl) were incubated for 1–48 h with trypsin at an enzyme/protein ratio of 1:10. After the digestion period and protease inactivation, the samples were centrifuged at 192000 *g* for 10 min. Both the supernatant and pellet were adjusted to give a final concentration of 2% SDS and 8 M-urea. The samples were then reduced and alkylated and finally analysed on a wide- M_r SDS/urea/PAGE system, as described in the Materials and methods section.

Fig. 1(b) shows that a 1 h trypsin digestion rendered major fragments of M_r 75000, 30000 and 8000 which remained attached to the membrane fraction. No radioactivity was recovered in the supernatant. Fig. 1(c) shows that in addition to these fragments, intermediate peptides of M_r 50000 and 18000 appear after 24 h of digestion (two enzyme additions). No radioactivity was found in the supernatant in either case. Increasing the incubation period up to 48 h with three additions of the enzyme

Fig. 1. Time course of the trypsinolysis of membrane-bound mAChR examined by wide- M_r SDS/urea/PAGE

Membranes labelled with [^3H]PrBCM were incubated either without (a) or with the addition of Tos-Phe- CH_2Cl -

treated trypsin for 1, 24 and 48 h (b, c and d respectively). At the end of the incubation the samples were centrifuged at 192000 *g*. Both the pellet (●) and the supernatant (○) were supplemented with SDS and urea at final concentrations of 2% and 8 M respectively. The samples were then reduced, alkylated and finally analysed by PAGE. Each sample contained 0.8 mg of protein. The pelleted and soluble fractions were applied to four and one lanes of the gel respectively. Each slice corresponds to 5 mm. The migration of 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; 7, myoglobin, M_r 16900; 8, myoglobin fragments I+II, M_r 14400; 9, myoglobin fragment I, M_r 8160; 10, myoglobin fragment II, M_r 6210; 11, myoglobin fragment III, M_r 2510; and 12, the tracking dye, Bromophenol Blue) are indicated.

resulted in a shift of the radioactivity pattern to the M_r -8000 region (Fig. 1d). The pattern underwent no further modifications, even when double the amount of enzyme was added, indicating that this fragment represents the limiting peptide of the trypsinolysis. Furthermore, it is possible to infer that this fragment was generated from that of M_r 30000, since the increase of the M_r -8000 peptide was accompanied by a concomitant decrease of the larger fragment (Fig. 1). Incubation for 48 h without addition of trypsin (Fig. 1a) did not produce noticeable changes in the M_r of the mAChR, thus indicating the tryptic origin of the fragments. It is worth noting that incubation of the membranes at 4 or 37 °C for 48 h did not result in modifications of the total number of atropine-sensitive sites recognized by [3 H]QNB. Thus no conformational changes at the level of the binding site were evident, since both ligand recognition and size of the mAChR were preserved during the incubation period.

Comparison of the trypsinolysis of membrane-bound or detergent-solubilized receptors (Alperin *et al.*, 1987) shows qualitative correlation of the labelled fragment sizes (Fig. 1). Moreover, in complementary experiments carried out as described previously (Alperin *et al.*, 1987), it was shown that fragments of higher M_r than 8000 bound to WGA-Sepharose, whereas the latter tryptic limiting peptide lacked sugars but contained thiol group(s) since it interacted with an immobilized organomercurial (Affi-Gel 501). Thus the peptides generated from the membrane-bound mAChR behave similarly to those found with the detergent-solubilized receptor (Alperin *et al.*, 1987), although they were produced at a slightly lower rate. The correlation suggests that most of the tryptic cleavage sites are outside the membrane bilayer, in accordance with the trans-membrane model (Kubo *et al.*, 1986).

The centrifugation conditions applied in the experiments described above (192000 *g* for 10 min) fulfil the general criteria of solubility and were less intense than those reported by Dohlman *et al.* (1987b) for similar experiments on the β -adrenergic receptor (450000 *g* for 30 min). Even when sedimentation of the peptides was tested using a much less intense force field (12000 *g* for 3 min), 64–72% ($n = 3$) of the total radioactivity was recovered in the pellet. A radioactivity pattern similar to that shown in Fig. 1(c) was found in both fractions.

Taken together, these results indicate that all labelled peptides remained associated with the pelleted membranes. Hence, according to the criteria of Dohlman *et al.* (1987b), each of the tryptic fragments may represent integral membrane domains anchored in the lipid bilayer.

