0026-895X/87/040443-07$02.00/0

MOLECULAR PHARMACOLOGY, 32:443-449

ACCELERATED COMMUNICATION

[^{3}H]Propylbenzilylcholine Mustard-Labeling of Muscarinic Cholinergic Receptors that Selectively Couple to Phospholipase C or Adenylate Cyclase in Two Cultured Cell Lines

MEINA LIANG, MICHAEL W. MARTIN, and T. KENDALL HARDEN

Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received May 27, 1987; Accepted July 21, 1987

SUMMARY

Although both second messenger response systems are fully functional in both cell lines, activation of muscarinic cholinergic receptors only results in inhibition of adenylate cyclase in NG108-15 neuroblastoma × glioma cells and stimulation of phosphoinositide hydrolysis in 1321N1 human astrocytoma cells. Muscarinic receptors on both cell types were covalently labeled with [^{3}H]propylbenzilylcholine mustard ([^{3}H]PBCM), and the mobilities of the [^{3}H]PBCM-labeled species of both cells were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. 1321N1 and NG108-15 cells each primarily expressed a single [^{3}H]PBCM-labeled species with an apparent size of approximately 92,000 and 66,000 Da, respectively. [^{3}H]PBCM labeling was completely inhibited by 1 μM atropine or by down-regulation of muscarinic receptors by an overnight incubation with carbachol. The apparent size of the [^{3}H]PBCM-labeled species of both cell lines was not altered by treatment with a series of protease inhibitors or by treatment with dithiothreitol and iodoacetamide. Since muscarinic receptors are glycoproteins, the contribution of carbohydrate groups to the difference in apparent size of the [^{3}H]PBCM-labeled proteins was determined by treatment of [^{3}H]PBCM-labeled membranes with endoglycosidase F, an enzyme that removes both complex and high mannose type N-linked carbohydrate chains. Endoglycosidase F treatment reduced the apparent size of the [^{3}H]PBCM-labeled species in 1321N1 cells from 92,000 to approximately 77,000 Da and in NG108-15 cells from 66,000 to 45,000 Da. Neuraminidase produced no further reduction of the apparent size of the [^{3}H]PBCM-labeled species from either cell after endoglycosidase F treatment, suggesting the absence of sialic acid containing O-linked carbohydrate chains on the muscarinic receptors of the two cell lines. The results suggest that different muscarinic receptor proteins may be responsible for the two different biochemical responses to muscarinic receptor activation.

Activation of muscarinic cholinergic receptors results in inhibition of adenylate cyclase (1–3) and an increase in phosphoinositide hydrolysis (3–5), apparently through separate guanine nucleotide regulatory proteins (6–8). To date, it is unclear whether different muscarinic cholinergic receptor proteins are responsible for these different second messenger responses. Putative M_1- and M_2-muscarinic cholinergic receptor subtypes have been differentiated based on relative receptor binding affinities of the antagonist pirenzepine, and attempts have been made to correlate a specific muscarinic cholinergic receptor subtype with either inhibition of adenylate cyclase or stimulation of phosphoinositide hydrolysis. Gil and Wolfe (9) found that in rat brain pirenzepine was 15-fold more potent for blockade of muscarinic receptor-mediated stimulation of phosphoinositide hydrolysis than for blockade of muscarinic receptor-mediated inhibition of adenylate cyclase. In contrast, pirenzepine was more potent as an antagonist of the adenylate cyclase response than the phosphoinositide response in chick heart cells (10). Both Lazareno *et al.* (11) and Fisher (12) have reported that there are regional differences in the affinity of pirenzepine for antagonism of muscarinic receptor-mediated stimulation of phosphoinositide hydrolysis in guinea pig brain. Thus it appears unlikely at this time that putative muscarinic cholinergic receptor subtypes identified on the basis of their affinity for pirenzepine can be clearly correlated with either of the second messenger responses.

We have previously utilized cultured cell lines to better understand muscarinic receptors and their mode of interaction

[1] J. M. May and T. K. Harden, unpublished observations.

ABBREVIATIONS: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; [^{3}H]PBCM, [^{3}H]propylbenzilylcholine mustard hydrochloride; [^{3}H]QNB, [^{3}H]quinuclidinyl benzilate; DMEM, Dulbecco's modified Eagle's medium; Endo F, endoglycosidase F; Tris, tris (hydroxymethyl) aminomethane; DTT, dithiothreitol; NP-40, Nonidet P-40; EDTA, ethylenediamino-tetraacetic acid; Hepes, (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

with second messenger response systems. Both cyclic AMP and inositol phosphate signaling systems are fully functional in NG108-15 neuroblastoma × glioma and 1321N1 human astrocytoma cells. However, muscarinic receptor activation only inhibits adenylate cyclase in NG108-15 cells (13), whereas it only stimulates phosphoinositide hydrolysis in 1321N1 cells (5, 13–15). One potential interpretation of these results is that there are different muscarinic receptor proteins that mediate different biochemical responses in NG108-15 and 1321N1 cells. In this study, we compare the properties of the muscarinic receptors of the two cell lines during SDS-PAGE after labeling with a covalent radioligand, [^{3}H]PBCM, and report evidence for the existence of two different muscarinic receptor proteins.

Experimental Procedures

Materials

[^{3}H]PBCM (39.9 Ci/mmol) and [^{3}H]QNB (33.4 Ci/mmol) were purchased from New England Nuclear (Boston, MA), and DMEM and fetal calf serum were from Grand Island Biological (Grand Island, NY). Leupeptin, pepstatin, and Endo F (from *Flavobacterium meningosepticum*) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and concanavalin A was from Calbiochem-Behring. Low molecular weight standards for SDS-PAGE, acrylamide, bis-acrylamide, SDS, Tris and glycine for SDS-PAGE, β-mercaptoethanol, and DTT were purchased from Bio-Rad Laboratories (Richmond, CA). Ultra-pure sucrose was obtained from Schwartz-Mann (Cambridge, MA). Atropine sulfate, carbachol, Tris, iodoacetamide, α-methylmannoside, lima bean trypsin inhibitor, benzamidine, phenylmethylsulfonyl fluoride, NP-40, and neuraminidase (from clostridium perfringens) were obtained from Sigma Chemical (St. Louis, MO).

