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Development of Antisera Selective for m4 and m5 Muscarinic Cholinergic Receptors: Distribution of m4 and m5 Receptors in Rat Brain

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SUMMARY

A portion of the cDNA sequence corresponding to the third intracellular loop of either the m4 or m5 muscarinic cholinergic receptor was ligated into the pRIT23 or pET-3a expression vector, respectively. The expressed fusion proteins were purified and used to develop selective polyclonal antisera to the m4 and m5 muscarinic receptors. These antisera were used in an immunoprecipitation protocol to examine quantitatively the distribution of receptor subtypes in regions of rat brain. The density of m4 receptors in rat brain increased in the caudal to rostral direction. The highest levels of m4 receptors were detected in the striatum (1280 fmol/mg) and olfactory tubercle (750 fmol/mg). Low levels of m5 receptors were detected in several brain regions (<25 fmol/mg). By combining the previously determined receptor densities for the m1, m2, and m3 receptors and results obtained with the newly developed antisera to m4 and m5 receptors, it was determined that 86–99% of the [^{3}H]quinuclidinyl benzilate binding sites in several brain regions were immunoprecipitated. In addition to measuring receptor densities in rat brain, the immunoprecipitation protocol was used to quantify muscarinic receptor levels in tissues reported to express mRNA encoding the m4 receptor. Thus, although only m4 mRNA has been detected in rabbit lung, NG108–15 cells, and N1E-115 cells, both rabbit lung and NG108–15 cells possess both m2 (rabbit lung, 27%; NG108–15 cells, 31%) and m4 (rabbit lung, 55%; NG108–15, 42%) receptors, whereas N1E-115 cells were found to have both m1 (15%) and m4 (65%) receptors. These antisera will be useful in studies of receptor regulation and in determining alterations in density that may occur after pharmacological or physiological manipulations and in various disease states.

Currently, five genes (m1-m5) have been cloned that encode distinct muscarinic cholinergic receptor subtypes (1–6). The mRNA for these five subtypes is expressed in the mammalian brain (1, 2, 7, 8), and the mRNA for at least the m1-m4 subtypes are expressed in other tissues (2, 9). Utilizing cells transfected with the cDNA encoding each of these receptors, the binding properties of these five subtypes have been examined extensively (10, 11). It is clear from these studies that drugs selective enough to permit distinction of the various subtypes do not currently exist.

The current investigation builds on previous work from this laboratory determining the localization and density of the m1, m2, and m3 muscarinic cholinergic receptors in the rat brain, peripheral tissues, and cell lines (12–15). In this study antisera for the m4 and m5 muscarinic cholinergic receptors have been developed, characterized, and used to complete a profile of muscarinic cholinergic receptor subtypes in the rat brain and to examine the receptor subtypes present in rabbit lung, the NG108-15 neuroblastoma × glioma cell line, and the N1E-115 neuroblastoma cell line, tissues in which only m4 mRNA has been observed (16–18).

In this manuscript, pharmacologically defined subtypes are denoted with upper case designations, M1-M4, whereas subtypes that have identified gene products are indicated with lower case designations, m1-m5, as has been suggested previously (19).

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Materials and Methods

Construction of expression vectors containing the i3 loops of the m4 and m5 receptors. A plasmid (pSP65) containing the cDNA

ABBREVIATIONS: i3 loop, putative third intracellular loop of guanine nucleotide-binding protein-coupled receptor; QNB, quinuclidinyl benzilate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; CHO, Chinese hamster ovary; D-MEM, Dulbecco's modified Eagle medium.

encoding most of the i3 loop (Ser-221 to Arg-393) of the rat m4 receptor was generously provided by Dr. Tom Bonner of the National Institute of Mental Health. The DNA was cut with *Apa*I at Gly-232 and the linker GGAATTCCGGCC was ligated into the vector, resulting in the addition of an *Eco*RI site positioned in-frame for the pRIT23 vector (20). Another *Eco*RI site was present at the 3′ end of the insert as part of the polycloning region of pSP65. The insert (Gly-232 to Arg-393) was removed with *Eco*RI, gel purified, and ligated into the *Eco*RI site of pRIT23 (a generous gift from Dr. Mathias Uhlen of the Royal Institute of Technology, Stockholm, Sweden), to yield the plasmid p23-rm4-232/393 (Fig. 1).

A plasmid (pSP73) containing the cDNA encoding most of the i3 loop of the rat m5 receptor was generously provided by Dr. Tom Bonner of the National Institute of Mental Health. The DNA was cut with *Bst*YI and ligated into the *Bam*HI site of pET-3a (generously provided by Dr. F. William Studier of Brookhaven National Laboratory), to yield a plasmid (p3a-rm5-251/418) encoding the i3 loop from Ser-251 to Asp-418 (Fig. 2). Both plasmids were partially sequenced to verify that the orientation and reading frames were correct.

Production of m4 and m5 fusion proteins. The general procedure to produce the m4 fusion protein by using the pRIT23 vector (20) and to purify it further by Sephacryl S-200 chromatography has been described previously for the m1 and m2 fusion proteins (13, 14). The yield of purified m4 fusion protein per liter of culture was lower than that obtained for the m1 and the m2 receptor fusion proteins and, on average, approximately 1 mg was obtained per 4.5-liter culture (13, 14).

The m5 pET-3a fusion protein was produced as described for the m2 receptor fusion protein (14). However, unlike the m2 receptor fusion protein produced by pET-3a (14), the m5 fusion protein was found to be insoluble. The yield of protein in the pellet appeared to be about 30 mg of protein/liter of culture. Four and one half liters of BL21 (DE) *pLysS* transformed with the p3a-rm5-251/418 plasmid were grown to an A_{600} of 0.6 absorbance units and induced with 1 mM IPTG. This

