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Production of Antisera Selective for m1 Muscarinic Receptors Using Fusion Proteins: Distribution of m1 Receptors in Rat Brain

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SUMMARY

A fragment of the cDNA encoding the third intracellular loop of the rat m1 muscarinic receptor was cloned, and the DNA was expressed in *Escherichia coli* as a fusion protein. The fusion protein was purified and utilized as an antigen to raise a polyclonal antiserum in rabbits. Chinese hamster ovary cells stably transfected with the cDNA encoding each of the five known subtypes of muscarinic receptor were used as tissue sources to test the antiserum. The antiserum was found to quantitatively immunoprecipitate m1 muscarinic receptors, while not precipitating m2, m3, m4, or m5 receptors. This selective antiserum was utilized to quantify the density of m1 muscarinic receptors in seven selected areas of the rat brain. Thus, cortex was found to contain approximately 0.8 pmol/mg of membrane protein, which represents 34% of the total density of muscarinic receptors. Similarly, hippocampus (1 pmol/mg; 47%), striatum (0.8 pmol/mg; 29%), and olfactory tubercule (0.9 pmol/mg; 35%) are rich in m1 receptors. In contrast, thalamus/hypothalamus contained only 0.15 pmol/mg, representing approximately 16% of the total density of muscarinic receptors, whereas pons/medulla (0.03 pmol/mg; 5%) and cerebellum (<0.01 pmol/mg; 2%) had very low levels of expression of m1 receptors. The development of a selective antiserum has provided a means for the quantification of a specific subtype of muscarinic receptor in tissues, such as the brain, that express multiple subtypes. This methodology will be applicable not only to the other subtypes of muscarinic receptor but also to the subtypes of several other neurotransmitter receptors that lack selective drugs with which to study them.

The existence of subtypes of muscarinic receptors was suspected as early as the 1960s (1). The strongest evidence in support of this hypothesis was obtained with the compounds 4-DAMP (2) and pirenzepine (3), muscarinic receptor antagonists that exhibit a degree of selectivity towards certain subtypes. Finally, cloning studies (4–9) confirmed the existence of at least five subtypes of muscarinic receptor, each with its own gene. Studies examining the distribution of the mRNA for each of the subtypes of muscarinic receptor have shown that the receptors are likely to coexist in the same tissues but, also, each is likely to have a unique distribution (10, 11). For example, mRNA for the m1 muscarinic receptor was reported to be highest in the hippocampus and cortex, with only slightly lower levels in the striatum. Conversely, levels in the midbrain and hindbrain were found to be very low.

Although it is very useful to be able to measure and map the mRNA encoding a given protein (e.g., m1 receptor), it is also useful to measure and map the resulting protein directly. Thus, location or amount of mRNA or changes in mRNA levels may, or may not, accurately reflect the status of the protein itself. However, the "selective" drugs currently available to study muscarinic receptor subtypes, such as pirenzepine, 4-DAMP, AF-DX116, etc., are only slightly selective for a given subtype (12). As such, they are limited in their ability to estimate accurately the density of a given subtype in a tissue that expresses multiple subtypes.

We, therefore, embarked on the project of raising selective antisera toward the m1 subtype of muscarinic receptor that could be used to estimate quantitatively the density of these receptors in various tissues. To this end, we cloned a unique part of the m1 receptor, corresponding to the third intracellular loop (i3 loop), and used this piece of DNA to express a fusion protein with which to raise antibodies. The antisera obtained appear to be highly selective and quantitatively immunoprecipitate m1 muscarinic receptors.

Materials and Methods

Fusion protein preparation. The expression vector pRIT23 was chosen to produce the fusion protein, because vectors similar to it had

