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Development of an Antiserum Against m3 Muscarinic Receptors: Distribution of m3 Receptors in Rat Tissues and Clonal Cell Lines

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SUMMARY

A synthetic oligopeptide (QCDKRKRRKQQYQQRQSV) corresponding to a carboxyl-terminal sequence of the rat m3 receptor (amino acids 561–578) was coupled to carrier proteins and used to generate a polyclonal antiserum. This serum selectively immunoprecipitates at least 90% of the m3 receptors expressed by A9 cells transfected with the cDNA encoding the m3 muscarinic receptor but does not precipitate receptors from cells transfected with cDNA encoding m1, m2, m4, or m5 receptors. Using this m3 antiserum, the density of m3 receptors in various regions of rat brain was quantified. Areas expressing the highest density of m3 receptors are the cortex, hippocampus, striatum, and olfactory tubercle, with 232 fmol/mg, 197 fmol/mg, 140 fmol/mg, and 130 fmol/mg, respectively. Hindbrain regions (i.e., cerebellum, thalamus/hypothalamus, and pons/medulla) expressed fewer m3 receptors, both as a percentage of total muscarinic receptors (5–6%) and in terms of absolute receptor density (12–70 fmol/mg). A panel of subtype-selective antisera (m1, m2, and m3) was used to determine receptor distribution in several peripheral tissues of the rat (lung, ileum, and bladder). The m2 receptor subtype constitutes the majority of total receptors in the bladder (86%), lung (91%), and ileum (69%). The m3 receptor was found at lower densities in these tissues (5–11%), whereas the m1 receptor is present in highest amounts in the ileum (17%). Human clonal cell lines, in which regulation of muscarinic receptors has been commonly studied, were also examined. The SK-N-SH neuroblastoma line, which has been reported to express M3 receptors, on the basis of pharmacology and molecular size, was found to express a mixture of subtypes (m1 = 31%, m2 = 21%, m3 = 43%). Interestingly, SH-SY-5Y and SH-IN cells, both derived from SK-N-SH cells, exhibit predominantly m3 receptors (74% for SH-SY-5Y; 58% for SH-IN), with lower levels of m1 and m2 receptors (5% and 8% for SH-SY-5Y; 4% and 23% for SH-IN, respectively.) Another commonly studied cell line, 132-1-N1 astrocytoma cells, reportedly expressing M3 receptors, based upon mRNA measurements and second messenger linkage, also expresses a predominance of m3 receptors (91% of total). This m3-selective antiserum should prove useful not only for localizing and quantifying m3 muscarinic receptors but also for examining mechanisms involved in the regulation of receptor expression in human tissues or animal models of disease, as well as in cell culture.

The genes for five subtypes of muscarinic cholinergic receptor have been cloned (1–3), and the expression of mRNA for each has been demonstrated in a number of tissues (4, 5). These receptors have been designated m1–m5. In contrast, pharmacological studies have demonstrated the likely existence of at least three distinct muscarinic receptors mediating functional responses in various tissues. These receptors have been termed M1–M3 and possess high affinity for the antagonists pirenzepine, AF-DX116, and 4-diphenylacetoxy-*N*-methylpiperidine methiodide, respectively. The relationship between the genetically defined receptors and the pharmacologically defined receptors is not entirely clear, although it is probable that m1 receptors are included in the M1 classification, m2 receptors are included in the M2 classification, and m3 receptors fall within the M3 classification (6). The drugs currently available for defining the subtypes are not highly selective and usually have high affinity for more than a single receptor subtype (7). Therefore, it is difficult to determine the levels of receptor subtypes expressed in a given tissue, particularly when a tissue expresses a mixed population of muscarinic receptor mRNAs.

In response to this problem, we have raised antisera to m1 and m2 muscarinic receptors, using either short synthetic peptides (8) or larger fusion proteins (9, 10). In this report, the development and characterization of an antiserum raised against a peptide whose sequence is found in the carboxyl

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ABBREVIATIONS: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; QNB, quinuclidinyl benzilate; TEDC, 10 mM Tris (pH 7.4), 1 mM EDTA, 1% digitonin, 0.2% cholic acid.

terminus of the rat and human m3 receptor are described. This antiserum is specific for the m3 muscarinic receptor, in that it can quantitatively immunoprecipitate m3 receptors, with no measurable precipitation of m1, m2, m4, or m5 receptors. Using this new tool, the density and distribution of m3 muscarinic receptors in various rat tissues were determined. Additionally, because several of the clonal cell lines commonly used to study the regulation of muscarinic receptors have been reported to express mainly M3 receptors (11, 12), the density of muscarinic receptor subtypes was determined in these cells.