Muscarinic receptors in neuronal perikarya

The occurrence of mAChR in neuronal bodies was first detected by binding assays with the reversible antagonist [3 H]QNB. The number of mAChR binding sites was found to be 0.61 ± 0.037 pmol/mg of protein (mean $\pm$ S.D., $n = 5$). Higher concentrations of the ligand resulted in a similar number of sites, thus indicating that saturation binding had been reached.

Specific binding with the irreversible antagonist [3 H]PBCM corresponded to approx. 65% of the total number of sites revealed by [3 H]QNB at a ligand concentration of 20 nM under the conditions described in the Materials and methods section. Phase-contrast microscopy revealed the presence of cells with the mor-

Fig. 2. Phase-contrast micrographs of neuronal perikarya from rat cerebral cortex

After isolation, the cells were washed in incubation medium, labelled with [3 H]PrBCM, centrifuged at 500 *g* for 5 min, resuspended in the same medium, incubated with trypsin for 3 h and photographed (top). A neuron representative of the population before the incubation is also shown (bottom). Magnification $\times 1012$.

phological appearance of neurons which were the most abundant elements observed at lower magnification, as shown by other authors (Ochoa *et al.*, 1982).

Trypsinolysis of mAChR in neuronal perikarya

The location of mAChR regions with respect to the membrane was studied by trypsin digestion of [3 H]PrBCM-labelled neurons. The use of neuronal bodies avoids artifacts that may be generated by homogenization and proteolytic treatment of membranes. In addition, the cells can be pelleted at very low speed. After labelling with the irreversible ligand, the neurons (1 mg of protein in 0.5 ml) were incubated for 3 h with or without trypsin (enzyme/protein ratio of 1:5) and then pelleted at 500 *g* for 5 min. Fig. 2 shows phase-contrast micrographs of labelled cells obtained at high magnification before and after the tryptic treatment. The cells share the characteristic appearance of neurons despite the 3 h of incubation. Fig. 3 shows the radioactive pattern of the pellet from control, labelled neurons. A major peak migrating at about M_r -70000–96000, corresponding to an atropine-sensitive component, was detected. This result indicates that even after an incubation period of 3 h (without trypsin), endogenous proteolysis, indicated by the appearance of a small amount of labelled protein in the mid- M_r region of the gel (above M_r 30000), was very slight. Fig. 3 also shows that 3 h of trypsinolysis rendered a major labelled fragment of M_r 8000 which remained attached to the cells. In three separate experi-

Fig. 3. Radioactivity profile of mAChR from neuronal perikarya analysed on wide- M_r SDS/urea/PAGE

[^3H]PrBCM-labelled neuronal perikarya (1 mg of protein) were incubated for 3 h in the presence ($\circ$, $\blacktriangle$) or absence ($\bullet$) of trypsin. At the end of the incubation period, the samples were centrifuged at 500 g for 5 min and the pellets ($\bullet$, $\blacktriangle$) and supernatant ($\circ$) were processed for SDS/PAGE as described in Fig. 1 legend. Each slice corresponds to 5 mm of gel. The numbers indicate the migration of the same electrophoretic standards described in the legend to Fig. 1. Each sample was applied to three lanes of the gel.

ments, performed with three different preparations of neurons, the radioactivity recovered in the 500 g supernatant was in all cases less than 20% of the total radioactivity recovered. When trypsinization was carried out for 1 h, the major labelled fragment detected was that of M_r 30000 (results not shown). The sharpness of the electrophoretic peaks was improved when smaller amounts of protein were analysed in the gels; however, the sensitivity for detection of the label in the supernatant fraction became too low.

The above results indicate that the trypsin limiting peptide, identified by site-specific labelling with [^3H]PrBCM, remains associated with the cells and therefore represents an integral membrane domain anchored in the lipid bilayer.

Hydrophobicity of the tryptic-limiting peptide

According to Kubo *et al.* (1986), the amino acid stretch 58–123 of the mAChR should be a hydrophobic membrane-bound region. This property was studied in the M_r -8000 fragment which has been tentatively assigned to the latter stretch (Alperin *et al.*, 1987; Wheatley *et al.*, 1988). The experiments were carried out on TCA-treated labelled membranes as described in the Materials and methods section.