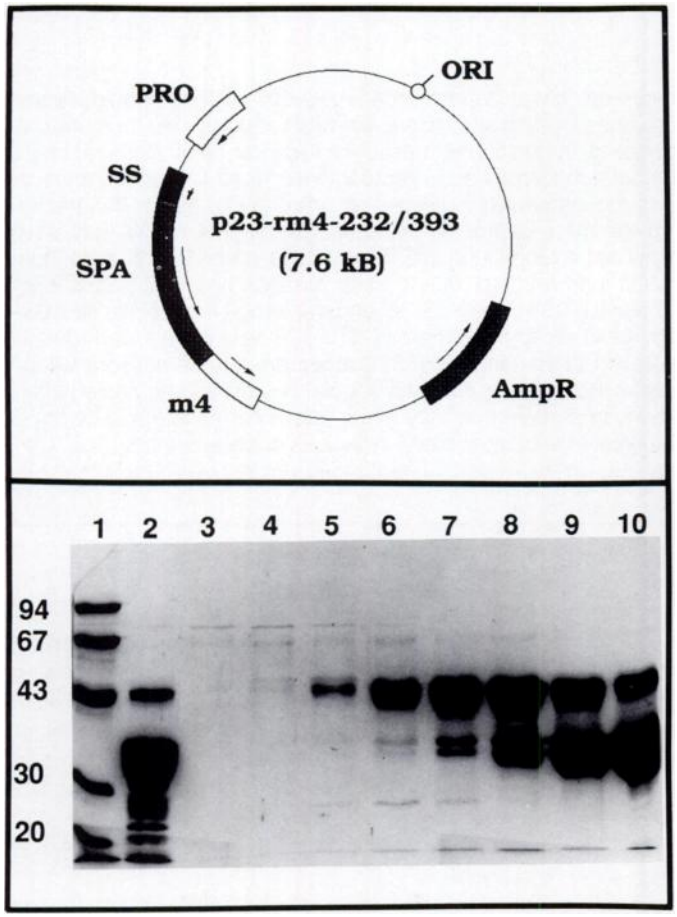

Fig. 1. Expression of the rat m4 fusion protein. *Top*, drawing of the plasmid that expresses the i3 loop of the rat m4 muscarinic cholinergic receptor. Part of the m4 receptor i3 loop was introduced into the unique *Eco*RI site in the expression vector pRIT23. *PRO*, staphylococcal Protein A promoter; *SS*, signal sequence; *SPA*, sequence for Fc binding domains of the Protein A gene; *m4*, sequence for part of the i3 loop of the rat m4 receptor; *AmpR*, sequence for β-lactamase; *ORI*, origin of replication in *E. coli*. *Bottom*, SDS-PAGE of the rat m4 fusion protein. The plasmid p23-rm4-232/393 was used to transform competent HB101 cells for expression of the rat m4 fusion protein. The fusion protein was further purified by affinity and molecular exclusion chromatography as described in the text. *Lane 1*, molecular mass standards (molecular masses are indicated on the *left*, in kilodaltons); *lane 2*, protein eluted from IgG-Sepharose column; *lanes 3-10*, Sephacryl S-200 column profile; protein in *lanes 4-7* was pooled and used to immunize rabbits.

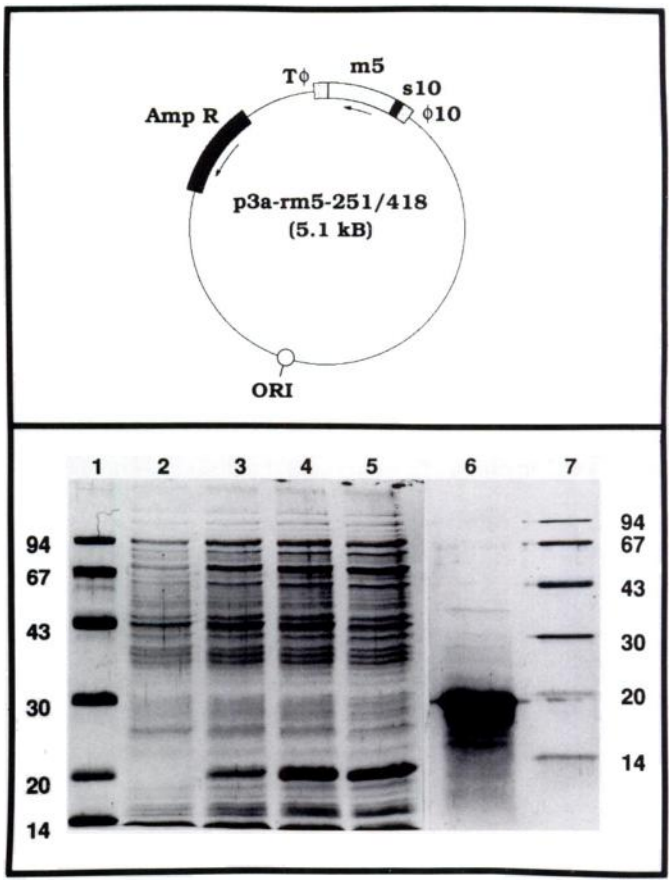

Fig. 2. Expression of the rat m5 fusion protein. *Top*, drawing of the plasmid that expresses the i3 loop of the rat m5 muscarinic cholinergic receptor. Part of the m5 receptor i3 loop was introduced into the unique *Bam*HI site in the expression vector pET-3a. *ϕ10*, promoter for the bacteriophage T7 gene *10* protein; *s10*, translation initiation region for bacteriophage T7 gene *10*; *m5*, sequence for part of the i3 loop of the rat m5 receptor; *Tϕ*, transcription terminator; *AmpR*, sequence for β-lactamase; *ORI*, origin of replication in *E. coli*. *Bottom*, SDS-PAGE of the proteins resulting from induction and purification of the m5 fusion protein. The plasmid p3a-rm5-251/418 was used to transform competent BL21 (DE) cells containing the plasmid *pLysS* for expression of the rat m5 fusion protein. *Lane 1*, molecular mass markers for *lanes 2-5* (molecular masses are indicated on the *left*, in kilodaltons); *lanes 2-5*, whole *E. coli* lysates at times 0, 1, 2, and 3 hr, respectively, after induction with IPTG; *lane 6*, sample of m5 fusion protein after purification by Sephacryl S-200 chromatography under denaturing conditions; *lane 7*, molecular mass markers for *lane 6* (molecular masses are indicated on the *right*, in kilodaltons). The SDS-PAGE gel seen in *lanes 1-5* was a 12% acrylamide gel, and the gel seen in *lanes 6* and *7* was a 17.5% acrylamide gel.

culture was incubated for 3 hr and then centrifuged at 1200 × *g* for 20 min. The pellet was resuspended in buffer (50 mM Tris·HCl, pH 8, 2 mM EDTA) and stored at −20°. The frozen bacterial suspension was thawed, sonicated, and centrifuged at 10,000 × *g* for 20 min at 4°. The pellet was resuspended and boiled for 10 min in a buffer containing 62.5 mM Tris·HCl, pH 6.8, 2% SDS, and 5% 2-mercaptoethanol. It was estimated that 10% of the total protein was fusion protein. Approximately 10 mg of fusion protein (100 mg of total protein) in a volume of 1.5 ml were loaded onto a 100-ml Sephacryl S-200 column (Pharmacia) that had been equilibrated with a buffer containing 62.5 mM Tris·HCl, pH 6.8, 2% SDS, and 0.5% 2-mercaptoethanol. The sample was cycled two times over this column to achieve better resolution. Fractions containing the purified fusion protein were pooled, extensively dialyzed against 50 mM ammonium bicarbonate, pH 7.9, and lyophilized.