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ABBREVIATIONS: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; bp, base pairs; QNB, quinuclidinyl benzilate; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methobromide

been reported to express fusion protein at high levels and because the resultant fusion proteins were easily purified (13, 14). This vector contains the coding region for the five IgG-binding domains of Protein A. This is preceded by a signal sequence that allows the expressed protein to be translocated through the bacterial inner cell membrane into the periplasmic space. This, in turn, is preceded by a strong constitutively active promoter directing high levels of expression of the protein. Thus, the resultant fusion protein, found in the periplasmic space, is easily purified via an IgG-Sepharose column, to which the Protein A portion of the fusion protein binds and the rest of the bacterial proteins do not. Isolation of m1 intracellular loop-Protein A fusion protein was achieved as described by Nilsson and Abrahmsen (14). Growth medium (5 ml of Luria broth supplemented with 0.1 mg/ml glucose and 0.25 mg/ml ampicillin) was inoculated with 5 μl of transformed bacterial stock (p23-rm1-220/346) and grown at 32°, at 300 rpm, for 4 hr. Bacteria were pelleted at 1200 × g for 10 min, resuspended in 400 ml of medium, and grown overnight at 32° at 300 rpm. The 400-ml culture was centrifuged at 1200 rpm for 10 min and the bacterial pellet was used to seed a 1500-ml preparation, which was grown at 37° for 3 hr at 300 rpm. The bacteria were pelleted, resuspended in 80 ml of ice-cold sucrose buffer (0.5 M sucrose, 0.1 M Tris·HCl, pH 8.2, 1 mM EDTA), and incubated for 10 min on ice. Lysozyme (0.52 ml of a 10 mg/ml stock) was added, followed immediately by the addition of 80 ml of H_2O and vigorous mixing. The preparation was allowed to stand on ice for 5 min, followed by the addition of 2.9 ml of $MgSO_4$ (1.0 M) and vigorous shaking. The lysed bacterial preparation was centrifuged at 12,000 × g (20 min at 4°). The supernatant was loaded (1.0 ml/min) onto a 10-ml IgG-Sepharose 6 Fast Flow column (Pharmacia) preequilibrated with TST buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20). Columns were prewashed with 100 ml of TST, followed by 50 ml of ammonium acetate (5 mM, pH 5.5). Bound proteins were eluted with 30 ml of ammonium acetate (0.5 M, pH 3.3). This fraction was lyophilized overnight and resuspended in a volume of 1.5 ml in H_2O. An aliquot was removed for SDS-PAGE and protein determination. The eluted protein (30 mg) was loaded (0.1 ml/min) onto a 100-ml Sephacryl S-200 column (Pharmacia) preequilibrated with ammonium acetate (0.1 mM, pH 7.9). An aliquot (15 μl) of each fraction (1.20 ml each) was removed for SDS-PAGE. Fractions identified as containing fusion protein (fractions 28–40) were pooled and lyophilized. The yield of fusion protein (m1 third intracellular loop/Protein A) was approximately 10 mg.

Antisera and Pansorbin preparation. Two rabbits (New Zealand females, 3 kg) were injected (15–20 sites, subcutaneously) with approximately 1–2 mg each of purified fusion protein suspended in 1.0 ml of H_2O mixed with an equal volume of either Freund's complete (first injection) or Freund's incomplete (booster injections) adjuvant. Booster injections were done at approximately 1-month intervals. The first bleed occurred 2 weeks after the first boost. Subsequent bleeds occurred 12–14 days after injection. Blood was allowed to clot at room temperature for 60 min, followed by centrifugation at 10,000 × g for 15 min at 4°. Antibodies were partially purified from serum by precipitation (10,000 × g for 15 min) with a 50% solution of ammonium sulfate at 4°. Pellets were resuspended in buffer (10 mM $NaPO_4$, pH 7.4) and the solution was dialyzed (10,000 molecular weight cutoff) against phosphate buffer (10 mM, pH 7.4, with 0.02% NaN_3) for 24 hr at 4°, with two solution changes. Antibody solution was then lyophilized and stored at −70°. Under these conditions, antibody activity has remained stable for at least 6 months.

Pansorbin (Calbiochem) was prepared by transferring the required volume to a 50-ml tube and centrifuging at 10,000 × g for 15 min. The supernatant was discarded, and the pellet was resuspended in a volume of 10% β-mercaptoethanol/3% SDS equivalent to the original Pansorbin volume. The suspension was boiled for 15 min, followed by centrifugation at 15,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in a volume of Tris-buffered saline (50 mM Tris, pH 7.4, 500 mM NaCl) equivalent to the starting volume. The washing and centrifugation steps were repeated five times. The final suspension in Tris-buffered saline was identical to the starting volume. Stripped Pansorbin slurry was stored at 4° and used within 1–3 days, although IgG binding activity appears to be stable for at least 5–7 days.