Materials and Methods

Development of antipeptide antisera. The m3 peptide was synthesized by Dr. Ruth Angeletti (Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, PA). The peptide was coupled to keyhole limpet hemocyanin, β-galactosidase, or soybean trypsin inhibitor, using either *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester or glutaraldehyde (13, 14). Free peptide was separated from conjugated peptide-carrier by two successive centrifugations with Centricon-10 microconcentrators (molecular weight cutoff, 10,000; Amicon Corporation, Danvers MA) or by dialysis (10-kDa cutoff) against phosphate buffer (10 mM, pH 7.4) for 48 hr, with three changes of buffer. Antiserum was generated in female New Zealand White rabbits as described previously (9).

Membrane preparation. Selected regions of brain and certain peripheral tissues (lung, bladder, and ileum) were dissected from male Sprague-Dawley rats (200 g), weighed, and stored at −70° until required. Brain regions were prepared as described previously (9). Peripheral tissues were immersed in liquid nitrogen and pulverized with mortar and pestle before subsequent homogenization, membrane isolation, labeling, and receptor solubilization. Typically, peripheral tissues were pooled for a single determination (i.e., tissue representing approximately seven bladders and four to six ileums were used in each experiment).

Transfected A9 cells or CHO cells carrying the cDNA encoding for the human m1, m2, m3, m4, or m5 subtypes of muscarinic cholinergic receptor were the generous gift of Dr. Mark Brann (National Institutes of Health). Receptor expression levels in CHO cells were determined to be m1 = 1650, m2 = 1830, m3 = 2850, m4 = 620, and m5 = 475 fmol/mg of membrane protein. Receptor levels in A9 cells were m1 = 500, m2 = 260, m3 = 530, and m4 = 170 fmol/mg of membrane protein. Mother flasks were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum (Hyclone Laboratories), 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. CHO cells transfected with cDNA encoding the human m1 or porcine m2 receptors were a generous gift of Dr. Daniel Capon (Genetech). Receptor expression levels in these cells were found to be m1 = 1100 and m2 = 12,000 fmol/mg of membrane protein. Mother flasks were grown in similar medium supplemented with 10% dialyzed fetal bovine serum (GIBCO Laboratories) and methotrexate (200 mM). SK-N-SH human neuroblastoma cells were the generous gift of Dr. Stephen Fisher (University of Michigan, Ann Arbor, MI). SH-SY-5Y neuroblastoma cells and SH-IN neuroblastoma cells were a generous gift from Dr. J. L. Beidler (Sloan Kettering, New York). All neuroblastoma mother flasks were grown in DMEM/F12 supplemented with 2 mM glutamine and 10% defined/supplemented bovine calf serum (Hyclone Laboratories), in a humidified atmosphere supplemented with 8% CO_2. Cells were plated on 150-mm dishes and grown for 7–10 days, with medium changes on day 5 and day 8. 132-1N1 human astrocytoma cells were received as a gift from Dr. Joan Heller Brown (University of California at San Diego, La Jolla, CA). Astrocytoma mother flasks were grown in DMEM supplemented with 2 mM glutamine and 5% fetal bovine serum (Hyclone Laboratories). 132-1N1 cells were grown for 21 total days in culture, because the density of muscarinic cholinergic receptors has been reported to increase with days grown after confluency.[1] Medium was changed on day 5, day 8, and every 2 days thereafter. Cells were harvested in 20 ml of ice-cold Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 7.4) with protease inhibitor cocktail (9), homogenized (Tekmar Tissuemizer, setting 6, 7 sec), and centrifuged at 32,000 × *g* for 15 min at 4°. It was observed that total precipitation from certain cell lines (e.g., 132-1-N1) was significantly diminished if cells were not harvested in the presence of protease inhibitors (71% versus 91%), suggesting that receptors are subject to proteolysis. Harvesting of cells, and all subsequent steps, were, therefore, routinely performed in the presence of protease inhibitors. The membrane pellet was resuspended in HEPES (10 mM, pH 7.4) EDTA (1 mM) buffer, aliquoted into 1.5-ml microfuge tubes, and stored at −70°. Protein present in harvested membranes was determined by the method of Lowry *et al.* (15).

Receptor labeling, solubilization, and immunoprecipitation. The methodology used for labeling with [^{3}H]QNB, solubilization with digitonin/cholate, and immunoprecipitation was described previously (8). Briefly, labeling of receptors was accomplished with the nonselective antagonist [^{3}H]QNB (1 nM), for 45 min at 37°, in approximately 20 ml of TE/PIC (10 mM Tris, 1 mM EDTA, pH 7.4, protease inhibitor cocktail). A parallel incubation was carried out in the presence of atropine (5 μM), to determine the percentage of nonspecific counts in the solubilized membrane preparation. Antisera were reconstituted in H_2O (0.1 ml) and incubated with a range of [^{3}H]QNB-labeled, solubilized receptor (5,500–40,000 cpm). Tissues with lower m3 receptor expression (percentage of total) required the addition of larger amounts of total receptor to attain a strong precipitation signal (approximately 2000 cpm/tube). Final volumes were brought up to 0.5 ml by the addition of 0.05 ml of TE/PIC and the required amount of 1.0% TEDC. In addition to the m3 antipeptide antisera described in this paper, incubations with cell lines and peripheral tissues were also carried out with m1 and m2 antisera (9, 10). Both of these antisera are able to precipitate selectively >90% of added solubilized receptors isolated from transfected CHO cells. Nonspecifically bound [^{3}H]QNB present in the solubilized fraction was determined with parallel labeling of tissue in the presence of atropine (5 μM), and this amount (approximately 5–10% for peripheral tissues, neuroblastoma cells, and astrocytoma cells; <5% for central nervous system tissues) was subtracted. Control antiserum was used to define nonspecific immunoprecipitation. This amount (typically ≤2.0%) was subtracted, to determine precipitation efficiency. Data were typically expressed as the mean ± standard error and, when applicable, standard errors were propagated (16).