To test whether SDS interacted with the tryptic limiting fragment, labelled membranes (1 mg of protein) were treated with TCA, solubilized in 0.075% SDS (2.5 ml) and trypsinized for 48 h (four regular additions, enzyme/protein ratio of 1:10). It was verified by gel electrophoresis that all of the material migrated with an M_r of 8000 (results not shown). The sample (250 μg of protein in approx. 0.6 ml) was chromatographed in two similar columns of Bio-Gel P-10 equilibrated and developed in either the absence or the presence of SDS. In the former case, about 85–90% ($n = 4$) of the material

was lost, presumably precipitated during the elution of the gel (results not shown). In contrast, in the presence of SDS, the M_r -8000 peptide was eluted near the void volume (Fig. 4a).

To determine the contribution of the amino acids interacting with SDS, the tryptic limiting fragment obtained as described above was further treated with proteinase for 48 h (four regular additions; enzyme/protein ratio of 1:20) and analysed in Bio-Gel P-10 in the presence and absence of SDS. In three experiments, more than 90% of the material was recovered, eluting with similar K_{av} in both the presence (Fig. 4a) and the absence (results not shown) of detergent. In both cases a change in migration, consistent with a reduction in size, was observed with respect to the tryptic limiting peptide (Fig. 4a). The behaviour of the M_r -8000 fragment in its interaction with the detergent may therefore be accounted for by its amino acid composition rather than the fact that it has a highly hydrophobic radioactive ligand attached to it.

In contrast to what is observed with the tryptic limiting fragment, the proteinase-treated peptide was retained in cation-exchange chromatography at pH 9.0 (Fig. 4b). A similar result was obtained when free ligand was chromatographed (results not shown). This suggests that the ligand onium group may account for this behaviour because of its high isoelectric point ($pI = 11$; Venter *et al.*, 1984) and that most of the amino acids of the tryptic limiting fragment were cleaved.

Curtis *et al.* (1989) have suggested that the tracer is covalently attached to an acidic amino acid through an ester linkage. The proteinase used in the above experiments is a mixture of several enzymes, some of them having esterolytic activity (Narahashi, 1970). Therefore the susceptibility of the labelled tryptic limiting fragment to these esterases may have also resulted in the splitting of the ligand. The latter possibility does not invalidate the observation that the hydrophobic properties of the M_r -8000 fragment are related to its amino acid composition.

Dadi & Morris (1984) described that alkaline treatment of [^3H]PrBCM-labelled mAChR resulted in the splitting of the label. To test the effect of alkali on the labelled tryptic limiting fragment, the pH of the solution containing the M_r -8000 species was raised to 12.0 with the addition of a few microlitres of conc. NaOH. The sample was then incubated for 6 h at 37 $^{\circ}\text{C}$, neutralized with HCl and analysed as before. The results obtained from two experiments were identical to those described above for the proteinase, either in Bio-Gel P-10 chromatography (with or without SDS) or by cation-exchange chromatography (results not shown). At the indicated pH, almost all of the M_r -8000 species was converted into the labelled derivative. At lower pH values, less conversion takes place and no products were detected migrating between the M_r -8000 species and its labelled derivative in SDS/Bio-Gel P-10 chromatography. This result suggests that the alkaline treatment may have acted on the M_r -8000 fragment in a similar way as proteinase. Since the alkaline treatment was mild, peptide linkages were possibly not affected, thus suggesting the existence of an ester linkage between the ligand and the peptide, and also supporting the view that the amino acids of the tryptic limiting fragment were involved in the hydrophobic properties.

The radioactive residue generated after the proteinase

Fig. 4. Gel exclusion and cation-exchange chromatography of [^3H]PrBCM-labelled mAChR fragments

(a) Migration on 0.075% SDS-Bio/Gel P-10 of the tryptic limiting fragment ($\bullet$) and of a similar sample that had been subsequently treated with proteinase ($\circ$). When a similar column was equilibrated and developed without the detergent, the tryptic limiting fragment was not recovered whereas the proteinase-derived residue and Phenol Red (arrow) eluted with the same K_{av} . (results not shown). The amount of protein was 0.25 mg in both cases. The V_0 and V_i were determined with Blue Dextran-2000 and Bromophenol Blue respectively. The M_r of Phenol Red, a more hydrophobic dye than Bromophenol Blue, is 354. (b) Behaviour of the same samples described for panel (a) on a Dowex 50W-X4 resin. Whereas the proteinase-derived residue was retained by the resin, the tryptic limiting fragment passed unretained. Identical results were obtained from the alkaline treatment of the tryptic limiting peptide. See the text for details.

treatment was not retained by a strong anion-exchange resin (Cellex-T, Bio-Rad) equilibrated with 5 mM-Tris at pH 9.5. This result suggests that carboxylate groups from residual amino acids are not present. It was verified that the label was not split from the tryptic limiting fragment at pH 9.5. The arrow in Fig. 4(a) indicates the migration of Phenol Red, a dye which is soluble in water but is more hydrophobic than Bromophenol Blue. Since the migration of the former dye is close to the residual label, this label should be, as expected, a small compound resulting from splitting the ligand from the peptide. The differences in migration between the two dyes may be the result of secondary interactions with the hydrophilic matrix of the gel.