Membrane preparation. CHO cells and A9 cells transfected with the cDNA encoding the human m1, m2, m3, m4, or m5 subtypes of muscarinic receptor were a generous gift of Dr. Mark Brann (National Institutes of Health). Receptor expression levels in CHO cells were determined to be m1 = 1,650, m2 = 1,830, m3 = 2,850, 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. CHO cells transfected with cDNA encoding the human m1 or porcine m2 receptors were a generous gift of Dr. Daniel Capon (Genentech). Receptor expression levels were m1 = 1,100 and m2 = 12,000 fmol/mg of membrane protein. Mother flasks were grown in 10% fetal bovine serum, Dulbecco's modified Eagles medium F12, supplemented with glutamine (2 mM), penicillin (100 units/ml), and streptomycin (0.1 mg/ml), at 37° in a humidified atmosphere supplemented with 8% CO_2. Cells from Genentech were grown in the same medium, supplemented with 200 nM methotrexate, and the serum was dialyzed before use. Cells to be harvested for experiments were plated on 150-mm culture dishes and grown for 7–10 days in 4% defined supplemented bovine calf serum (GIBCO), in Optimem medium (GIBCO Laboratories) supplemented with glutamine and penicillin/streptomycin. Medium was changed on day 5 and on the day before harvesting (approximately day 7). Cells were harvested in 25 ml of ice-cold TE/PIC (10 mM Tris, pH 7.4, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml α_2-macroglobulin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml bacitracin, 1 μg/ml pepstatin, and 500 μM phenylmethylsulfonyl fluoride). Membranes were homogenized (Tekmar Tissuemizer; setting 6, 7 sec) and centrifuged for 15 min at 32,000 × g at 4°. Membranes were resuspended in a volume of TE (10 mM Tris, 1 mM EDTA, pH 7.4), aliquoted, and stored at −70°. Brain regions (cortex, hippocampus, striatum, olfactory tubercule, thalamus/hypothalamus, pons/medulla, and cerebellum) were dissected on ice from adult (200 g) male rats (Sprague-Dawley), weighed, and immediately frozen at −70°. At the time of the experiment, brain regions were thawed on ice and transferred to 10 ml of TE/PIC buffer. The tissue was homogenized (Tekmar Tissuemizer; setting 6, 7 sec), and an aliquot was removed for labeling and solubilization. The homogenate was centrifuged at 32,000 × g for 15 min at 4°, and the membrane pellet was resuspended in ice-cold TE/PIC buffer. For determination of receptor density, 100–250 μg of membrane protein were incubated with the nonselective muscarinic receptor antagonist [^{3}H]QNB (1 nM) for 45 min at 32° in TE/PIC buffer (1 ml final volume). Nonspecific binding was determined in the presence of atropine (5 μM). Ice-cold wash buffer (10 ml of 10 mM Tris, 0.9% NaCl, pH 7.4) was added to each binding tube, followed by filtration through glass fiber filters (Schleicher and Schuell no. 30). Filters were washed with two 5-ml aliquots of cold wash buffer, transferred to scintillation vials, and incubated overnight with 4 ml of Ecolite (ICN) scintillation cocktail before counting for 5 min. Protein concentrations were determined by the method of Lowry *et al.* (15).

Receptor labeling and solubilization. Membranes were incubated with [^{3}H]QNB (25 ml, 1.5 nM) at 32° for 45 min, followed by centrifugation at 30,000 × g for 15 min at 4°. The supernatant was discarded, and the pellet was washed twice in cold TE/PIC. For receptor solubilization, a volume of cold TEDC buffer (10 mM Tris, 1 mM EDTA, 1.0% digitonin, 0.2% cholic acid, pH 7.4) was added to yield a final concentration of approximately 3 mg of membrane protein/ml of solubilization buffer. Pellets were resuspended with a Tissuemizer (setting 6, 4 sec) and transferred to an ice bath for 30 min with occasional trituration. Samples were centrifuged at 80,000 × g for 45 min. Receptor solubilization efficiency under these conditions was 40–60%. No consistent difference was observed in solubilization efficiency between receptor subtypes.