Results

Production and characterization of m3 receptor antisera. The development of antibodies was assessed by determining the efficiency of precipitation of solubilized m3 muscarinic cholinergic receptors from transfected A9 cells. As shown in Fig. 1, successive challenges with the peptide resulted in an increased ability of antisera to precipitate added receptor. By the fourth bleed, isolated antibody was capable of precipitating approximately 95% of added receptors. Maximum precipitation was achieved after 45 hr of incubation and remained stable for up to 60 hr at 4° (data not shown). In experiments examining distribution of m3 receptors in rat tissues or cell lines, a concentration of antiserum was utilized that precipitated added receptor at an efficiency of ≥90%. At this concentration (0.6 mg/ml), uniform precipitation efficiency was observed across a range of added receptor (25–150 fmol/tube) (data not shown). For bleeds 4–8, the approximate EC_{50} value was 20 μg/ml, corresponding to a 1/1000 dilution of original serum.

To determine the selectivity of the antiserum for m3 receptors, parallel incubations were carried out with solubilized

[1] T. K. Harden, personal communication.

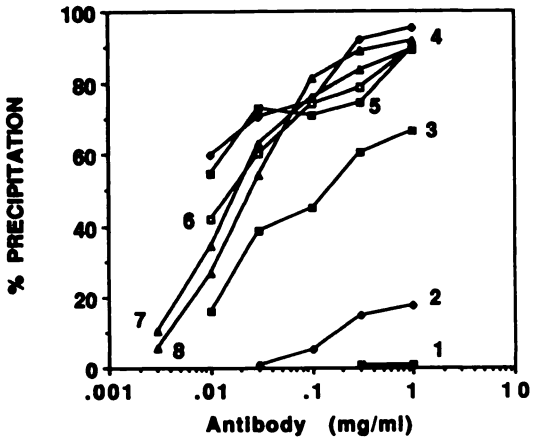

Fig. 1. Titer curves for the first eight bleeds. Lyophilized antisera raised against a portion of the carboxyl terminus of the m3 receptor were incubated with 100 fmol (4000 cpm) of solubilized m3 receptor from A9 cells that had been labeled with [^{3}H]QNB, as described in Materials and Methods (500-μl final volume). Incubations were carried out at 4° for 45 hr before loading on Sephadex G-50 columns, incubation with Pansorbin, and immunoprecipitation, as described. Data are expressed as a percentage of total labeled receptor added per tube that was precipitated. Data shown represent means of duplicate or triplicate determinations at each concentration.

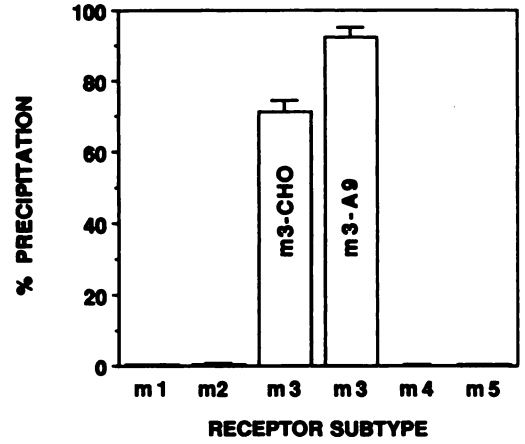

Fig. 2. Specificity of precipitation with m3 antibody. Antibody (1.0 mg/ml) was incubated for 45 hr at 4° with 100 fmol of [^{3}H]QNB-labeled m1, m2, m3, m4, or m5 receptors solubilized from transfected CHO cells or with m3 receptors solubilized from A9 cells. Data shown represent the mean ± standard deviation of triplicate determinations, with nonspecific precipitation (preimmune serum) subtracted.

receptors from CHO cells tranfected with cDNAs encoding m1–m5 muscarinic receptor subtypes. These tissues were selected for the experiments because receptor expression in CHO cells lines was found to be approximately 5–10 times greater than that in the transfected A9 cells. As can be seen in Fig. 2, for m1, m2, m4, and m5 receptors the anti-m3 antiserum precipitated <2.0% of added receptor. The antiserum was able to precipitate maximally approximately 70% of m3 receptors solubilized from transfected CHO cells, while at the same time precipitating 90–95% of added receptors from transfected A9 cells.