Methods

Cell culture. NG108-15 cells were grown in DMEM containing 5% fetal calf serum, 4.5 g/l glucose, 0.1 mM hypoxanthine, 16 μM thymidine, 1 μM aminopterin, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated in a humidified atmosphere of 92% air and 8% CO_2 at 37°C; 1321N1 cells were grown in DMEM containing 5% fetal calf serum and no antibiotics.

Membrane preparations. Confluent NG108-15 cells were rinsed once with lysis buffer (2 mM EDTA, 1 mM Tris, pH 7.5) at 4°, detached by scraping the dish with a rubber policeman, and homogenized using a Dounce homogenizer. The homogenate was centrifuged at 400 × *g* for 5 min to remove nuclei and whole cells, and the resultant supernatant was centrifuged at 40,000 × *g* for 15 min. The membrane pellet was washed once with lysis buffer and once with 50 mM phosphate buffer (pH 7.0) at 4° and then suspended with the same phosphate buffer.

Confluent 1321N1 cells were routinely used for experiments 10–15 days after seeding. Cells were incubated with 0.25 mg/ml concanavalin A in Hepes-buffered DMEM (25 mM Hepes, pH 7.4) for 40 min at 4° before incubation in lysis buffer on ice for 40 min (16). The lysate was layered on a gradient consisting of sucrose steps of 20, 35, and 60% and centrifuged at 100,000 × *g* for 1 hr at 4°. Membranes were removed from the 35–60% sucrose interface, treated with 0.5 M α-methylmannoside for 2 hr at 37°, and applied to a second gradient consisting of 20, 40, and 50% sucrose steps. Centrifugation was at 100,000 × *g* for 1 hr. Membranes recovered at the 20–40% sucrose interface were diluted and washed once with 50 mM phosphate buffer (pH 7.0) at 4°. The washed membranes were suspended with the same phosphate buffer described above.

For brain membranes, an adult rat was killed, the brain quickly removed, and the cerebral cortex and cerebellum were isolated and homogenized in a Brinkman Polytron (setting 6.5) in 50 mM phosphate buffer, pH 7.4, containing 0.32 M sucrose. After centrifugation of the homogenate at 400 × *g*, the supernatant was collected and centrifuged at 40,000 × *g* for 15 min. The resultant membrane pellet was washed once with 50 mM phosphate buffer (pH 7.0 at 4°) and resuspended in the same buffer.

Protease inhibitors, 3 μg/ml leupeptin, 0.1 mM benzamidine, 1 μg/ml lima bean trypsin inhibitor, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 3 μg/ml pepstatin were included in each step of all membrane preparations.

Covalent labeling of membrane muscarinic receptors with [^{3}H]PBCM. [^{3}H]PBCM was cyclized by dilution with 50 mM phosphate buffer (pH 7.4) and incubation at room temperature for 30 min. The cell or brain membranes were initially incubated with or without 1 μM atropine at 30° for 15 min and then labeled with precyclized [^{3}H]PBCM (final concentration, 10 nM) at 30° for 30 min. The labeling was terminated by either filtration through a glass fiber filter or centrifugation at 15000 × *g*.

The conditions of [^{3}H]PBCM cyclization and labeling were chosen to label a high percentage of the total muscarinic cholinergic receptors with a minimum of nonspecific [^{3}H]PBCM labeling (detected in the presence of 1 μM atropine). [^{3}H]PBCM at final concentrations of 10 and 50 nM labeled approximately 80 and 100%, respectively, of the specific binding sites identified by [^{3}H]QNB. Most experiments were performed using 0.2–0.5 mg of membrane protein and 10 nM [^{3}H]PBCM.

Covalent labeling of muscarinic receptors on intact cells with [^{3}H]PBCM. Muscarinic receptors were labeled on intact cells as previously described by Hunter and Nathanson (17). 1321N1 or NG108-15 cells, grown on 150-mm tissue culture plates, were incubated with labeling buffer (115 mM NaCl, 10 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 1.0 g/l *d*-glucose, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.0) with or without 10 μM atropine at 30° for 15 min. Precyclized [^{3}H]PBCM (as described above) was added at a final concentration of 10 nM, and the cells were incubated at 30° for 30 min. The labeling was terminated by washing cells twice with labeling buffer followed by detaching cells from the plate by either directly suspending NG108-15 cells off the plate with 20 ml of the above labeling buffer or incubating 1321N1 cells with 20 mM EGTA (in labeling buffer) at 37° for 50 min and then suspending as above. The cells were then centrifuged at 500 × *g*, and the cell pellet was washed once with labeling buffer before addition of 1.5 ml of SDS sample buffer [62.5 mM Tris (pH 6.8), 4% SDS, 0.02% Bromophenol blue, 5% β-mercaptoethanol, 10% glycerol]. After incubation at room temperature for 2 hr, SDS-PAGE was conducted as described below.

Endo F and neuraminidase treatment. [^{3}H]PBCM-labeled membranes (200–500 μg of membrane protein) were suspended in 30 μl of buffer A (50 mM EDTA, 38 mM Na_2HPO_4, 1% β-mercaptoethanol, 1% NP-40, 0.1% SDS, pH 6.1) and incubated at room temperature for 1 hr with periodic mixing. Endo F (250 mU/0.2–0.5 mg protein in 20 mM Na_2HPO_4 and 50 mM EDTA, pH 7.2) or neuraminidase (130 mU/0.2–0.5 mg protein in distilled water containing 1 mg/ml bovine serum albumin) was added and incubated (usually for 2 hr) at 37° with periodic mixing. In some experiments, fresh Endo F was added every 30 min (total 3500 mU Endo F), and the incubation was conducted at 37° for 9 hr 15 min. Results were the same as that obtained with one addition of Endo F (250 mU) and incubation for 2 hr. The reaction was terminated with DTT and iodoacetamide treatment (see below) followed by an equal volume of SDS-sample buffer [125 mM Tris (pH 6.8), 4% SDS, 0.04% Bromophenol blue, 10% β-mercaptoethanol, 21% glycerol].