Immunoprecipitation. The methodology employed is a revised version of a previously described protocol (16). Antisera were reconsti-

tuted in TE and incubated with aliquots of labeled solubilized receptor, in a final volume of 0.5 ml at 4°. Typically, 25–200 fmol of receptor (1000–8000 cpm) were added per tube, and incubations were carried out for 44–50 hr. At the end of this period, samples were loaded onto Sephadex G-50 columns (3 ml) that were preequilibrated with column buffer (0.1% digitonin, 0.02% cholic acid, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.9% NaCl). A 0.9-ml wash of column buffer was then applied, followed by a second 1.6-ml aliquot of buffer. The 1.6-ml fraction eluted 89 ± 4% of added radioactivity. Free [^{3}H]QNB (<5% of added counts) eluted in the later fractions. The 1.6-ml solubilized receptor preparation was then divided into two fractions (A and B). Fraction A (1.20 ml) was incubated with Pansorbin for 2 hr at 4° in 1.5-ml microfuge tubes, with constant rotation. Samples were then centrifuged at 15,000 × *g* at 4° for 3 min, and the supernatants were discarded. Pellets, consisting of labeled receptor-antibody-Pansorbin complexes, were surface washed with 1.0 ml of cold TE buffer. The wash was discarded, and the pellet was recentrifuged. Wash volume remaining above the pellet was removed. The pellet was incubated with 0.10 ml of NaOH/DOC (0.10 N NaOH, 3% sodium deoxycholate) for 60 min at 22° and resuspended with 0.30 ml of column buffer, and the radioactivity was quantified. NaOH/DOC (0.1 ml) was added to 0.3 ml of fraction B, and radioactivity was quantified. The counting efficiency for both fractions was approximately 40%. Precipitation efficiency was calculated by dividing counts present in the pellet from fraction A by added counts (fraction B × 4). Nonspecific precipitation determined with either preimmune antisera or antiserum from animals injected with truncated Protein A (i.e., protein isolated from *Escherichia coli* transformed with pRIT23) represented 0.5–2.0% of added counts, and this value was routinely subtracted. Additionally, precipitation in samples that had been labeled in the presence of 5 μM atropine yielded less than 5% of the counts found in samples labeled in the absence of atropine. Quantitative immunoprecipitation (>90%) was attained with antibodies isolated from both rabbits injected with m1-Protein A fusion protein, and sera were used interchangeably in subsequent experiments.

Results

Cloning of cDNA encoding the i3 loop of rat m1 receptor and construction of p23-rm1-220/346. Using the published sequence for the porcine m1 receptor (4), an oligonucleotide was constructed, taking into consideration potential (and unknown at the time) differences in nucleotide sequence between porcine and rat m1 receptors. Thus, a portion of the i3 loop of the porcine receptor, corresponding to Ile^{302} to Lys^{316}, was identified as having a relatively low level of codon degeneracy, and the 256-fold degenerate oligonucleotide 5′-TA-ITT(TC)TACGG(IC)TACCA(IC)CTIGG(IC)CT(TC)CG(IC)-GT(TC)CG(IC)GGICGITT was synthesized. Nucleotides in parentheses indicate positions in which more than one nucleotide was incorporated and I indicates inosine. Inosine was utilized if the likely complementary nucleotides were T, C, or A, whereas inosine and cytosine were utilized if the possible complementary nucleotides were G, T, C, or A (17). The oligonucleotide was 5′ labeled with T4 polynucleotide kinase, purified by ethanol precipitation, and utilized as a probe to screen a rat brain cDNA library in λgt11 (Clonetech). Approximately 10^6 plaques were screened, and the nitrocellulose filters were washed at 59° in tetramethylammonium chloride (18). Three positive clones were isolated that contained 1900-bp inserts, each of which appeared to be identical to the other on the basis of restriction maps. The insert was excised from λgt11 with *Eco*RI and ligated into pBR322. Sequencing confirmed that the insert coded for the rat m1 receptor, because the predicted amino acid sequence was nearly identical to that of the porcine receptor (4) and the nucleotide and amino acid sequences agreed nearly perfectly with the report by Bonner *et al.* (8). Thus, of the 127 amino acids of the i3 loop, only one residue appeared to differ (i.e., Ser^{307} in Bonner's sequence corresponded to Cys^{307} in our sequence). The 3′ end of the insert corresponded to Lys^{346} via a cloning artifact, presumably occurring during library construction. Thus, an *Eco*RI site existed after Lys^{346}. The *Sma*I site at Arg^{220} was opened and the linker 5′-GGAATTCC was ligated into the plasmid. The resulting plasmid produced a 380-bp and a 1500-bp fragment upon digestion with *Eco*RI. The 380-bp fragment was ligated into pRIT23 (14), HB101 *E. coli* were transformed with the ligation mix, and several of the resulting clones were partially sequenced to determine the orientation of the insert and to verify that only a single linker had been inserted. A clone in the proper orientation (p23-rm1-220/346) was selected for production of fusion protein (Fig. 1).