Determination of m3 receptor density in selected regions of rat brain. The distribution of m3 receptors was examined in seven regions of adult rat brain (cortex, hippocampus, olfactory tubercle, striatum, thalamus/hypothalamus, pons/medulla, and cerebellum). [^{3}H]QNB-labeled receptors were solubilized from these tissues and incubated with antibody as described. As a percentage of total muscarinic receptors expressed, the m3 receptor was found in highest amounts (10–12%) in the cortex and hippocampus (Fig. 3). Lower and more uniform distribution (5–6%) was seen in the remaining brain areas. When these data were expressed as densities (fmol/mg of membrane protein), highest levels of m3 receptor were found in forebrain areas, i.e., cortex (232 fmol/mg), hippocampus (197 fmol/mg), olfactory tubercle (130 fmol/mg), and striatum (140 fmol/mg). Comparatively fewer m3 receptors were found by moving in a rostral to caudal direction in the rat brain, i.e., thalamus/hypothalamus (66 fmol/mg), pons/medulla (36 fmol/mg), and cerebellum (12 fmol/mg).

The possibility existed that the actual m3 receptor density in these brain regions was underestimated, due to the presence of an endogenous proteolytic activity and/or factor that inhibited the antibody-receptor interaction. We addressed this hypothesis in the experiments presented in Fig. 4. A range of solubilized unlabeled protein (isolated from the cortex, cerebellum, nontransfected CHO cells, or m1-transfected CHO cells) was coincubated with solubilized [^{3}H]QNB-labeled m3 receptors from transfected cells, to try to compete with or inhibit

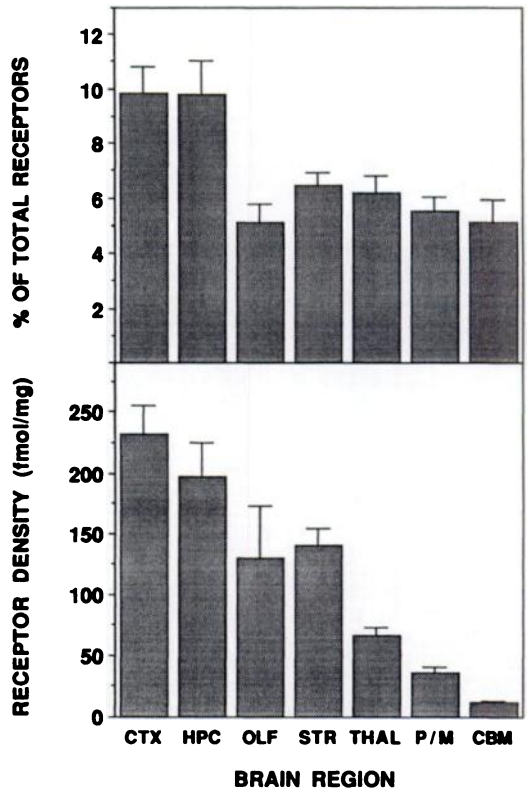

Fig. 3. Distribution and density of m3 muscarinic receptors in isolated regions of rat brain. *Upper*, seven regions of rat brain were dissected and prepared as described in Materials and Methods. Percentage of precipitation in each experiment was obtained from triplicate samples, with nonspecific precipitation (<2% of total) subtracted. Data shown are the mean ± standard error of multiple independent experiments (olfactory tubercle, three; thalamus, four; and remaining tissues, five). *Lower*, receptor density for each brain region was determined as described in Materials and Methods. The value for percentage of m3 receptor for a given region, as shown in *upper*, was multiplied by the B_{max} value corresponding to that brain experiment, to obtain the m3 receptor density. Data shown are the mean ± standard error. *CTX*, cortex; *HPC*, hippocampus; *OLF*, olfactory tubercle; *STR*, striatum; *THAL*, thalamus/hypothalamus; *P/M*, pons/medulla; *CBM*, cerebellum.