Production of polyclonal antibodies. Two rabbits (female, New Zealand White, 3 kg) were used for m4 antisera production and two rabbits were used for m5 antisera production. Primary and secondary subcutaneous injections of approximately 1 mg of fusion protein and preparation of antisera were carried out as described earlier (13, 14).

[^{3}H]QNB labeling, solubilization of receptor, and immunoprecipitation. CHO cells stably transfected with the m1 and m2 receptors were a generous gift of Dr. Daniel Capon (Genetech), and CHO cells that were stably transfected with the m3, m4, and m5 receptors were a generous gift of Dr. Mark Brann (University of Vermont). NG108-15 cells were a generous gift of Dr. Marshall Nirenberg (National Institutes of Health). N1E-115 cells were a generous gift of Dr. Michael McKinney (Mayo Clinic, Jacksonville, FL). [^{3}H]QNB binding assays to determine B_{max} values, [^{3}H]QNB labeling, and receptor solubilization procedures for cell line membranes and animal tissue membranes have been described previously (13–15).

The immunoprecipitation was done as described earlier (12–15), with the following modifications. Membranes were radiolabeled with 2 nM [^{3}H]QNB (50 Ci/mmol; Amersham) for 45 min at 32° in TE buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA) with the following protease inhibitors (in μg/ml): soybean trypsin inhibitor, 10; bacitracin, 1; pepstatin A, 1; leupeptin, 1; α_2-macroglobulin, 1; and phenylmethylsulfonyl fluoride, 100. These protease inhibitors were included in all buffers used in this immunoprecipitation procedure. The labeled membranes were washed three times in TE buffer and then solubilized in 1% TEDC buffer [10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 1% digitonin (Gallard-Schlessinger), 0.2% sodium desoxycholate (Fisher)] for 45 min at 4°. The solubilized membrane preparation was centrifuged at 80,000 × *g* for 45 min at 4°. The supernatants were diluted in 1% TEDC buffer such that there were 30–760 fmol of [^{3}H]QNB-labeled receptors per 350 μl. The assay volume was brought up to 500 μl with the appropriate antiserum in H_2O, at concentrations indicated in each figure. The samples were incubated at 4° for about 44 hr and then loaded over 4-ml Sephadex G-50 columns that had been equilibrated with 0.1% TEDC buffer, to separate bound [^{3}H]QNB from free [^{3}H]QNB. The column was eluted with 0.1% TEDC buffer and fractions (1.3 ml) containing bound [^{3}H]QNB were collected directly into 1.5-ml microcentrifuge tubes that contained 200 μl of stripped Pansorbin (Calbiochem) (13, 14). Samples were incubated at 4° with rotation (40 rpm) for about 30 min and the samples were pelleted at 10,000 × *g* for 5 min in a refrigerated microcentrifuge at 4°. The Pansorbin pellet was washed with 200 μl of TE buffer and pelleted at 10,000 × *g* for 5 min, and the amount of [^{3}H]QNB in the pellet was determined by liquid scintillation counting. The amount of [^{3}H]QNB in the supernatant (i.e., not immunoprecipitated) was determined by combining the initial Pansorbin supernatant and the supernatant from the 200-μl TE wash step. The percentage of immunoprecipitation was calculated by dividing the amount of [^{3}H]QNB found in the Pansorbin pellet by the sum of the [^{3}H]QNB in the total supernatant and the [^{3}H]QNB in the Pansorbin pellet. Nonspecific immunoprecipitation was determined using antiserum from a rabbit that had been given injections of the pRIT23-Protein A product.

The percentage of receptors that could be effectively solubilized in cultured cells and dissected brain regions ranged from 44 to 60%. The five receptor subtypes expressed in CHO cells appeared to be solubilized similarly, because no systematic differences in the solubilization efficiency between these receptors were observed. Similarly, there was no evidence for differential solubilization efficiency in the seven rat brain regions examined. The only differences consistently observed in solubilization efficiency were due to different lots of digitonin and a decrease in solubilization efficiency when the protein concentration was increased above 3 mg/ml.

Cell culture. NG108-15 cells were grown in D-MEM containing 10% fetal bovine serum, supplemented with 0.1 mM hypoxanthine, 1 μM aminopterin, and 12 μM thymidine. Some plates of NG108-15 cells also included 100 units/ml penicillin and 0.1 mg/ml streptomycin. N1E-115 cells were grown in D-MEM containing 10% fetal bovine serum, supplemented with 7.3 ng/ml biotin, 200 ng/ml lipoate, and 1.35 μg/ml vitamin B_{12}. CHO cells obtained from Dr. Mark Brann were grown in medium containing 45% D-MEM, 45% F-12 nutrient mixture, and 10% fetal bovine serum, supplemented with 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. CHO cells obtained from Dr. Daniel Capon were grown in medium containing 45% D-MEM, 45% F-12, and 10% dialyzed fetal bovine serum, supplemented with 2 mM glutamine, 200 nM methotrexate, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. All cell lines were grown at 37° in humidified air supplemented with 8% CO_2.

Statistical analysis. Data were analyzed by one-way analysis of variance and the Newman-Keuls multiple comparison test or the Student *t* test, using the computer program of Tallarida and Murray (21).

Results

Production of fusion proteins. The production of fusion proteins was accomplished by the use of two expression vectors, pRIT23 (20) and pET-3a (22). DNA coding for part of the i3 loop of the rat m4 receptor was ligated into the pRIT23 vector to make a fusion protein composed of amino acids 232–393 of the m4 receptor and a truncated Protein A with the five Fc IgG binding domains intact. Fig. 1 (*top*) shows a schematic map of the pRIT23 vector. A lysate of a 4-hr culture of *Escherichia coli* containing the p23-rm4-232/393 plasmid was loaded onto an IgG-Sepharose column. The column was washed and the fusion protein was eluted from the column by low pH. The proteins in this eluate are shown in the SDS-PAGE gel in Fig. 1, *bottom, lane 2*. The largest protein eluted from the IgG-Sepharose column had a molecular mass of about 45 kDa, which is less than the calculated size of the fusion protein, 57 kDa. Because the molecular mass of the truncated Protein A that is expressed by pRIT23 is about 39 kDa, some of these proteins between 30 and 43 kDa (seen in Fig. 1, *bottom, lane 2*) could be degraded proteins that contain little or no m4 receptor. To reduce the amount of these proteins of lower molecular mass, the fractions that were eluted by low pH from the IgG-Sepharose column were pooled, concentrated, and loaded onto a Sephacryl S-200 size-exclusion column. Fig. 1, *bottom, lanes 3-10*, represents the elution profile from this column. Fractions corresponding to Fig. 1, *bottom, lanes 4-7*, which contained the highest percentage of the largest molecular mass species, were pooled and used for the production of polyclonal antibodies in rabbits.