Production and purification of fusion proteins. This clone was found to express approximately 15–20 mg/liter protein that bound to and was eluted from an IgG-Sepharose column. As can be seen in Fig. 2, approximately half of the protein had the molecular size expected (M_r 53,500) of the fusion protein, whereas the other half appeared to be smaller than expected (Fig. 2, *lane 2*). It was assumed that the lower molecular weight species (M_r 42,000, 35,000, and 33,000) represented proteolyzed fusion protein that may be missing some or all of the third intracellular loop. Thus, the protein that was purified over IgG-Sepharose was further purified using a Sephacryl S-200 sizing column (Fig. 2, *lane 3*), and the material with the highest molecular weight was used for injections into rabbits to raise antisera.

Production and characterization of antisera. Rabbits were administered protein at monthly intervals, and serum was isolated about 12–14 days after each booster injection. Immunoprecipitation of solubilized radiolabeled m1 muscarinic receptors was used as a measure of antibody production. As can be seen in Fig. 3, no measurable precipitation was elicited by serum from the first bleed. On the other hand, all subsequent bleeds were able to immunoprecipitate radiolabeled m1 muscarinic receptors. The titer of the sera improved with each

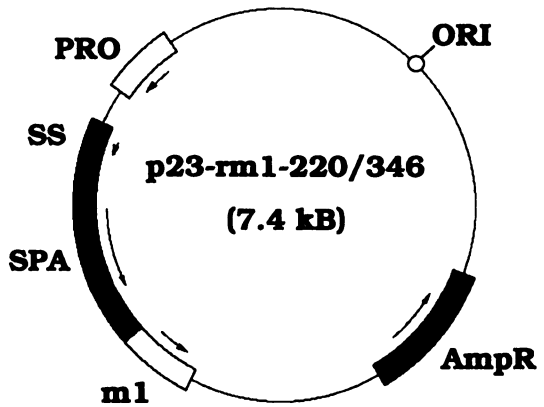

Fig. 1. Expression vector p23-rm1-220/346. The plasmid pRIT23 was modified by introduction at the *Eco*RI site of a cDNA encoding 127 amino acids of the proposed third intracellular loop of the rat m1 muscarinic receptor (Arg^{220} to Lys^{346}). *PRO*, the staphylococcal Protein A promoter; *SS*, the signal sequence; *SPA*, the truncated portion of Protein A; *m1*, the portion of the rat m1 receptor included in the construct; *ORI*, the origin of replication in *E. coli*; *AmpR*, the ampicillin resistance gene. The resultant plasmid p23-rm1-220/346 was utilized to transform competent HB101 cells for expression of the receptor-Protein A fusion protein.