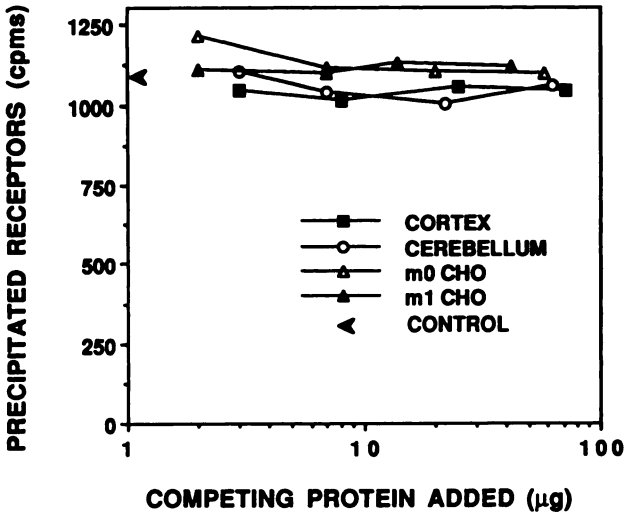

Fig. 4. Precipitation efficiency as a function of competing protein. Unlabeled membrane preparations from cerebellum, cortex, CHO cells transfected with cDNA encoding the m1 muscarinic receptor (*m1*), or nontransfected CHO cells (*m0*) were solubilized at 3 mg of membrane protein/ml of 1.0% TEDC. Increasing amounts of protein were added to [^{3}H]QNB-labeled, solubilized, m3 receptors (10 μg of protein) and m3 antiserum (0.75 mg/ml). Incubations and subsequent processing of receptor were as described in Materials and Methods. Data shown are the means of duplicate determinations.

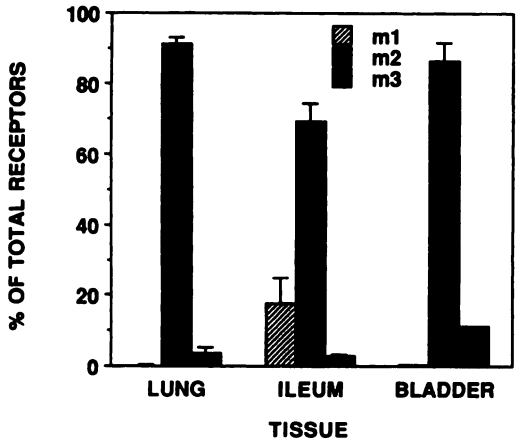

Fig. 5. Distribution of muscarinic receptors in selected rat peripheral tissues. Tissues were isolated and prepared as described in Materials and Methods. Lyophillized antisera raised against m1, m2, or m3 muscarinic receptor epitopes were incubated for approximately 45 hr at 4° with 50–200 fmol of [^{3}H]QNB-labeled, solubilized receptors (2000–8000 cpm). Fractions were applied to G-50 columns, incubated with Pansorbin, and immunoprecipitated as described in Materials and Methods. Tissue receptor B_{max} values were lung, 47 fmol/mg; ileum, 180 fmol/mg; and bladder, 145 fmol/mg. Total receptors accounted for lung, 95.0 ± 2%; bladder, 98 ± 5%; and ileum, 89 ± 9%. Data shown represent the mean ± standard error of three experiments, each performed with triplicate determinations.

antibody-m3 receptor interactions. Even at high levels of competing protein and receptor, the m3 receptor precipitation was unaffected (Fig. 4).

Determination of m1, m2, and m3 receptor densities in rat peripheral tissues. The distribution of muscarinic receptors was examined in preparations of lung, ileum, and bladder (Fig. 5). Solubilized receptors from each of the tissues listed were incubated with antisera recognizing m1, m2, or m3 receptors. Calculated receptor densities in peripheral tissues typically fell within the range of 45–170 fmol/mg of membrane protein. The predominant receptor found to be expressed in lung, bladder, and ileum was of the m2 subtype, accounting for 70–90% of expressed receptors. The m3 receptor accounted for between 5% and 11% of the muscarinic receptors in these tissues. The m1 receptor subtype comprised 17% of ileal muscarinic receptors and was undetectable in the other tissues.

Distribution of m1, m2, and m3 receptors in clonal cell lines. A number of cell lines have been reported to express muscarinic receptors, based upon their ligand-binding profile. On the basis of this pharmacology, receptors in the 132-1N1 human astrocytoma cells and the SK-N-SH human neuroblastoma line have been reported to express receptors falling into the "glandular" or M3 class (11, 17). The distribution of m3 receptors was examined in these two lines and, additionally, in two subclones of the SK-N-SH cells, the SH-IN and SH-SY-5Y lines (Fig. 6).