The cDNA encoding amino acids 251–418 of the i3 loop of the rat m5 receptor was inserted into the pET-3a vector to make a fusion protein that includes the first 11 amino acids of gene 10 (the major capsid protein of bacteriophage T7), amino acids 251–418 of the m5 receptor i3 loop, and an 18-amino acid

tail. The molecular mass of this protein is predicted to be about 20 kDa. Fig. 2, *top*, illustrates the construct of the i3 loop of the m5 receptor ligated into pET-3a. Fig. 2, *bottom*, shows the protein pattern from the lysate of *E. coli* containing p3a-rm5-251/418, upon induction of the m5 receptor fusion protein with 1 mM IPTG, at 0, 1, 2, and 3 hr (Fig. 2, *bottom, lanes 2-5*, respectively). The resulting fusion protein (seen at 20 kDa) was insoluble. Attempts to solubilize this protein in 8 M urea were not successful. Therefore, in order to further purify the fusion protein, the *E. coli* lysate was solubilized by boiling in the presence of SDS and 2-mercaptoethanol and was loaded on a Sephacryl S-200 column that had been equilibrated with SDS and 2-mercaptoethanol. Fractions containing the largest amounts of the 20-kDa protein were collected and pooled. Fig. 2, *bottom, lane 6*, shows Sephacryl S-200-purified fusion protein that was used to raise polyclonal antibodies.

Characterization of m4 and m5 receptor antibodies. A titer curve of the fourth bleed from a rabbit that had been given injections of the m4 fusion protein is shown in Fig. 3A. The antiserum was tested using membranes from CHO cells that expressed only the human m4 receptor. Because the antisera were partially purified by 50% ammonium sulfate precipitation and then lyophilized (13), the weights indicated in Fig. 3A reflect the dry weight of the lyophilized antisera. There were approximately 20 mg of dry antiserum in each milliliter of rabbit serum. Both rabbits gave a similar antigenic response. The antisera could immunoprecipitate 50% of the receptors at a concentration of 0.03 mg/ml, which represents a >600-fold dilution of the original serum. Most experiments were done with 0.6 mg/ml or a 33-fold dilution of the original antisera. The maximal amount of m4 receptors that these antisera were able to immunoprecipitate in CHO membranes varied from 83 to 94%. The reason for this variability is uncertain, but variability was not typically observed in any of the rat tissues examined. When the m4 antisera were tested for cross-reactivity against other subtypes of muscarinic receptors, they were found to be completely selective for the m4 receptor, as seen in Fig. 3B. The ability of the m4 antisera to immunoprecipitate m1, m2, m3, or m5 receptors was negligible (<1%). Therefore, these antisera provide a quantitative and selective assay for determining the density of m4 receptors even in tissues expressing multiple subtypes of muscarinic receptors.

The titer curve for the antiserum from the fourth bleed from a rabbit that had been given injections of the m5 fusion protein is shown in Fig. 4A. This antiserum was prepared as described for the m4 antiserum and was tested against the human m5 receptor expressed in CHO cells. It was more potent than the m4 antiserum and immunoprecipitated 50% of the m5 receptors at a concentration of about 0.01 mg of dry weight/ml, which is equivalent to a 2000-fold dilution of the antiserum. The titers from both rabbits were very similar and, as with the m4 antisera, most experiments with the m5 antisera were done with a 33-fold dilution of antisera. Variability in the maximal amount of m5 receptors that could be immunoprecipitated from transfected CHO cells was also observed and ranged from 83 to 95%. The m5 antisera were selective for only the m5 receptor and did not precipitate m1-m4 receptors (Fig. 4B). Thus, like the m4 receptor antisera, the m5 receptor antisera are quantitative and selective for m5 receptors.

Distribution of m4 and m5 receptors in rat brain. Using these reagents to quantify the densities of m4 and m5 receptors,

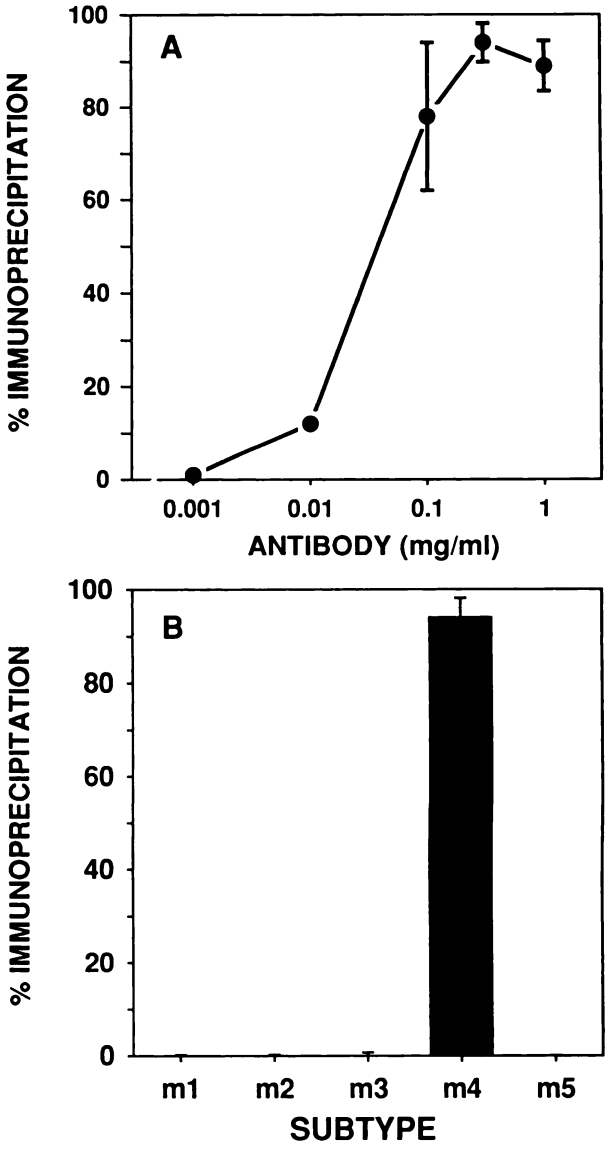

Fig. 3. Characterization of the m4 fusion protein antisera. A, Antiserum titer. Immunoprecipitation of [^{3}H]QNB-labeled membranes from m4 CHO cells with indicated concentrations of the m4 antisera. B, Antiserum specificity. Immunoprecipitation with the m4 antisera (0.6 mg/ml) performed with [^{3}H]QNB-labeled m1, m2, m3, m4, and m5 CHO membranes was done to determine specificity of the antisera. The preparation of membranes and the immunoprecipitation procedures are described in Materials and Methods. Data are presented in A and B as the percentage of the total [^{3}H]QNB-labeled receptors that were found in the pellet and show the mean ± standard deviation for a representative experiment. These experiments were done at least twice with similar results.