DTT and iodoacetamide treatment. The membranes or detergent-solubilized proteins were treated with 750 μM DTT in 10% SDS at room temperature for 30 min followed by 240 mM iodoacetamide at room temperature for 15 min.

SDS-PAGE. Electrophoresis was performed using 9% acrylamide linear slab gels unless indicated in the figure legend (18). Gels were stained with Coomassie blue and sliced into 3- or 5-mm slices, which were then solubilized with 300 μl of 30% H_2O_2 at 60–70°. After incubation with 4 ml of scintillation fluid overnight, the gel slices were

counted in a scintillation counter for 10 min (counting efficiency was 20%).

The protein standards used were phosphorylase B (92,500 Da), albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da), and lysozyme (14,400 Da). The apparent size of [^{3}H]PBCM-labeled species was estimated from a linear plot of logarithm of molecular weight versus relative mobility.

Protein was determined by the method of Bradford using bovine serum albumin as the standard (19).

Results

Comparison of [^{3}H]PBCM-labeled muscarinic cholinergic receptors of 1321N1 and NG108-15 cells by SDS-PAGE. Muscarinic cholinergic receptors of each cell line were covalently labeled with [^{3}H]PBCM, and their comparative mobilities on SDS-PAGE were examined (Fig. 1). Radioactivity migrated primarily as a single major peak in each case (but see Fig. 5, below). However, the apparent size of the labeled species from 1321N1 cells ($M_r \cong 92{,}000$) was larger than that from NG108-15 cells ($M_r \cong 66{,}000$). Labeling of the major species was blocked by atropine in a concentration-dependent manner in each case with maximal inhibition observed in the presence of 1 μM atropine (Fig. 1 and data not shown). Although data presented in Fig. 1 involved the use of membranes prepared from 1321N1 cells by sucrose density gradient centrifugation, the [^{3}H]PBCM labeling profile observed with washed membranes from 1321N1 cells was the same (data not shown) as that presented in Fig. 1A, indicating that a difference in the methods of membrane preparation was not responsible for the different [^{3}H]PBCM labeling profiles observed with the two cell lines.

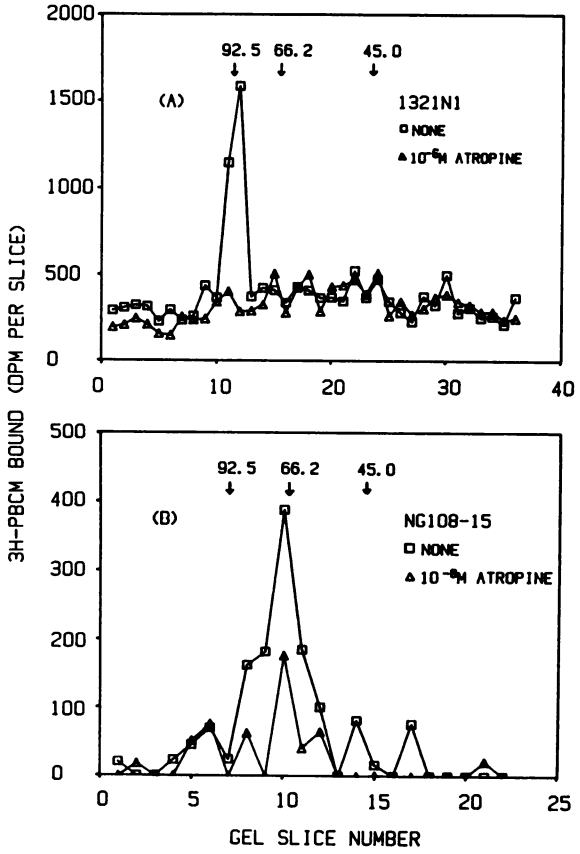

Fig. 1. SDS-PAGE of [^{3}H]PBCM-labeled membrane proteins from 1321N1 (A) and NG108-15 cells (B). Membranes were prepared in the presence of protease inhibitors as described under Experimental Procedures. The purified membranes were labeled with precyclized [^{3}H]PBCM at 30° for 30 min and then solubilized with 2% SDS followed by SDS-PAGE on a linear 9% gel. After Coomassie blue staining, gels were sliced into 3- (A) or 5-mm (B) slices, and slices were solubilized with 30% H_2O_2 before quantitation of ^{3}H in a scintillation counter. Nonspecific labeling was defined as [^{3}H]PBCM labeling in the presence of 1 μM atropine. In NG108-15 cells, nonspecific [^{3}H]PBCM labeling was subtracted from total binding in each fraction. Standards proteins used in each gel are phosphorylase B (92,500 Da), albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da), and lysozyme (14,400 Da). The sizes of the [^{3}H]PBCM-labeled peaks were estimated from a linear plot of the logarithm of molecular weight versus relative mobility. These data are representative of results obtained with 3 and 15 separate preparations of NG108-15 and 1321N1 membranes, respectively.

Iodoacetamide treatment after DTT treatment has been reported (20) to prevent disulfide bond-associated protein aggregation. However, sequential DTT-iodoacetamide treatment did not change the profile of [^{3}H]PBCM labeling in either cell line (data not shown). A series of protease inhibitors (see Materials and Methods) also had no effect on the [^{3}H]PBCM labeling profile of 1321N1 and NG108-15 cells. However, the possibility that some other unanticipated factors or a protease insensitive to the inhibitors used in this study was responsible for the smaller apparent size of the [^{3}H]PBCM-labeled species of NG108-15 cells was examined. Membranes from 1321N1 and NG108-15 cells were prepared in the absence of protease inhibitors as indicated under *Methods*. The membranes from 1321N1 cells then were mixed with either NG108-15 membranes or a cytosolic fraction prepared from NG108-15 cells, and the mixtures were labeled by [^{3}H]PBCM, solubilized, and their properties compared on SDS-PAGE (Fig. 2). Two radioactive species were observed in the mixed membranes that corresponded to the peaks of [^{3}H]PBCM-labeling observed with 1321N1 and NG108-15 membranes alone (Fig. 2A). The cytosolic fraction obtained from NG108-15 cells also did not change the [^{3}H]PBCM labeling profile observed with 1321N1 cell membranes (Fig. 2B).