The 132-1N1 cells express a relatively low level of muscarinic

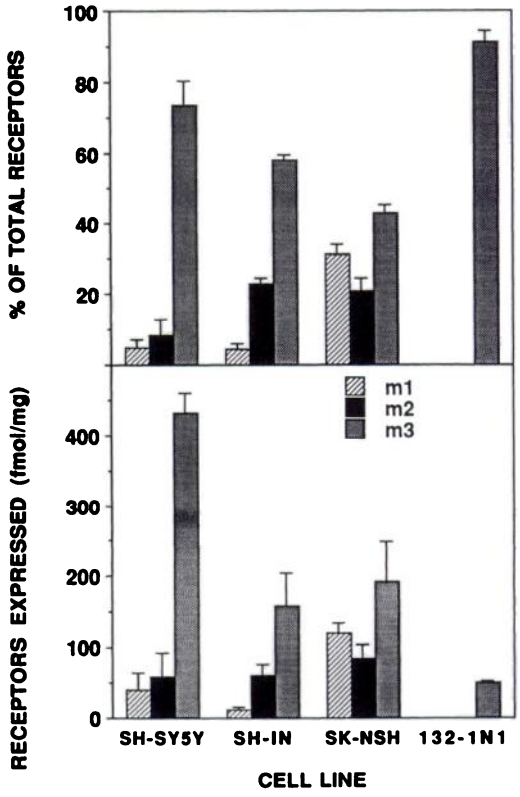

Fig. 6. Muscarinic receptor profile in clonal cell lines. *Upper*, cell membranes were harvested and prepared as described in Materials and Methods. Incubations with antisera selective for m1, m2, or m3 receptor subtypes were carried out for approximately 55 hr at 4°. Data shown for neuroblastoma are the mean ± standard error of three or four experiments, each performed with triplicate determinations, for each antibody. Data shown for 132-1-N1 astrocytoma are the mean ± standard deviation of four determinations. Total percentages of receptors accounted for by immunoprecipitation with m1, m2, and m3 antisera were SH-SY-5Y, 87 ± 9%; SH-IN, 85 ± 3%; SK-N-SH, 95 ± 5%; and 132-1N1, 91 ± 3%. *Lower*, receptor density was determined for each cell line as described in Materials and Methods. The percentage of receptor subtype calculated (*upper*) was multiplied by the B_{max} value for each cell line, to determine the expression levels for m1, m2, and m3 receptors. Data shown for neuroblastoma are the mean ± standard error of three or four experiments, each done with triplicate determinations. Data for astrocytoma are the mean ± standard deviation of four determinations. Total receptor density, as fmol/mg, was, SK-N-SH, 396 ± 60; SH-SY-5Y, 601 ± 86; SH-IN, 269 ± 71; and 132-1-N1, 39 ± 5.

receptors (39 fmol/mg), and these are predominantly m3 receptors (91%), with no detectable m1 or m2 receptors. With respect to the neuroblastoma cells, pharmacological data reported for the SK-N-SH cell line are consistent with the majority of the receptors being of the m3 subtype (11). In our hands, this cell line expresses at least three subtypes of muscarinic cholinergic receptors, with m3 and m1 receptors being found at higher levels (43% and 31%, respectively) than m2 receptors (21%). As a percentage of total receptors, the two subclones derived from SK-N-SH cells, SH-IN and SH-SY-5Y, express a significantly greater percentage of m3 receptors than the mother line from which they were isolated, SH-IN with 58% m3 receptors and SH-5Y-SY with 74% of total receptors of the m3 subtype.

The percentage receptor composition data were used to calculate levels of protein expressed in terms of receptor density (Fig. 6, *lower*). Levels of m2 receptors appear to be relatively constant, at 60–80 fmol/mg of membrane protein, in each neuroblastoma line. In contrast, levels of m3 receptors are up-regulated in SH-SY-5Y cells, to 2–3 times the levels observed in the other subclones (425 fmol/mg). In contrast, m1 receptors apparently are down-regulated, relative to SK-N-SH cells, amounting to 5% of expressed receptors in both of the SH-IN and SH-SY-5Y subclones.

Discussion

This report describes the successful production and isolation of an antiserum that is capable of selectively and quantitatively (>90%) immunoprecipitating an individual member of the muscarinic receptor family. An immediate concern, however, was the apparent inability of the antibody to recognize all m3 receptors when expressed in CHO cells (Fig. 2). When titer curves were produced using these cells, precipitation routinely plateaued at 65–70% of total added receptor, regardless of bleeds 4–8. This is in contrast to the data shown in Fig. 1 and Fig. 2, *bar 4*, which were obtained with membranes from transfected A9 cells. There are several possible explanations for this observation.

The 18 amino acids synthesized for immunization, according to the presently accepted model, are found intracellularly at the carboxyl-terminal tail of the m3 receptor (1). Proteolytic cleavage of this tail region would be expected to remove the epitope required for antibody recognition and may account for the lower precipitation in transfected CHO cells. However, enhanced immunoprecipitation from CHO membranes was not achieved, despite cooling of cells to 4° before harvesting of membranes on ice, or harvesting in the presence of a cocktail of protease inhibitors. These findings suggest that, if proteolysis is the reason for the decreased precipitation efficiency, it occurs either before membrane harvesting or as a result of a protease activity that is resistant to the protease inhibitor cocktail. Given that m3 receptors isolated from A9 cells can be quantitatively precipitated, it would appear that any such hypothesized protease activity is not found uniformly in all tissues.