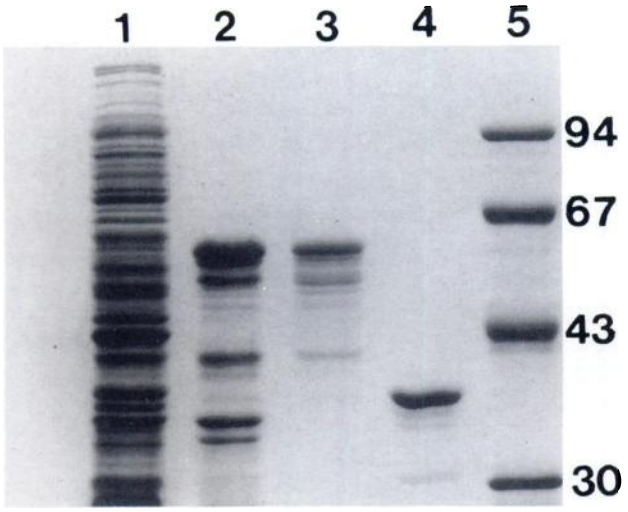

Fig. 2. SDS-PAGE of isolated purified fusion protein. *Lane 1*, the bacterial pellet corresponding to 25 ml of an overnight culture of HB101 transformed with p23-rm1-220/346 was solubilized in SDS/β-mercaptoethanol, loaded onto a 10% polyacrylamide gel, and subjected to SDS-PAGE, followed by staining with Coomassie blue. *Lane 2*, protein was bound to IgG-Sepharose Fast Flow columns and eluted with ammonium acetate (0.5 M, pH 3.3), as described in Materials and Methods. Three major proteins, presumably containing the Protein A moiety required for binding to the IgG-Sepharose column, are visible (M_r 53,000, 42,000, and 35,000), as are two minor proteins (M_r 51,000 and 33,000), presumably representing proteolysis products derived from the major band. *Lane 3*, purified rat m1 fusion protein. Proteins eluted from the IgG-Sepharose column were subjected to Sephacryl S-200 column chromatography to allow separation of fusion protein from proteolysis products. Fractions containing the major band at M_r 53,000, corresponding to m1 fusion protein, were pooled and utilized for injection into rabbits. *Lane 4*, truncated Protein A mother protein. HB101 bacteria were transformed with pRIT23, and the resulting protein was purified over IgG-Sepharose as in *lane 2*, resulting in a M_r 38,000 truncated portion of Protein A. Yields of protein from pRIT23 approximate those obtained with p23-rm1-220/346 (15–20 mg/liter). *Lane 5*, molecular weight standards.

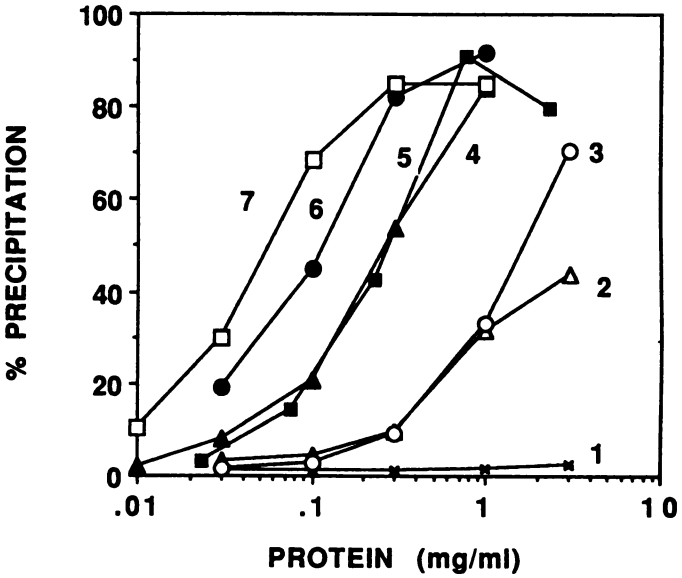

Fig. 3. Antibody titer of the first seven bleeds. Lyophilized antisera raised against the third intracellular loop of the m1 receptor were reconstituted in TE, as 5× stocks. Antibody (100 μl) was added to 1.5-ml microfuge tubes containing 100–140 fmol of solubilized m1 receptor from CHO cells, which had been labeled with [^{3}H]QNB as described. The final volume was 500 μl. Incubations were carried out for 40–55 hr at 4° before loading on Sephadex G-50 columns, incubation with Pansorbin, and immunoprecipitation, as detailed in Materials and Methods. Data are expressed as a percentage of total receptors added per tube that were precipitated. Similar titers developed in both rabbits. Data shown represent mean of triplicate determinations at each concentration for antiserum obtained from rabbit B47120. Nonspecific precipitation, assessed with control serum from rabbits injected with truncated Protein A isolated from pRIT23-transfected bacteria (Fig. 2, *lane 4*) or preimmune antiserum, was less than 2% and was subtracted.

bleed, such that at the seventh bleed the EC_{50} was approximately 50 μg/ml. This corresponds to an approximate 1:400 dilution of original serum. It is also important to note that by the fourth bleed, but not sooner, the antiserum was able to precipitate greater than 90% of the labeled receptors in the tube. Thus, it appears that the antisera generated can quantitatively immunoprecipitate m1 muscarinic receptors.