Hunter and Nathanson (17) have reported that [^{3}H]PBCM labeled a lower molecular weight species ($M_r \cong 48{,}000$) in homogenates from cultured heart cells than with intact cells ($M_r \cong 70{,}000$) and that the change of molecular weight of [^{3}H]PBCM-labeled species after homogenization could not be prevented by a series of protease inhibitors. To further examine whether differences in apparent size of the receptors on both cell lines were due to artifactual contributions during membrane preparation, [^{3}H]PBCM was used to label muscarinic receptors on intact 1321N1 and NG108-15 cells. The [^{3}H]PBCM-labeled cells then were directly solubilized with SDS-sample buffer, and SDS-PAGE was performed as before (Fig. 3). The apparent sizes of the labeled species from 1321N1 cells ($M_r \cong 92{,}000$) and NG108-15 cells ($M_r \cong 66{,}000$) revealed by intact cell labeling were essentially identical to those obtained if the labeling step was performed with purified membranes (Fig. 1).

Deglycosylation of muscarinic cholinergic receptors in 1321N1 and NG108-15 cells. Since muscarinic cholinergic receptors are glycoproteins (21, 22), the apparent difference in size of [^{3}H]PBCM-labeled muscarinic receptors on 1321N1 and NG108-15 cells could result from differences in the carbohydrate groups on the respective receptor proteins.

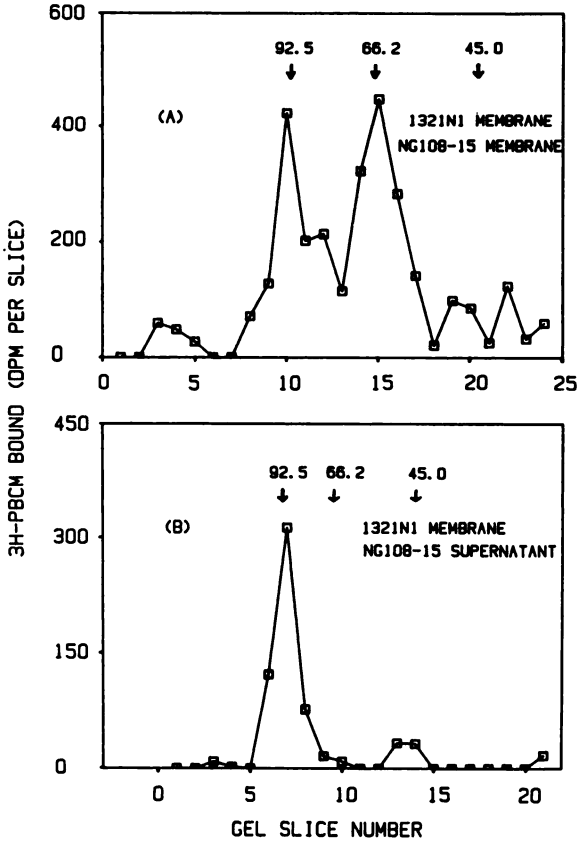

Fig. 2. SDS-PAGE of [^{3}H]PBCM-labeled mixtures of samples from 1321N1 and NG108-15 cells. A, 1321N1 membranes mixed with equal volume of NG108-15 membranes. B, 1321N1 membranes mixed with equal volume of a cytosolic fraction of NG108-15 cells. Membranes from 1321N1 and NG108-15 cells were separately prepared as described in Fig. 1 in the absence of protease inhibitors. Before [^{3}H]PBCM labeling, membranes from 1321N1 cells were mixed with either membranes from NG108-15 cells or a cytosolic fraction of NG108-15 cells (supernatant after 40,000 × *g* centrifugation of a NG108-15 cell homogenate). The [^{3}H]PBCM-labeled membranes were solubilized and subjected to linear 9% SDS-PAGE as described in Methods. Gels were stained with Coomassie blue and sliced into 5-mm fractions. Data are represented as specifically bound radioactivity (dpm), and results represent typical profiles from three experiments.

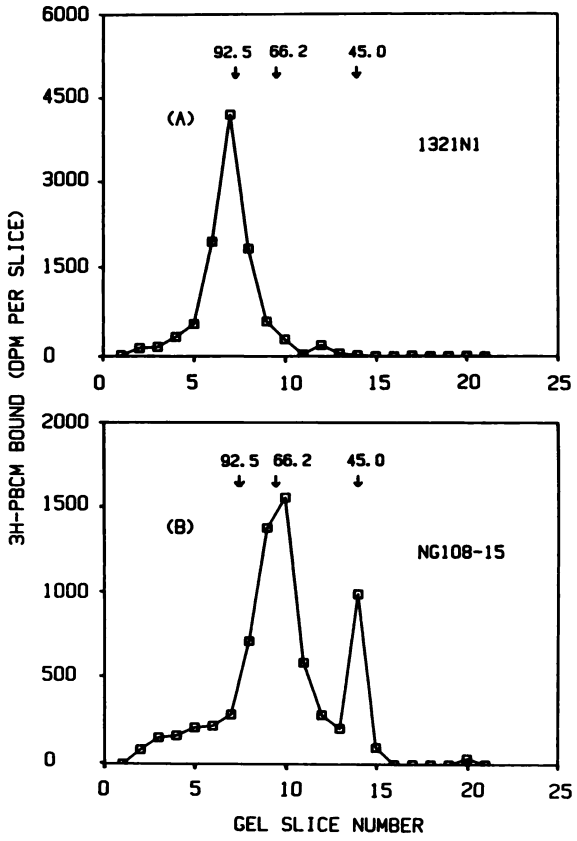

Fig. 3. SDS-PAGE of [^{3}H]PBCM-labeled intact 1321N1 (A) and NG108-15 (B) cells. One confluent 150-mm tissue culture plate of intact 1321N1 or NG108-15 cells was labeled by [^{3}H]PBCM and directly solubilized in 4% SDS-sample buffer at room temperature for 2 hr as described in Experimental Procedures, followed by SDS-PAGE on a linear 9% gel. Gel was stained with Coomassie blue and sliced into 5 mm slices. Nonspecific labelling was defined as [^{3}H]PBCM labeling in the presence of 10 μM atropine. Data are represented as specifically bound radioactivity (dpm).