The stability of the interaction of SDS with the tryptic limiting fragment was further established by carrying out a series of dilutions (100–200-fold) of a 0.075%-SDS-solubilized M_r -8000 residue (100 μg of protein). Upon centrifugation at 165000 g for 1 h, 74–76% ($n = 3$) of the label was recovered in the supernatant. Similar results were obtained when identical samples were filtered through 0.2 μm membranes. In all cases, almost all of the labelled material passed through the filter. These results indicate that the interaction of SDS with the peptide is very strong, since it remains stable despite large dilutions [to 0.000375% SDS, which is well below the critical micelle concentration (Welling *et al.*, 1987)]. Very large dilutions and long time periods should promote dissociation from the detergent, as may be the case with the Bio-Gel P-10 chromatography in the absence of SDS.

Analysis of the labelled tryptic-limiting fragment by reverse-phase h.p.l.c. on a C_{18} column, under the conditions used by Kubo *et al.* (1986), resulted in low recoveries of the labelled peptide; furthermore, its elution volume varied from one experiment to another. Such behaviour has been described for highly hydrophobic residues (Welling *et al.*, 1987). Since the solvent system

used was acetonitrile/water, we attempted to test the effect of acetonitrile on the behaviour of the SDS-dissolved M_r -8000 residue. A sample of the SDS-solubilized tryptic limiting fragment (100 μg of protein) was diluted with the same buffer to 0.00075% SDS. Increasing amounts of acetonitrile were then added to separate tubes. The detergent remained soluble in all cases. It was found that at a final concentration of 20% acetonitrile, most of the labelled peptide sedimented upon centrifugation (73–92%, $n = 3$) and was retained by the filters (about 85%), with a recovery higher than 90% in both cases. The material retained by the membrane was further eluted from the filters with a solution of 2% SDS at 60 $^{\circ}\text{C}$, whereas 8 M-urea failed to resolubilize the retained material. Acetonitrile may act on the SDS-peptide complex promoting its dissociation (the effect of this solvent in reverse-phase h.p.l.c.). Once produced, the radioactive fragment becomes insoluble in the medium.

Taken together, these results suggest that the [^3H]PrBCM-labelled tryptic limiting fragment exhibits properties characteristic of a hydrophobic peptide.

DISCUSSION

Physiologically relevant properties of the mAChR have been characterized in synaptosomal membranes; for instance, its binding heterogeneity (Birdsall *et al.*, 1978), coupling to guanine-nucleotide-binding proteins (G-proteins) (Dunlap & Heller Brown, 1984), agonist stimulation of phosphatidic acid synthesis (Smith & Yamamura, 1985) and desensitization (Burgoyne, 1983). From these studies it is apparent that the structure of the receptor is well preserved in the membranes.

The availability of the amino acid sequences deduced from cloning studies has made possible the application of methods which predict the location of the mAChR

protein with respect to the membrane (Kubo *et al.*, 1986). However, experimental confirmation of such theoretical postulates is difficult because of the small amount of native mAChR. In the present work, we found that [³H]PrBCM-labelled fragments of M_r 50 000, 30 000, 18 000 and 8000, produced by limited trypsinolysis of the native receptor, were recovered with the homogenized membranes, thus suggesting that all of them are anchored to the bilayer. All peptides contain at least part of the binding site, as indicated by the presence of the covalently bound radioligand which permits the precise site-specific labelling of the mAChR in a region located a few Angstroms from the onium group of the aziridinium-reactive form of the ligand.

Binding assays with [³H]QNB in isolated neuronal perikarya revealed that a large proportion of the mAChR occurs in neuronal bodies, which contain about 60 % of the number of sites detected in rich synaptosomal membranes.

Topological experiments carried out with neuronal perikarya, which can be sedimented at low speed, avoid artifacts that may result from homogenization and/or trypsinolysis of membranes. The results obtained with the use of these cells reinforce the view that the M_r -8000 labelled residue represents an authentic membrane-bound domain of the mAChR.