the distribution of these proteins was determined in rat brain (Fig. 5). The general distribution of m4 receptors is somewhat similar to that found for m1 receptors (13). That is, the largest amounts (Fig. 5B) and percentages (Fig. 5A) of receptors were located rostrally in the telencephalic regions and declined as one proceeded caudally to the metencephalon. The largest numbers of m4 receptors were found in the striatum (1280 fmol/mg of protein) and the smallest numbers of receptors were in the cerebellum (9 fmol/mg of protein). The olfactory tubercle also appeared to have a high concentration of m4 receptors (750 fmol/mg of protein). This is in contrast to m2 receptors, for which the largest percentage of muscarinic receptors oc-

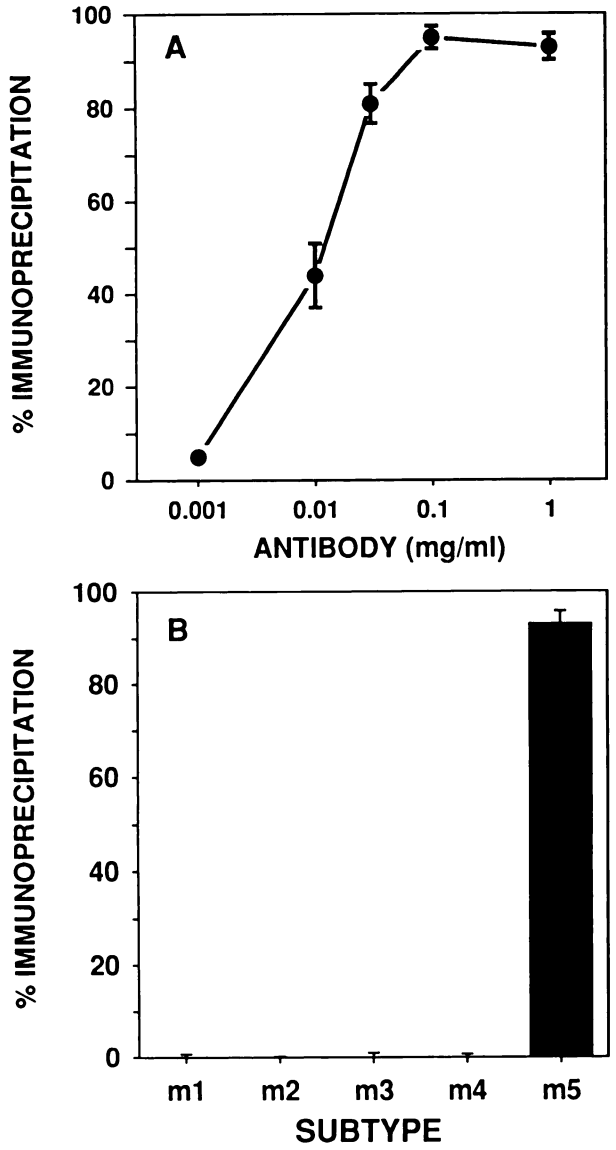

Fig. 4. Characterization of the m5 fusion protein antisera. A, Antiserum titer. Immunoprecipitation of [^{3}H]QNB-labeled membranes from m5 CHO cells with indicated concentrations of the m5 antisera. B, Antiserum specificity. Immunoprecipitation with the m5 antisera (0.6 mg/ml) performed with [^{3}H]QNB-labeled m1, m2, m3, m4, and m5 CHO membranes was done to determine specificity of the antisera. The preparation of membranes and the immunoprecipitation procedures are described in Materials and Methods. Data are presented as in Fig. 3.

curred in the cerebellum and the absolute receptor density changed little across the brain (14). It also differs from the distribution of m3 receptors, for which the percentage of receptors did not change dramatically across the brain but the amount decreased as one moved caudally in the brain (15).

Although Fig. 4 demonstrates that the m5 receptor antisera are selective and quantitative, the percentage of m5 receptors found in all rat brain areas examined was <2% of the total number of muscarinic receptors (Fig. 5C). This indicated that the receptor density of m5 receptors was <25 fmol/mg in all of the areas of rat brain that were dissected (Fig. 5D) and suggested that the amount of m5 receptors may be near the level of detection of the immunoprecipitation assay. Two criteria were used to determine whether these low m5 receptor densities

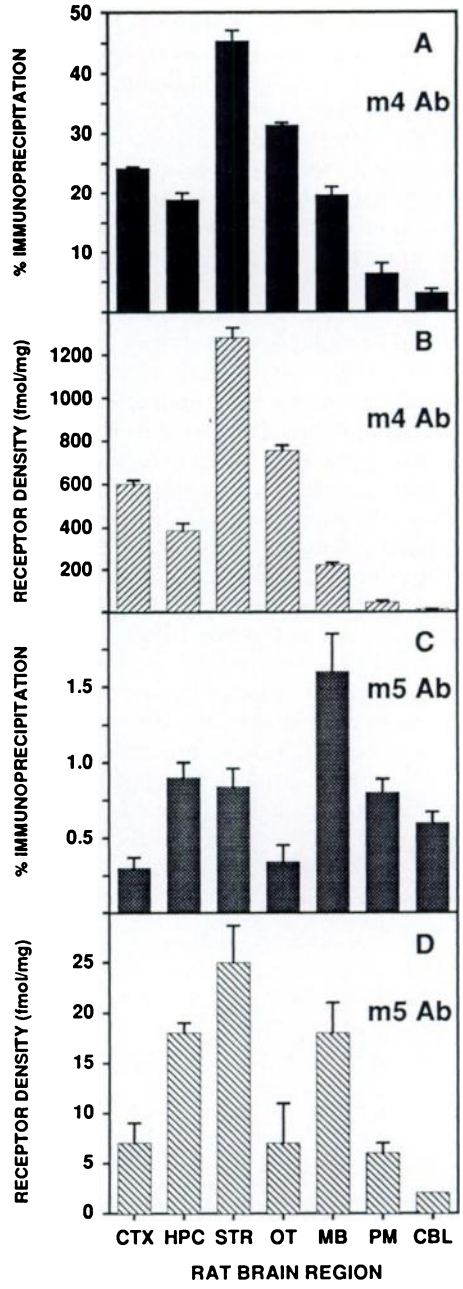

Fig. 5. Distribution of m4 and m5 muscarinic receptors in rat brain areas. Solubilized [^{3}H]QNB-labeled rat brain membrane regions were immunoprecipitated with either m4 or m5 antisera as described in Materials and Methods. A, Percentage of m4 muscarinic receptors in cortex (*CTX*), hippocampus (*HPC*), striatum (*STR*), olfactory tubercle (*OT*), midbrain (*MB*), pons-medulla (*PM*), and cerebellum (*CBL*) is shown. The total receptor number (pmol/mg of protein; mean ± standard error of three experiments except for striatum, where there were eight experiments) for each brain region was 2.47 ± 0.06, 2.03 ± 0.08, 2.93 ± 0.10, 2.41 ± 0.10, 1.13 ± 0.04, 0.69 ± 0.04, and 0.30 ± 0.01, respectively. B, m4 muscarinic receptors in rat brain regions, expressed as fmol/mg of protein. The amount of the m4 receptor subtype in each rat brain area was calculated by multiplying the fraction of total [^{3}H]QNB bound that was immunoprecipitable times the B_{max} for the corresponding brain area. C, Percentage of m5 muscarinic receptors in the same tissues used to determine m4 muscarinic receptor percentages. D, m5 muscarinic receptors in rat brain regions, expressed as fmol/mg of protein.