Thus [^{3}H]PBCM-labeled membrane preparations were treated (see Methods) with Endo F, an enzyme that removes both complex and high mannose type N-linked carbohydrate chains (23). For both types of chains, the site of cleavage is between the di-N-acetylglucosamine core linkage, and therefore a glycoprotein should be essentially deglycosylated in terms of N-linked carbohydrate chains after Endo F treatment. The effect of Endo F on [^{3}H]PBCM-labeled muscarinic receptors of rat cerebellum was first tested (Fig. 4). The apparent size of the major [^{3}H]PBCM-labeled species was reduced from $M_r \cong 66{,}000$ to $M_r \cong 52{,}000$ by removal of the N-linked carbohydrate chains. A similar result was obtained for [^{3}H]PBCM-labeled muscarinic receptors of rat cerebral cortex after Endo F treatment (data not shown). Treatment with Endo F reduced the apparent size of the [^{3}H]PBCM-labeled species of 1321N1 cells from $M_r \cong 92{,}000$ to $M_r \cong 77{,}000$ (Fig. 5). Higher concentrations of Endo F, longer incubation times, or readdition of fresh enzyme did not result in a labeled species with greater mobility during SDS-PAGE than the $M_r \cong 77{,}000$ protein. Endo F treatment of NG108-15 membranes resulted in a single [^{3}H]PBCM-labeled species with an apparent size of approximately 45,000 Da (Fig. 6C). Higher concentrations of Endo F or longer incubation times did not produce a further reduction in apparent size of the labeled protein (data not shown).

[^{3}H]PBCM labeled a species in NG108-15 cells with an apparent size of $M_r \cong 66{,}000$ (Fig. 1B). However, in some preparations (10 of 13 experiments) from NG108-15 cells a second radioactive species with an apparent size of $M_r \cong 45{,}000$ was also labeled (Figs. 3B and 6A and B). Both [^{3}H]PBCM-labeled peaks disappeared after down-regulation of muscarinic receptors by treatment of NG108-15 cells with 100 μM carbachol at 37° overnight (Fig. 6A), and labeling of both species was completely blocked by 1 μM atropine (data not shown). Endo F treatment of NG108-15 membranes that expressed both [^{3}H]PBCM-labeled species (Fig. 6B) resulted in production of a single radioactive species of $M_r \cong 45{,}000$ (Fig. 6C), suggesting that the [^{3}H]PBCM-labeled second species ($M_r \cong 45{,}000$) may represent muscarinic receptors that are not glycosylated.

Muscarinic receptors from the two cell lines could also contain O-linked carbohydrate chains. These O-linked carbohydrate chains would most likely contain sialic acid residues, which can be cleaved with neuraminidase (24, 25). NG108-15 or 1321N1 membrane preparations were treated with Endo F

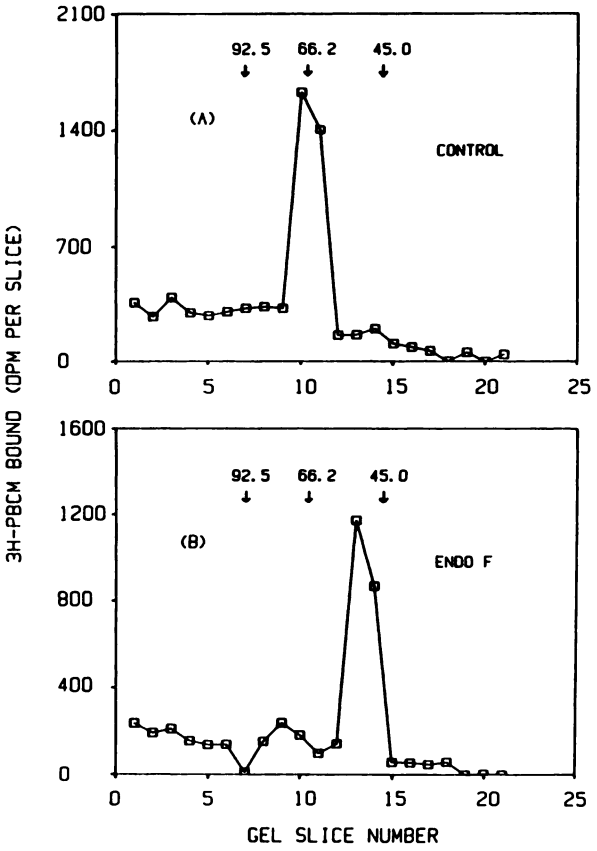

Fig. 4. Effect of Endo F on the apparent size of the [^{3}H]PBCM-labeled membrane proteins of rat cerebellum membranes. Membranes from rat brain cerebellum were prepared in the presence of protease inhibitors and labeled with [^{3}H]PBCM as described in Experimental Procedures. After suspension in NP-40 buffer A, [^{3}H]PBCM-labeled membranes were treated without or with Endo F (treated with 250-mU of Endo F at 37° for 1 hr followed by a second 250 -mU Endo F treatment at 37° for additional 2 hr) and then treated with DTT and iodoacetamide before solubilization and SDS-PAGE (linear 9% gel). The gels were stained, sliced into 5-mm fractions, and specific counts bound (dpm) are presented. The size of the [^{3}H]PBCM-labeled species was estimated as in Fig. 1 and was approximately 66,000 Da with control membranes (A) and 52,000 Da with Endo F treated membranes (B).