It has not been possible to determine in this work whether trypsin has had access to one or both sides of the perikarya membrane, as reported by other authors in similar cases (Hootman & Picado-Leonard, 1985; Hootman *et al.*, 1985; Dohlman *et al.*, 1987b). If the membranes were 'leaky' to trypsin, the M_r -8000 fragment could have been generated by cleavage in the cytoplasmic side, and this result would be consistent with the putative transmembrane disposition. However, this may not be the only possibility; although the membrane-bound behaviour is consistent with the hydrophobic profile, alternative dispositions may also be possible as reported in another case (Maelicke *et al.*, 1989). It should be pointed out that the isolated neurons in these experiments constitute a minimally disturbed system in which native receptors can be found in their natural environment.

Hootman & Picado-Leonard (1985) found that upon papain digestion of acinar pancreatic cells, a [³H]PrBCM-labelled fragment of M_r 45 000 derived from mAChR remained attached to the cells. More recently, Cho & Klein (1988) showed that [³H]PrBCM-labelled mAChR of retinal and cardiac origin generated labelled fragments of different sizes upon cleavage with *Staphylococcus aureus* V8 protease and that they remained bound to the membranes after centrifugation. No quantitative studies were performed, however, in the latter case. Biochemical studies of mAChRs from rat brain membranes suggest that about half of the protein containing [³H]PrBCM can be enzymically released as a water-soluble fragment (Venter, 1983). In the latter case about one half of the radioactivity, which cannot be assumed to be non-degraded receptor, remained bound to the membranes. According to the results shown in the present work, all labelled fragments generated by proteolytic cleavage of mAChR are insoluble in aqueous media (Fig. 1). The centrifugal force field used (192 000 *g* for 10 min) is less than that used for the β -adrenergic receptor (450 000 *g* for 30 min; Dohlman *et al.*, 1987b). Furthermore, even reducing the centrifugal force to 12 000 *g* for 3 min (instead of 42 000 *g* for 20 min as used by Venter, 1983)

we found that 68 % of the labelled peptides were recovered with the membranes. Thus, under all sets of conditions used in the present work, most of the labelled peptides were recovered bound to the membranes, suggesting that they constitute mAChR domains anchored in the bilayer (Dohlman *et al.*, 1987b).

Upon extensive trypsinolysis, the membrane-bound [³H]PrBCM-labelled receptor in either homogenized membranes or neurons could be degraded to a limiting peptide of M_r 8000 which lacks sugars, possesses thiol groups, and reveals a very hydrophobic behaviour. These data concur with those obtained with SDS-unfolded mAChR, in which the amino acid stretch of 58–123 of the m1 subtype of mAChR was tentatively assigned to the region involved with the binding site (Alperin *et al.*, 1987). According to this assignment, the latter fragment should remain attached to the membranes since Arg and Lys residues, the trypsin cleavage sites, are postulated to occur outside the bilayer (Kubo *et al.*, 1986). Furthermore, this fragment should be very hydrophobic.

Wheatley *et al.* (1987) reported that purified [³H]PrBCM-labelled mAChR precipitated from a SDS solution by ethanol is insoluble in aqueous media and that its tryptic digestion yields a water-insoluble fragment of M_r 11 000, in general agreement with the results reported in the present work. The difference in size of the tryptic fragments may be accounted for by the SDS/PAGE systems and the behaviour of small peptides (Dunker & Rueckert, 1969), the protein standards used and perhaps by selection of some mAChR subclass during purification (Wheatley *et al.*, 1988).

The nature of the bond between the tracer and the protein through an ester linkage was also reported by Curtis *et al.* (1989), based on the effect of nucleophiles, acids and bases. Our proteolysis data support this view and provide the first evidence about the nature of the bond using enzymic digestion. From CNBr cleavage experiments, Curtis *et al.* (1989) assigned the (theoretically postulated) transmembrane helix 3 as the site of [³H]PrBCM attachment. This could indeed be the case, as the labelled tryptic limiting fragment, which should contain the latter region, was shown in this work to be membrane-bound and highly hydrophobic.

We are greatly indebted to Dr. J. Azcurra and P. Berninsone from the University of Buenos Aires for kindly providing implements for neuronal isolation and help. The help of E. Buzzi and H. Caldironi from our Institute in carrying out some of the experiments is also acknowledged. C.B.B. is a fellow of the Argentinian Scientific Research Council (CONICET).