in rat brain regions were real. The first criteria was whether these receptor densities were statistically significantly different from nonspecific samples (i.e., samples that were immunoprecipitated by antisera to the pRIT23-Protein A product). From the data in Fig. 5D, the striatum, hippocampus, midbrain, pons-medulla, and cerebellum had amounts of m5 receptors that were statistically different from nonspecific samples at $p < 0.01$. The second criterion was to see whether known amounts of m5 receptors at percentages similar to those found in rat brain regions could be detected. Fig. 6 presents three experiments where known amounts of m5 receptors (0–3%) were added to a constant amount of m2 receptors (0.12, 0.26, and 0.72 pmol) in each experiment. Fig. 6 plots the actual percentage of m5 receptors added to the assay mixture versus the amount experimentally determined. The line of identity is shown to indicate where points should fall if 100% of the m5 receptors were precipitated. The Newman-Keuls multiple comparison test was performed with each experimental set of determinations to see which points were significantly different from zero. Statistical significance was conservatively set at $p < 0.01$. At 0.12 pmol (4400 cpm), 0.26 pmol (9860 cpm), and 0.72 pmol (27,500 cpm) of total receptor, the m5 percentages were significant at 1.5%, 0.82%, and 0.75%, respectively. If one uses these as strict limits to determine whether m5 receptors are really detected, then the only brain regions where statistically significant levels of m5 receptors were found were the striatum, hippocampus, and midbrain. In the data presented in Fig. 5D, there were 0.38 pmol of total receptors in the rat hippocampus, of which 0.9% were m5 receptors, 0.88 pmol of total receptors in the rat striatum, of which 0.8% were m5 receptors, and 0.41 pmol of total receptors in the rat midbrain, of which m5 antisera immunoprecipitated 1.6%. Therefore, the only brain regions that met both criteria were the striatum, hippocampus, and midbrain.

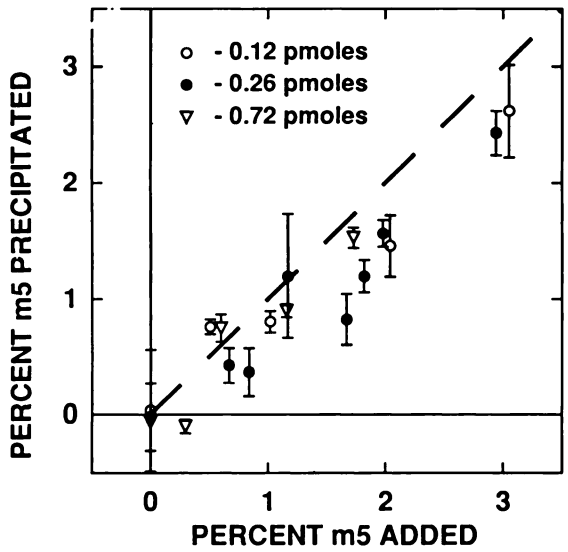

Fig. 6. Determination of the level of detection for muscarinic m5 receptors. Immunoprecipitations were performed on solubilized preparations composed of a fixed amount of m2 receptors and a range of m5 receptors from 0 to 3% of the total receptors. This experiment was done three times with various total numbers of receptors and all data are shown in this figure. The actual percentage of m5 receptors added to the tube is shown on the *x*-axis and the percentage of m5 receptor determined by the immunoprecipitation assay is shown on the *y*-axis. The total number of receptors in each experiment was 0.12 pmol (4400 cpm) (○), 0.26 pmol (9860 cpm) (●), and 0.72 pmol (27,600 cpm) (▽). The percentage of m5 receptors added was 0–3%, 0–2.9%, and 0–1.7%, respectively. - - -, Line of identity.

Distribution of m1-m5 receptors in rat lung, rabbit lung, NG108–15 cells, and N1E-115 cells. A recent paper from this laboratory (15) demonstrated that the muscarinic receptor subtype expressed by rat lung is predominantly (91%) m2. No receptors in rat lung could be immunoprecipitated with the m4 or m5 antisera (data not shown). In contrast to rat lung, rabbit lung expresses both m2 (27%) and m4 (55%) receptors and low levels of m1 (3%) and m3 (4%) receptors (Fig. 7A). These data are in agreement with the recent findings of Dörje *et al.* (23), who showed the presence of m2 and m4 receptors in rabbit lung.

Because rabbit lung has been shown to be pharmacologically similar to the NG108–15 neuroblastoma × glioma cell line (16), and both have been suggested to be model M4 tissues (24, 25), the subtypes of muscarinic receptors in NG108–15 cells were also examined. Based on the pharmacology of the muscarinic receptors in NG108–15 cells, Lazareno *et al.* (16) have suggested that there may be an M2 component present in this cell line. Fig. 7B demonstrates the presence of significant amounts of m2 (31%) and m4 (42%) receptors in NG108–15 cells. No significant levels of m1, m3, or m5 receptors were detected.

N1E-115 neuroblastoma cells are another cell line in which only the mRNA for the m4 receptor has been found (18). Although the presence of a muscarinic receptor-mediated inhibition of cAMP accumulation that was inhibited with moderate affinity (K_d = 93 nM) by pirenzepine is consistent with the presence of m4 receptors, McKinney *et al.* (26) also demonstrated that these cells express muscarinic receptors that stimulate guanylate cyclase and are inhibited potently (K_d = 6 nM) by pirenzepine, suggesting the presence of m1 receptors. The data in Fig. 7C clearly demonstrate that both m1 (15%) and m4 (65%) receptors are present in N1E-115 cells.