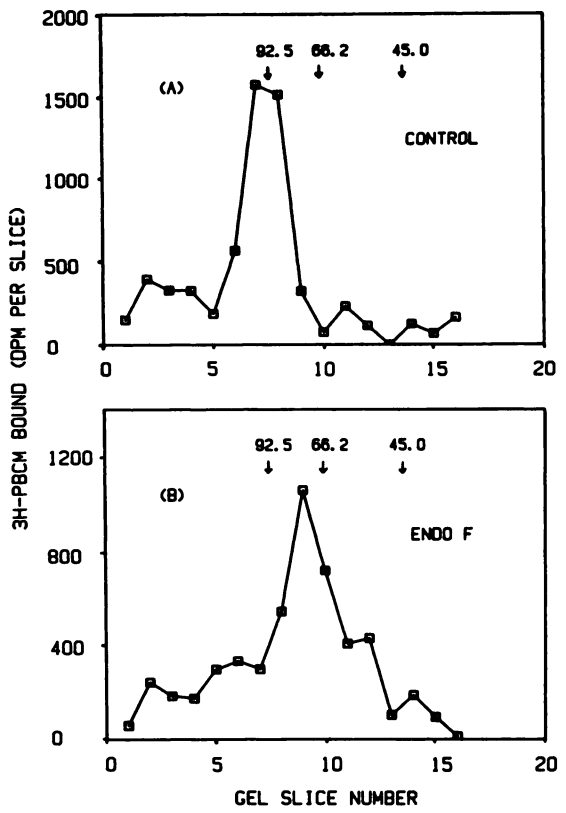

Fig. 5. Effect of Endo F on the apparent size of the [^{3}H]PBCM-labeled membrane proteins from 1321N1 cells. Membranes were purified in the presence of protease inhibitors and labeled with [^{3}H]PBCM before treatment with or without Endo F at 37° as described in Experimental Procedures. Membranes then were treated with DTT and iodoacetamide, solubilized in 2% SDS, and subjected to 8–12.5% gradient SDS-PAGE. Data are presented as specific binding (dpm) and are typical results obtained in five experiments. The size of the [^{3}H]PBCM-labeled peak was estimated as in Fig. 1 and was approximately 92,000 Da in control membranes (A) and 77,000 Da in Endo F-treated membranes. (B).

to remove N-linked carbohydrate chains and then treated with neuraminidase. The effectiveness of the neuraminidase treatment was demonstrated by treating rat brain cortex membranes under identical conditions and observing an increased mobility of the muscarinic receptors on SDS-PAGE. No change in mobility of labeled species on both cell lines was observed after Endo F and neuraminidase sequential treatment (data not shown), suggesting the absence of sialic acid containing O-linked carbohydrate chains on the muscarinic receptors of the two cell lines.

Discussion

Cloning, deduction of amino acid sequence, and expression of complementary DNA should allow identification of muscarinic receptor structural heterogeneity and eventual association of different cloned receptor sequences with the different functional correlates of cholinergic action. To date, the muscarinic receptor from porcine cerebral cortex (26) and heart (27, 28) has been cloned and the predicted amino acid sequences of each protein reported. Based on the apparent affinity of the cerebral receptor for pirenzepine after expression in *Xenopus* oocytes (26), it was proposed that the cloned porcine brain receptor was of the putative "M_1" subtype. By analogy, the relative preponderance in heart and medulla pons of RNA hybridizing with cDNA for the heart muscarinic receptor lead to the suggestion that the cloned cardiac muscarinic receptor was of the putative "M_2" subtype (27, 28). The functional significance of these data are not yet clearly established, however, due to the continued difficulty of convincingly and consistently associating a putative receptor subtype identified on the basis of relative affinity for pirenzepine with a given class of physiological and/or second messenger responses. The recent introduction of new selective muscarinic receptor antagonists provides expectation that some of the problems inherent with receptor classification based primarily on the relative affinity of a single drug, pirenzepine, can be overcome. Indeed, data obtained with at least one of these drugs, AF-DX 116, suggest that more than two muscarinic receptor subtypes exist (29).

Multiple receptor subtypes also can be delineated on the basis of their selective interaction with different second messenger response systems. One of the clearest examples of this approach is the α-adrenergic receptor subtypes; α_1-receptors couple through a yet-to-be-identified guanine nucleotide regulatory protein to activate phospholipase C, whereas α_2-receptors

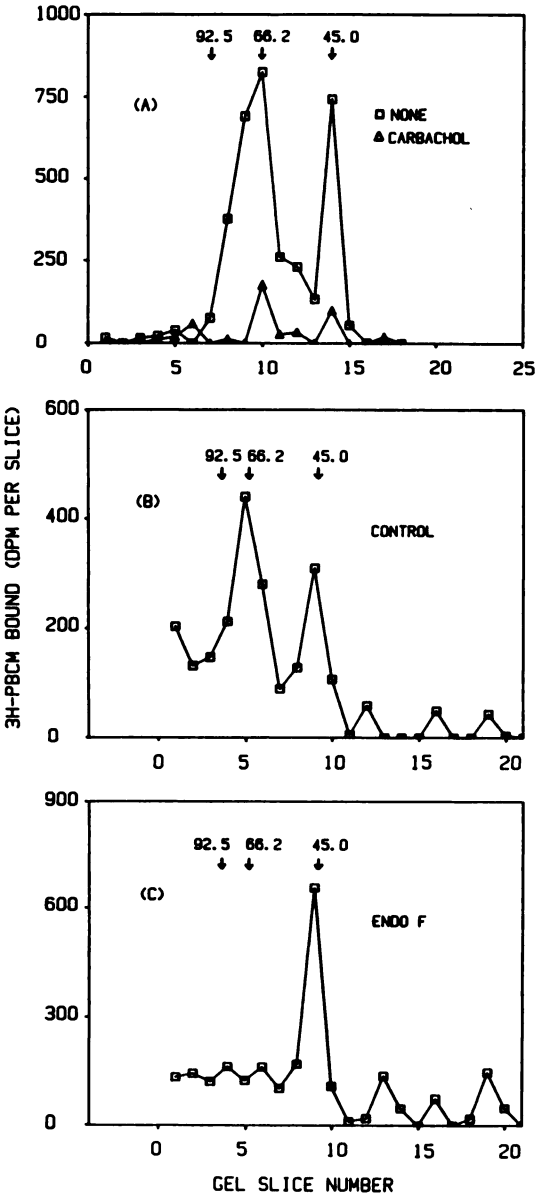

Fig. 6. Effect of Endo F on apparent size of the [^{3}H]PBCM-labeled membrane proteins from NG108-15 cells. (A) Membranes were purified in the presence of protease inhibitors from NG108-15 cells that had been incubated without (□) or with (△) 100 μM carbachol at 37°C for 24 hr. Membranes were labeled with [^{3}H]PBCM in the presence or absence of 1 μM atropine. Data are presented as specific dpm, and the results are typical of those obtained in 10 experiments. (B) and (C) NG108-15 membranes were purified in the presence of protease inhibitors, labeled with [^{3}H]PBCM in the presence or absence of 1 μM atropine, and treated without (B) or with (C) 250 mU Endo F at 37°C for 2 hr. Data are presented as specific dpm and are typical of those obtained in five experiments.