REFERENCES

- Alperin, D. M., Bouzat, C. B. & Barrantes, F. J. (1987) *Biochem. J.* **251**, 657–665
- Birdsall, N. J. M., Burgen, A. S. V. & Hulme, E. C. (1978) *Mol. Pharmacol.* **14**, 723–736
- Blow, D. M., Birktoft, J. J. & Hartley, B. S. (1969) *Nature (London)* **221**, 337–340
- Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. (1987) *Science* **237**, 527–532
- Bonner, T. I., Young, A. C., Brann, M. R. & Buckley, N. J. (1988) *Neuron* **1**, 403–410
- Burgoyne, R. D. (1983) *J. Neurochem.* **40**, 324–331
- Cho, N. J. & Klein, W. L. (1988) *J. Neurochem.* **50**, 1403–1411

- Curtis, C. A. M., Wheatley, M., Bansal, S., Birdsall, N. J. M., Eveleigh, P., Pedder, E. K., Poyner, D. & Hulme, E. C. (1989) *J. Biol. Chem.* **264**, 489–495
- Dadi, H. K. & Morris, R. J. (1984) *Eur. J. Biochem.* **144**, 617–628
- Dohlman, H. G., Caron, M. C. & Lefkowitz, R. J. (1987a) *Biochemistry* **26**, 2657–2664
- Dohlman, H. G., Bouvier, M., Benovic, J. L., Caron, M. G. & Lefkowitz, R. J. (1987b) *J. Biol. Chem.* **262**, 14282–14288
- Dohlman, H. G., Caron, M. G., Strader, C. D., Amlaiky, N. & Lefkowitz, R. J. (1988) *Biochemistry* **27**, 1813–1817
- Dunker, A. K. & Rueckert, R. R. (1969) *J. Biol. Chem.* **244**, 5074–5080
- Dunlap, J. & Heller Brown, J. (1984) *J. Neurochem.* **43**, 214–219
- Hootman, S. R. & Picado-Leonard, T. M. (1985) *Biochem. J.* **231**, 617–622
- Hootman, S. R., Picado-Leonard, T. M. & Burnham, D. B. (1985) *J. Biol. Chem.* **260**, 4186–4194
- Kubo, T., Fukuda, K., Mikami, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Kojima, J., Matsuo, H., Hirose, T. & Numa, S. (1986) *Nature (London)* **323**, 411–416
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 262–275
- Maelicke, A., Plümer-Wilk, R., Fels, G., Spencer, S. R., Engelhard, M., Veltel, D. & Conti-Tronconi, B. M. (1989) *Biochemistry* **28**, 1396–1405
- Narahashi, I. (1970) *Methods Enzymol.* **19**, 653–655
- Ochoa, E. L. M., Brusco, A., Idoyaga-Vargas, V. P. & Carminatti, H. (1982) *J. Neurochem.* **38**, 1236–1240
- Sellinger, O. Z., Azcurra, J. M., Johnson, D. E., Ohlsson, W. G. & Lodin, Z. (1971) *Nature (London)* **230**, 253–256
- Smith, T. L. & Yamamura, H. I. (1985) *Biochem. Biophys. Res. Commun.* **130**, 282–285
- Strader, C. D., Sigal, J. S., Candelore, M. R., Rands, E., Hill, W. S. & Dixon, R. A. F. (1988) *J. Biol. Chem.* **263**, 10267–10271
- Venter, J. C. (1983) *J. Biol. Chem.* **258**, 4842–4848
- Venter, J. C., Eddy, B., Hall, L. M. & Fraser, C. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 272–276
- Welling, G. W., Van Der Zee, R. & Welling-Wester, S. (1987) *J. Chromatogr.* **418**, 223–243
- Wheatley, M., Birdsall, N. J. M., Curtis, C., Eveleigh, P., Pedder, E. K., Poyner, D., Stockton, J. M. & Hulme, E. C. (1987) *Biochem. Soc. Trans.* **15**, 113–116
- Wheatley, M., Hulme, E. C., Birdsall, N. J. M., Curtis, C. A. M., Eveleigh, P., Pedder, E. K. & Poyner, D. (1988) *Trends Pharmacol. Sci. Suppl.* **3**, 19–24
- Yanagahira, T. (1979) *J. Neurochem.* **32**, 169–177

Received 4 April 1989/14 June 1989; accepted 26 June 1989