Discussion

The strategy adopted for the development of antisera that would selectively recognize a single subtype of muscarinic receptor relied on the fact that most of the i3 loop of each subtype possesses a unique primary sequence. On the other hand, because the m2 and m4 receptors have been shown to be coupled preferentially to the inhibition of adenylate cyclase and the m1, m3, and m5 receptors to the stimulation of phosphoinositide hydrolysis (6, 17, 27), certain portions of this i3 loop that interact with a given guanine nucleotide-binding protein to affect a specific second messenger system are conserved. The specificity of this coupling is conferred, in part, by a peptide sequence at the amino-terminal region of the i3 loop of each receptor subtype (28). Thus, both the m4 and m5 antisera were produced using fusion proteins containing the sequence of the i3 loop of each respective receptor subtype, excluding only the sequence that is conserved between m2 and m4 receptors and between m1, m3, and m5 receptors. It was expected, therefore, that little cross-reactivity among these antisera would be seen. This is clearly shown in the data presented in Figs. 3B and 4B. The titers of these antibodies (Figs. 3A and 4A) were similar to those reported previously (13–15), with EC_{50} values of approximately 500–2000-fold dilutions of the sera.

The immunoprecipitation data using the m4 antisera in rat brain suggest that receptors are expressed at high levels in

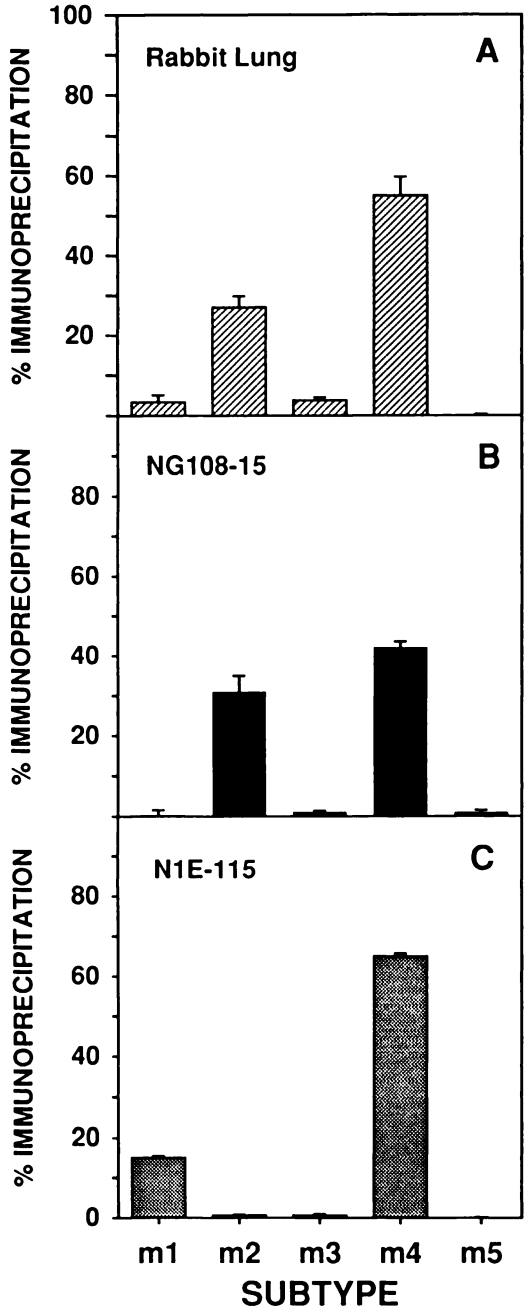

Fig. 7. Immunoprecipitation of m1-m5 muscarinic cholinergic receptors in rabbit lung, NG108-15 cells, and N1E-115 cells. B_{max} (fmol/mg of protein) values (mean ± standard error) were 126 ± 31 (four experiments), 46.7 ± 14 (three experiments), and 23.6 ± 1.6 (three experiments), respectively. Immunoprecipitations and B_{max} determinations were performed as described in Materials and Methods.

many brain regions and have a distribution generally similar to that seen for m1 receptors (13). This agrees with the conclusions of McKinney *et al.* (18), who suggested that most of the inhibition of cAMP accumulation in rat cortex and striatum is mediated by m4 receptors. The corresponding immunoprecipitation data for m5 receptors shown in Figs. 5 and 6 demonstrate that this subtype is expressed at measurable but very low levels in certain parts of the rat brain.

To estimate the relative amounts of each muscarinic receptor subtype in different brain regions, the percentages of m4 and m5 receptors are shown together with the percentages previously determined for the m1, m2, and m3 receptor subtypes in each brain region (13–15). Fig. 8 demonstrates how well the percentages of the five muscarinic cholinergic receptor subtypes account for the total binding to muscarinic cholinergic receptors. At worst, 86% of the receptors were immmunoprecipitated in the olfactory tubercle and the cerebellum, whereas, at best, nearly all (99%) of the muscarinic cholinergic receptors were immunoprecipitated in the striatum. These data indicate that, although it is possible that there may be more muscarinic receptor subtypes present in rat brain, this does not appear likely because the amounts of total receptors that can be immunoprecipitated from transfected CHO cell lines (83–95%) are similar to the values shown in Fig. 8.

In situ hybridization histochemistry has demonstrated that m4 mRNA is found at high levels in the rat caudate putamen, the cerebral cortex, and the pyramidal cell layer of the hippocampus (7), which agrees with the immunoprecipitation of m4 receptors observed in this study as well as in the recent investigation by Levey *et al.* (29). The relative distribution of m4 receptors seems to be similar to the distribution of m4 mRNA in these brain regions. That is, there are relatively more m4 receptors in the telencephalic regions (striatum, cortex, olfactory tubercle, and hippocampus) of the brain and the levels decrease caudally towards the diencephalon (thalamus and hypothalamus), mesencephalon (midbrain), and metencephalon (pons-medulla and cerebellum).

Recently, Waelbroeck *et al.* (25), using sophisticated but complicated radioligand binding techniques, showed the presence of four pharmacologically defined muscarinic cholinergic receptor binding sites in the rat cortex, hippocampus, and striatum. They were able to accomplish this by taking advantage of the fact that *N*-[^{3}H]methylscopolamine dissociated

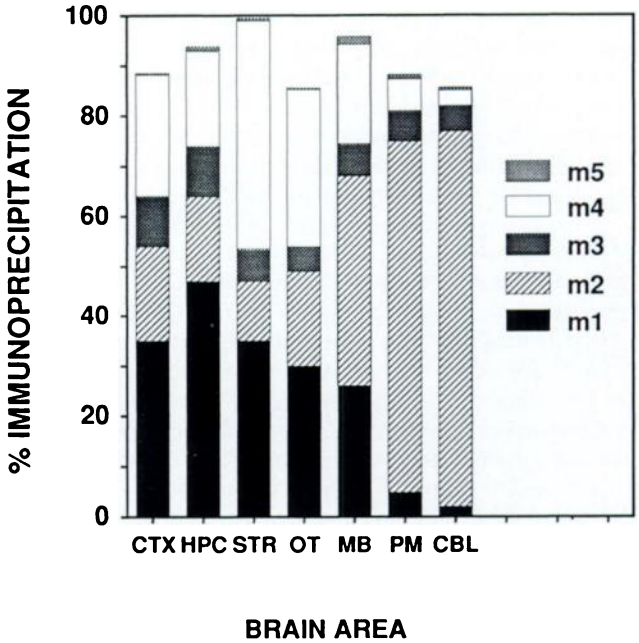

Fig. 8. Percentage of each muscarinic cholinergic receptor in regions of the rat brain. Percentage of m1-m5 muscarinic receptors in cortex (*CTX*), hippocampus (*HPC*), striatum (*STR*), olfactory tubercle (*OT*), midbrain (*MB*), pons-medulla (*PM*), and cerebellum (*CBL*) is shown. This figure is a compilation of rat brain immunoprecipitation experiments from previous reports (13–15) and this manuscript.