couple through the inhibitory guanine nucleotide regulatory protein G_i to inhibit adenylate cyclase (30). The availability of an extensive list of subtype-selective ligands has clearly aided in the confident subclassification of α-receptor subtypes. Work with NG108-15 and 1321N1 cells has suggested that putative muscarinic receptor subtypes may be associated with separate second messenger response systems. That is, the receptor of NG108-15 cells appears to selectively interact with G_i and adenylate cyclase, whereas the receptor of 1321N1 cells selectively interacts with a putative guanine nucleotide regulatory protein that regulates phospholipase C. Unfortunately, studies attempting pharmacological distinction of the muscarinic receptor subtypes on the two cell lines have been disappointing in that pirenzepine (31) and drugs proposed to have receptor selectivity in other tissues have failed to show any remarkable selectivity between the two cell lines.[1] Thus, to date we can only propose that the muscarinic receptors of the two cell lines differ in that portion of their amino acid sequence important for interaction with their respective guanine nucleotide regulatory proteins but not for receptor ligands.

[^{3}H]PBCM has been reported to label specifically a single species of $M_r \cong 65$–80,000 in various tissues, such as brain (20, 22, 32), heart (32), guinea pig ileum (32), and NG108-15 cells (33). [^{3}H]PBCM labeled a single band of M_r greater than 80,000 Da in mammalian exocrine glands (33) and IM-9 lymphocytes (33). The apparent size of the [^{3}H]PBCM-labeled species in NG108-15 cells ($M_r \cong 66{,}000$) is similar to that in brain and heart, and the [^{3}H]PBCM-labeled species in 1321N1 cells ($M_r \cong 92{,}000$) is similar to that in exocrine glands.

Taken together, the [^{3}H]PBCM-labeling data are consistent with the idea that different muscarinic receptor proteins are expressed in the two cell lines. As predicted by previous studies on the muscarinic receptors of mammalian tissues (21, 22), the receptors from NG108-15 and 1321N1 cells are glycoproteins, the apparent size of which could be reduced markedly by treatment with Endo F. Despite the marked deglycosylation-induced modification of the mobility of receptors from both cell lines, the differences in apparent size of the [^{3}H]PBCM-labeled species was maintained. Nothing in the data can completely eliminate an additional factor unaffected by Endo F or O-linked carbohydrate chains without sialic acid that renders the muscarinic receptors of the two cells differentially mobile during SDS-PAGE. However, the most logical interpretation of the observed differences is that dissimilar amino acid sequences of muscarinic receptor subtypes have functional significance in their selectivity of interaction with guanine nucleotide regulatory proteins. Based on precedence established for other receptor subtypes that couple to multiple second messenger response systems, it would be unlikely to expect that this would be the only difference in the receptors.

The apparent size of the deglycosylated muscarinic receptors from NG108-15 cells is similar to the size ($M_r \cong 51{,}000$) predicted for the muscarinic receptors cloned from both porcine cerebral cortex (26) and heart (27, 28). From a functional point of view, i.e., inhibition of adenylate cyclase, the muscarinic receptors expressed in NG108-15 cells are most closely related to those expressed in heart (27, 28). However, it has become clear that muscarinic receptors in heart activate multiple second messenger response systems (3, 34), so it is clearly premature to associate the cloned heart receptor with a given effector response. The muscarinic receptors of 1321N1 cells would appear to be a protein larger than either of the receptors that have been cloned. It will be important to determine if cDNA libraries from various sources possess a member that predicts a muscarinic receptor of this size. Based on the existing knowledge of the biochemical responses to muscarinic receptor activation in 1321N1 cells, these cells together with NG108-15 cells could prove useful in establishing the association of second messenger responses with muscarinic receptor structures.

Acknowledgments

The authors thank Margaret Tapp for excellent work in preparation of the manuscript. The advice and encouragement provided by Drs. G. A. Scarborough and K. D. McCarthy is gratefully acknowledged. This research was supported by Public Health Service Grants GM38213 and NS23019.