Alternatively, a posttranslational modification may limit access of antibodies to the carboxyl tail of the receptor. One possibility concerns a carboxyl-terminal cysteine residue conserved across the superfamily of guanine nucleotide-binding protein-linked neurotransmitter receptors (including β-adrenergic, α-adrenergic, serotonergic, dopaminergic, and muscarinic receptors). In the human β-$_2$-adrenergic receptor, Cys-341 is covalently modified by thioesterification with palmitic acid (18, 19). The palymitoylated tail is required for maximal activation of adenylyl cyclase and high affinity, GTP-sensitive, agonist binding. The tail has been proposed to form a fourth intracellular loop for the receptor, facilitating interaction with guanine nucleotide-binding proteins. In addition to the β-adrenergic receptor, palmitoylation of corresponding cysteine residues in rhodopsin (Cys-322 and Cys-323) and the α_{2A}-adrenergic receptor (Cys-442) has been reported (20, 21). Palmitolyation of Cys-560 and/or Cys-562 in a population of m3 receptors may result in the association of this carboxyl-terminal region with the plasma membrane. It is possible that our antiserum cannot recognize a palmitoylated form of the m3 receptor. The observed quantitative precipitation (>90%) observed in A9 cells would suggest that the hypothesized palmitolyation may not be occurring or may occur to a lesser degree than in CHO cells.

With respect to m3 receptor distribution in rat brain, *in situ* hybridization analysis has identified the highest levels of m3 mRNA in the external and deep layers of the cerebral cortex and, to a lesser degree, within the central layers of the cortex (4). In addition, receptor message is observed at relatively high levels in the CA1–CA3 regions of the hippocampus. Diffuse but lower labeling of the striatum is also observed, with little labeling of hindbrain structures. These findings are consistent with the levels of m3 receptors observed in these tissues (Fig. 3). Although the percentage of m3 receptors is low, relative to the total density of muscarinic receptors, the receptor densities are comparable to, for example, reported densities of β_1-adrenergic receptors in forebrain tissues, which are approximately 50–100 fmol/mg.

The presence of tissue-specific proteolysis was examined to determine whether the apparent m3 receptor density in brain regions reflects the actual receptor distribution or is influenced by protease activity. Varying amounts of unlabeled solubilized receptors and protein from cortex, cerebellum, nontransfected CHO cells, and m1 receptor-transfected CHO cells were coincubated with [^{3}H]QNB-labeled m3 receptors and antibody. In no case did this coincubation inhibit the ability of antibody to precipitate solubilized m3 receptors (Fig. 4), suggesting that any degradation of m3 receptors during the assay is minimal. Moreover, our immunoprecipitation data agree closely with the percentages of m3 receptors determined in a recent study using radioligand binding (22). In that study, four antagonists were used to generate competition curves against 1-[*N-methyl*-^{3}H] scopolamine. These data were used to estimate the percentages of four muscarinic receptor subtypes in three brain regions. The percentages of m3 receptors calculated (cortex, 11%; hippocampus, 11%; and striatum, 8%) are nearly identical to the values presented in Fig. 3 (cortex, 10%; hippocampus, 10%; and striatum, 5%). Therefore, we do not believe that proteolysis is resulting in a marked underestimation of m3 receptor densities. In addition, the m3 antiserum described in this report is one of several antisera available in our laboratory against receptor subtypes (9, 10). The recent development of antibodies directed against the third intracellular loops of the m4 and m5 receptors (23) enables us to immunoprecipitate >90% of solubilized receptors from each of the seven brain areas described (24), which also argues against an underestimation of m3 receptor density.

Data reported here indicate that the m2 gene product is the predominant receptor expressed in peripheral smooth muscle-containing tissues. Message for the m2 receptor has been reported at high levels in such tissues (25) and, accordingly, the

m2 receptor constitutes 86% of receptors in bladder. The bladder also contained mRNA for m3 receptors, which accounted for 10–15% of total receptors by immunoprecipitation. Also, in agreement with these findings, binding studies with a panel of seven antagonists indicated that the predominant receptor expressed in bladder was of the cardiac (M2) type, whereas a smaller population of glandular M3 receptors was also present (26). Our results indicate that the molecularly defined m2 receptor constitutes 91% of solubilized muscarinic receptors in rat lung, with m3 receptors being present at significantly lower levels (4%). In agreement with these data, airway smooth muscle has been reported to express m2 mRNA and m3 mRNA (25), and analysis of radioligand binding to rat lung indicated both M2 and M3 receptors were expressed, (27).

Our finding that the m2 receptor predominates in the rat ileum (69%) is not unexpected, given the presence of smooth muscle in this tissue. The identification of m1 and m3 receptors also agrees with certain reported functional pharmacological properties of longitudinal muscle strips of the guinea pig ileum. Thus, pirenzepine had negligible effects on muscle contractions induced by pilocarpine but significantly decreased pilocarpine-enhanced [^{3}H]acetylcholine release (28). These data would suggest that a population of excitatory m1 receptors (17%, by precipitation) is located presynaptically on neurons of the myenteric plexus, which arborize through the ileum. In a later study, chloride ion secretion by enterocytes of ileal mucosa and ileal smooth muscle contraction were most effectively antagonized by ligands selective for M3 receptors (29). In light of the levels of identified m3 receptors (7% by precipitation) in the ileum, it is instructive to note that the immunoprecipitation protocol does not allow the assignment of a functional role subserved by a given receptor subtype. It is possible that a very few receptors may be responsible for generating a robust physiological response measured in a functional assay. Discrete localization of a given receptor subtype at the cellular level, by immunocytochemistry, would add to our understanding of processes mediated by specific subtypes. However, our preliminary attempts with this technique in transfected cells have not proven successful. In contrast to data presented for smooth muscle-containing tissues, additional immunoprecipitation experiments conducted with parotid gland indicate that this tissue expresses almost exclusively the m3 subtype of muscarinic receptor (30).