more slowly from M3 and M4 binding sites than from M1 and M2 binding sites. This allowed those investigators to evaluate no more than two receptor subtypes at a time. A comparison of the receptor subtype densities determined with antisera in our studies (Fig. 8) and those determined using pharmacological tools (25) shows strikingly similar profiles for the m1-m4 receptor subtypes and the M1-M4 binding sites defined by Waelbroeck *et al.* (25). The subtype-selective antisera show that the m1, m2, m3, and m4 receptors are 34, 19, 10, and 24%, respectively, of the muscarinic cholinergic receptors in the rat cortex. With correction for the percentage of receptors occupied at equilibrium, Waelbroeck *et al.* (25) found that the M1, M2, M3, and M4 binding sites are 34, 22, 10, and 34%, respectively, of the muscarinic cholinergic receptors in rat cortex. Similarly, in hippocampus our data show that the m1, m2, m3, and m4 receptors are 47, 17, 10, and 19%, respectively, whereas those authors reported the M1, M2, M3, and M4 binding sites to be 51, 23, 10, and 16%, respectively. In striatum we find 35, 12, 6, and 45% for the m1, m2, m3, and m4 receptors, respectively, whereas they found 27, 19, 9, and 45% for the M1, M2, M3, and M4 binding sites, respectively. Thus, although these percentages are not identical they are quite close in these three rat brain areas and suggest that the pharmacologically defined binding sites, as measured by the techniques of Waelbroeck *et al.* (25), fairly accurately represent the molecularly defined receptor subtypes.

The m5 antisera that were developed could quantitatively and selectively immunoprecipitate m5 receptors in solubilized CHO membranes (Fig. 4). However, the percentage of m5 receptors in the rat brain regions examined was <2% of total muscarinic receptors and represented <25 fmol/mg of protein (Fig. 5). Because it was unclear whether these values were "real," two criteria were established. First, it was determined whether these m5 receptor densities were significantly different from nonspecific samples. It was found that the striatum, hippocampus, midbrain, pons-medulla, and cerebellum had values for m5 receptor density that were statistically significantly different from blank samples at a $p < 0.01$. Second, the level of detection of known percentages of m5 receptors was determined. There were three separate experiments done with varying amounts of total receptors (0.12–0.72 pmol). When very low (<0.4%) levels of m5 receptors were added, some of the experimental values were not statistically different from zero (Fig. 6). As more m5 receptors were added, the experimentally determined values began to fall closer to the predicted line (Fig. 6). These values generally fall slightly below the predicted line because the amount of receptors immunoprecipitated is somewhat less than 100% (e.g., see Fig. 4). Based on these two established criteria, only the striatum, hippocampus, and midbrain had a clear statistical indication of m5 receptors being present (Fig. 5), where m5 receptors represent 0.8%, 0.9%, and 1.6% of the total receptors (with nonspecific binding having been subtracted), respectively. In the case of the midbrain, one could speculate that much of this m5 receptor protein is in the substantia nigra, because this area is contained within the midbrain homogenate and mRNA for the m5 receptor has been reported in the substantia nigra, pars compacta (8). Furthermore, one could speculate that at least some of the m5 receptors in the striatum may reside on terminals originating in the substantia nigra. The total picomoles of receptors used for other brain regions yielded percentages of m5 receptors that were below the level of detection for this immunoprecipitation assay, even though some of these areas may have m5 receptors present. In contrast to our data, Levey *et al.* (29) have shown no evidence of detectable m5 receptors in the rat brain.

Before the development of selective antisera for the muscarinic receptors, the only way to examine the distribution of a molecularly defined receptor subtype was by determining where the mRNA was found. However, this methodology can miss mRNA transcripts that are expressed at low levels but are translated with high efficiency. Therefore, the expression of muscarinic receptor subtypes was also measured in two cell lines and in rabbit lung. These tissues were chosen because only mRNA encoding the m4 receptor had been found in each of these tissues but the pharmacological profiles suggested the presence of another receptor subtype as well (16–18). Additionally, Lazareno *et al.* (16) noted that rabbit lung and NG108–15 cells possessed a similar pharmacological profile. Fig. 7 clearly demonstrates the presence of both m2 and m4 receptor subtypes in rabbit lung and NG108–15 cells. These data make it clear why NG108–15 cells and rabbit lung appear similar pharmacologically.

N1E-115 cells express both m1 and m4 receptors (Fig. 7C). The presence of these two subtypes agrees with pharmacological and biochemical data of McKinney *et al.* (18, 26). The pharmacological profile of the cAMP inhibition in N1E-115 cells and the fact that the m4 transcript is present in N1E-115 cells supports the presence of m4 receptors (18, 26). Although no mRNA transcript for the m1 receptor has been observed (18), McKinney *et al.* (26) showed that the receptor mediating stimulation of [^{3}H]cGMP formation had high affinity for pirenzepine (K_d = 6.5 nM) and the receptor mediating the inhibition of adenylate cyclase had lower affinity for pirenzepine (K_d = 68 nM), which corresponds to the affinities of pirenzepine for m1 and m4 receptors, respectively (10, 11). Thus, even though m1 mRNA transcripts could not be found (26), the pharmacology clearly demonstrates the presence of an M1 site coupled to the stimulation of cGMP formation. The present study agrees with those earlier studies and identifies the receptor mediating cGMP formation as being the m1 muscarinic receptor.

These studies demonstrate the utility of antibodies selective for subtypes of muscarinic receptors. It is apparently not always possible to detect mRNA transcripts (e.g., NG108–15 cells, N1E-115 cells, and rabbit lung), and this can lead to erroneous conclusions regarding the composition of receptor subtypes in a given tissue. Understanding the distribution of muscarinic cholinergic receptor subtypes in the brain may be important in determining the role of each receptor subtype in pathological states such as Alzheimer's disease and Parkinson's disease. By targeting the specific receptor subtypes that may be involved in these disorders, selective drugs can be designed to minimize untoward side effects while delivering therapeutic relief.

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