References

1. Murad, F., Y.-M Chi, T. W. Rall, and E. W. Sutherland. Adenyl cyclase. III. The effect of catecholamines and choline esters on the formation of adenosine 3′, 5′-phosphate by preparations of cardiac muscle and liver. *J. Biol. Chem.* **237**:1223–1238 (1962).
2. Lichtshtein, D., G. Boone, and A. Blume. Muscarinic receptor regulation of NG-108-15 adenylate cyclase: requirement for Na^+ and GTP. *J. Cyclic Nucleotide Res.* **5**:367–375 (1979).
3. Brown, J. H., and S. B. Masters. Muscarinic regulation of phosphatidylinositol turnover and cyclic nucleotide metabolism in the heart. *Fed. Proc.* **43**:2613–2617 (1984).
4. Michell, R. H. Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta* **415**:81–147 (1975).
5. Masters, S. B., T. K. Harden, and J. H. Brown. Relationships between phosphoinositide and calcium responses to muscarinic agonists in astrocytoma cells. *Mol. Pharmacol.* **26**:149–155 (1984).
6. Hughes, A. R., M. W. Martin, and T. K. Harden. Pertussis toxin differentiates between two mechanisms of attenuation of cyclic AMP accumulation by muscarinic cholinergic receptors. *Proc. Natl. Acad. Sci. USA* **81**:5680–5684 (1984).
7. Evans, T., M. W. Martin, A. R. Hughes, and T. K. Harden. Guanine nucleotide-sensitive, high affinity binding of carbachol to muscarinic cholinergic receptors of 1321N1 astrocytoma cells is insensitive to pertussis toxin. *Mol. Pharmacol.* **27**:32–37 (1985).
8. Masters, S. B., M. W. Martin, T. K. Harden, and J. H. Brown. Pertussis toxin does not inhibit muscarinic-receptor-mediated phosphoinositide hydrolysis or calcium mobilization. *Biochem. J.* **227**:933–937 (1985).
9. Gil, D. W., and B. B. Wolfe. Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinositide breakdown and inhibition of adenylate cyclase. *J. Pharmacol. Exp. Ther.* **232**:608–616 (1985).
10. Brown, J. H., D. Goldstein, and S. B. Masters. The putative M_1 muscarinic receptor does not regulate phosphoinositide hydrolysis. *Mol. Pharmacol.* **27**:525–532 (1985).
11. Lazareno, S., D. A. Kendall, and S. R. Nahorski. Pirenzepine indicates heterogeneity of muscarinic receptors linked to cerebral inositol phospholipid metabolism. *Neuropharmacology* **24**:593–595 (1985).
12. Fisher, S. K. Inositol lipids and signal transduction at CNS muscarinic receptors. *Trends Pharmacol. Sci. Suppl.* **7**:61–65 (1986).
13. Harden, T. K., L. I. Tanner, M. W. Martin, N. Nakahata, A. R. Hughes, J. R. Hepler, T. Evans, S. B. Masters, and J. H. Brown. Characteristics of two biochemical responses to stimulation of muscarinic cholinergic receptors. *Trends Pharmacol. Sci. Suppl.* **7**:14–18 (1986).
14. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated activation of phosphodiesterase. *Mol. Pharmacol.* **22**:310–319 (1982).
15. Martin, M. W., T. Evans, and T. K. Harden. Further evidence that muscarinic cholinergic receptors of 1321N1 astrocytoma cells couple to a guanine nucleotide regulatory protein that is not Ni. *Biochem. J.* **229**:539–544 (1985).
16. Lutton, J. K., R. C. Frederich, Jr., and J. P. Perkins. Isolation of adenylate cyclase-enriched membranes from mammalian cells using concanavalin A. *J. Biol. Chem.* **254**:11181–11184 (1979).
17. Hunter, D. D., and N. M. Nathanson. Biochemical and physical analyses of newly synthesized muscarinic acetylcholine receptors in cultured embryonic chicken cardiac cells. *J. Neurosci.* **6**:3739–3748 (1986).
18. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
19. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
20. Dadi, H. K., and R. J. Morris. Muscarinic cholinergic receptor of rat brain. *Eur. J. Biochem.* **144**:617–628 (1984).
21. Herron, G. S., and M. I. Schimerlik. Glycoprotein properties of the solubilized atrial muscarinic acetylcholine receptor. *J. Neurochem.* **41**:1414–1420 (1983).
22. Rauh, J. J., M. P. Lambert, N. J. Cho, H. Chin, and W. L. Klein. Glycoprotein properties of muscarinic acetylcholine receptors from bovine cerebral cortex. *J. Neurochem.* **46**:23–32 (1986).
23. Elder, J. H., and S. Alexander. Endo-β-N-acetylglucosaminidase F:Endoglycosidase from Flavobacterium meningosepticum that cleaves both high-mannose and complex glycoproteins. *Proc. Natl. Acad. Sci. USA* **79**:4540–4544 (1982).
24. Spiro, R. G., and V. D. Bhoyroo. Structure of the O-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.* **249**:5704–5717 (1974).
25. Lotan, R., and G. L. Nicolson. Purification of cell membrane glycoproteins by lectin affinity chromatography. *Biochim. Biophys. Acta* **559**:329–376 (1979).
26. Kubo, T., K. Fukuda, A. Mikami, A. Maeda, H. Takahashi, M. Mishina, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature (Lond.)* **323**:411–416 (1986).
27. Kubo, T., A. Maeda, K. Sugimoto, I. Akiba, A. Mikami, H. Takahashi, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, H. Matsuo, T. Hirose, and S. Numa. Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence. *FEBS Lett.* **209**:367–372 (1986).
28. Peralta, E. G., J. W. Winslow, G. L. Peterson, D. H. Smith, A. Ashkenazi, J. Ramachandran, M. I. Schimerlik, and D. J. Capon. Primary structure and biochemical properties of an M_2 muscarinic receptor. *Science* **236**:600–605 (1987).
29. Hammer, R., E. Giraldo, G. B. Schiavi, E. Monferini, and H. Ladinsky. Binding profile of a novel cardioselective muscarinic receptor antagonist, AF-DX 116, to membranes of peripheral tissues and brain in the rat. *Life Sci.* **38**:1653–1662 (1986).
30. Fain, J. N. and J. A. Garcia-Sainz. Role of phosphatidylinositol turnover in $alpha_1$ and of adenylate cyclase inhibition in $alpha_2$ effects of catecholamines. *Life Sci.* **26**:1183–1194 (1980).
31. Evans, T., M. M. Smith, L. I. Tanner, and T. K. Harden. Muscarinic cholinergic receptors of two cell lines that regulate cyclic AMP metabolism by different molecular mechanisms. *Mol. Pharmacol.* **26**:395–404 (1984).
32. Venter, J. C. Muscarinic cholinergic receptor structure. *J. Biol. Chem.* **258**:4842–4848 (1983).
33. Hootman, S. R., T. M. Picado-Leonard, and D. B. Burnham. Muscarinic acetylcholine receptor structure in acinar cells of mammalian exocrine glands. *J. Biol. Chem.* **260**:4186–4194 (1985).
34. Pfaffinger, P. J., J. M. Martin, D. D. Hunter, N. M. Nathanson, and B. Hille. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature (Lond.)* **317**:536–538 (1985).

Send reprint requests to: Meina Liang, Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514