To determine optimal conditions for the precipitation assay, a number of parameters were varied and the effect on percentage of precipitation was measured. Experiments were carried out at 4° in order to minimize ligand off-rate and receptor proteolysis. It was determined that, for samples processed much before 45 hr of incubation with antiserum, less than maximal precipitation was attained (Fig. 4). Therefore, experiments were routinely carried out by incubating soluble receptors and antisera for 45 to 55 hr at 4°. The effect of altering the time of incubation with Pansorbin was also determined, and it was found that incubation times ranging from 15 to 180 min all gave the same percentage of precipitation (data not shown). In addition, precipitation was not significantly affected by varying assay volume between 0.15 and 0.5 ml (data not shown). To determine whether the amount of receptor added to the assay would influence the results, experiments were carried out in which the amount of solubilized labeled m1 muscarinic receptors per tube was varied from 35 to 140 fmol (1400–5600 cpm) (0.5 ml final volume). No effect of varying labeled receptors was found, and the precipitation typically ranged from 90 to 100% (data not shown). Thus, all experiments were carried out with m1 receptor concentrations in the range of 35–140 fmol/tube.

Pilot experiments indicated that tissue-specific differences in m1 receptor density would necessitate the addition of more total solubilized muscarinic receptors from hindbrain regions than forebrain regions. (i.e., 100–200 fmol or 4,000–8,000 added cpm/tube for cortex, hippocampus, olfactory tubercule, and striatum; 200–400 fmol or 8,000–16,000 added cpm/tube for thalamus/hypothalamus, cerebellum, and pons/medulla) to achieve the desired m1 receptor density (35–140 fmol/tube). To address the possibility that the presence of a tissue-specific factor or protease was masking true m1 receptor density in certain brain regions (e.g., cerebellum), unlabeled tissue from cortex, cerebellum, untransfected CHO cells, or m3 CHO cells was solubilized as described in Materials and Methods and incubated with m1 antibody and labeled solubilized m1 receptors (50 fmol of receptor, 20 μg of solubilized protein). For all tissues listed, and across a range of competing protein concentrations (3–80 μg/tube), precipitation of labeled m1 receptors was within 5% of control precipitation (data not shown). Thus, it appears likely that the quantity of m1 receptors found in the precipitate represents the actual amount of m1 receptors expressed in a given tissue.

To determine the selectivity of the antisera, membranes were prepared from five cell lines, each expressing a single defined subtype of muscarinic receptor (12). Receptors from each cell

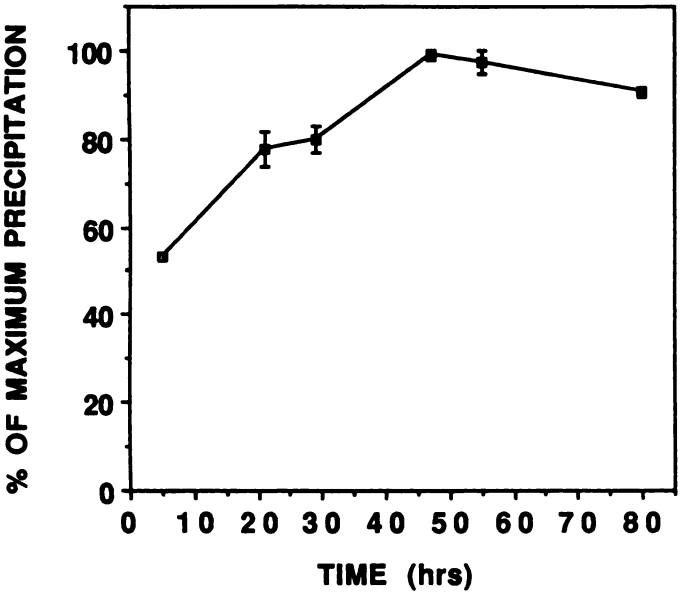

Fig. 4. Time course of immunoprecipitation. Two concentrations of antibody (0.1 and 0.3 mg/ml) were utilized for incubation with solubilized labeled m1 muscarinic receptor. Data obtained at each time point were normalized for maximum immunoprecipitation obtained at each concentration (28 and 77%, respectively). Times shown indicate the sum of the time required for primary incubation of receptor with antibody, subsequent passage of the mixture over Sephadex G-50 columns, and incubation with Pansorbin, with the latter two steps requiring approximately 3 hr. Data shown are the mean ± standard error of average precipitation efficiencies attained at each of the two antibody concentrations (triplicate determinations).