In 132-1N1 human astrocytoma cells, the m3 peptide antiserum was able to precipitate 91% of [^{3}H]QNB-labeled receptors, whereas no detectable precipitation was observed using either the m1 or m2 receptor antibodies. Northern analysis of receptor message in 132-1N1 cells indicates that m3 mRNA is present in this cell line (12). In addition, muscarinic agonist stimulation of 132-1N1 cells elicits an increase in phosphoinositide metabolism, with no inhibition of cyclase activity, consistent with m3 receptor pharmacology (31). The immunoprecipitation data reported here are consistent with the m3 receptor being the principal subtype found in this cell line.

Neuroblastoma tumors, derived from stem cells of the autonomic nervous system, are able to differentiate to several distinct phenotypic lineages. Accordingly, they have been used for the study of neuronal development and cell differentiation (32). The SK-N-SH cell line is one of a number of human neuroblastoma cell lines identified. In addition to interest generated because of its tumor properties, this cell line has proven to be a useful model system for the study of muscarinic receptor-mediated phosphoinositide hydrolysis. Covalent labeling of muscarinic receptors in SK-N-SH cells with [^{3}H]propylbenzilylcholine mustard and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis indicate that the predominant species present has a molecular mass in the 80–90-kDa range, consistent with its assignment as an m3 receptor and not m1, m2, or m4. In the same report, the pharmacological profile of SK-N-SH cells was consistent with the majority of the receptors being of the m3 subtype (11). Thus, it was not unexpected that the SK-N-SH cell line expresses the m3 receptor. However, it was of interest that both the m1 and m2 receptors were also present at levels not predicted by pharmacological analysis. Northern analysis of SK-N-SH muscarinic receptor message indicates that the m3 and m2 receptors would be the dominant species expressed (12). It should be noted that, in our hands, the amounts of m1, m2, and m3 receptors varied from preparation to preparation. This leads us to believe that subtle differences in culturing conditions may regulate the density of individual subtypes in distinct ways. Hence, differences in culturing conditions may complicate comparison and interpretation of results from different laboratories.

With the panel of m1, m2, and m3 antisera, we are able to account for the majority of muscarinic receptors found in the three neuroblastoma variants, SK-N-SH (95 ± 5%), SH-SY-5Y (87 ± 9%), and SH-IN (85 ± 3%). Variability in the absolute density of the three receptor subtypes in a given subclone was evident with multiple passages of cells. This is interesting, in light of the observation that the three subclones of neuroblastoma have been reported to interconvert at slow rates when maintained in tissue culture (33). An m1 and m3 inversion would account for the recent report in which SH-SY-5Y neuroblastoma was reported to be an M1 clonal cell line (34). Thus, agonist-stimulated phosphoinositide turnover was blocked with high affinity by pirenzepine (K_i of 11 nM), and [^{3}H]pirenzepine was shown to bind to a population of sites with a profile (K_d of 13 nM) consistent with m1 receptors comprising the major subtype expressed in these cells. In contrast, Lambert *et al.* (35) evaluated antagonist inhibition of *N*-[^{3}H]methylscopolamine binding to SH-SY-5Y membranes and reported K_i values of 2 nM for 4-diphenylacetoxy-*N*-methyl piperidine methiodide, 245 nM for pirenzepine, and 1510 nM for AF-DX116, concluding that expressed receptors were of a homogeneous M_3 subtype. Moreover, Adem *et al.* (36) identified both high (34%) and low (66%) affinity sites for pirenzepine in SH-SY-5Y cells cultured in their laboratory, suggesting that both M1 and M2 classes are present. These data argue that considerable plasticity exists in the regulation of receptor subtypes in these cell lines. Factors regulating the phenotypic interconversion process, and presumably affecting expression of m1, m2, and m3 receptors in these human neuroblastomas, are as yet undefined.

In conclusion, an antiserum raised against a unique epitope of the m3 muscarinic receptor was shown to be capable of selectively recognizing and immunoprecipitating solubilized receptors. Using a panel of three such antisera, the distribution of m3 receptors in the rat brain was defined, as were the levels of m1, m2, and m3 receptors in selected peripheral tissues. In addition, the profile of the m1, m2, and m3 receptor expression in four human clonal cell lines was determined. This approach should be useful for the examination of subtype-selective changes in muscarinic receptor density as a consequence of

pharmacological or physiological manipulations, as well as in aging or disease states. In addition, the SK-N-SH cell line and its subclones may represent potential tools for the study of human muscarinic receptor expression and mechanisms of regulation at the transcriptional and translational level.

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