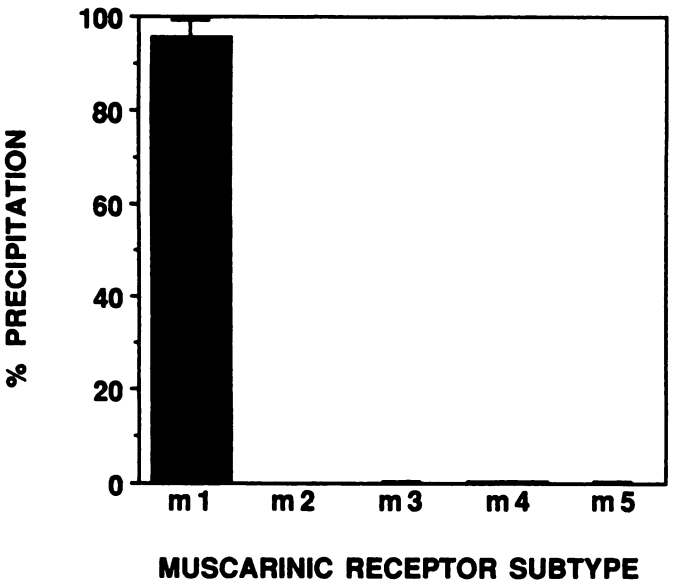

Fig. 5. Specificity of precipitation with m1 antibody. Antibody (0.75 mg/ml) was incubated for 46 hr at 4° with 100–135 fmol of [^{3}H]QNB-labeled muscarinic receptors, solubilized from transfected CHO cells, as described in Materials and Methods. Control antisera (see legend to Fig. 3) (0.75 mg/ml) were utilized in parallel precipitations for determination of nonspecific precipitation. Data shown represent mean ± standard deviation of triplicate determinations, with nonspecific precipitation (<2.0%) for each subtype subtracted. Precipitation of m2, m3, m4, and m5 receptors with m1 antibody did not exceed 1.0% of added receptor. In contrast, 96 ± 3% of added m1 receptors were precipitated.

line were labeled, solubilized, and precipitated as described. As can be seen in Fig. 5, [^{3}H]QNB binding sites from cells expressing m1 muscarinic receptors were quantitatively immunoprecipitated. However, [^{3}H]QNB binding sites from cells expressing either m2, m3, m4, or m5 receptors were not precipitated to any extent. Therefore, the antisera generated against the fusion protein appear to be both quantitative and selective, regarding immunoprecipitation of muscarinic receptor subtypes.

Determination of m1 receptor density in selected regions of rat brain. To determine the density of m1 muscarinic receptors, rat brains were dissected into seven sections, cortex, hippocampus, striatum, olfactory tubercule, thalamus/hypothalamus, pons/medulla, and cerebellum. Receptors were labeled with [^{3}H]QNB in membrane preparations and solubilized as described. The soluble receptors were incubated with anti-m1 serum, and the percentage of precipitation was determined for each preparation. As can be seen in Fig. 6, m1 receptors comprise a significant portion of the total density of muscarinic receptors in the forebrain (cortex, hippocampus, striatum, and olfactory tubercule), with the hippocampus having the highest percentage (47%). In contrast, the percentage of m1 receptors drops off markedly as one moves caudally in the brain, with the thalamus/hypothalamus expressing approximately 16% m1 receptors, pons/medulla expressing 4–6%, and m1 receptors in the cerebellum constituting less than 2% of the total receptor density. This gradient, in conjunction with the fact that the total density of muscarinic receptors decreases as one moves caudally in rat brain, results in extremely low densities of m1 receptors in the hindbrain (Fig. 6).

Other factors were taken into consideration when the calculated percentage of total receptor density represented by the m1 subtype was evaluated. In each experiment, a standard curve of precipitation efficiency versus a range of added m1 receptor (fmol/tube) was generated. The purpose was 2-fold, to assess the stability and/or potential variability in frozen antibody aliquots and to determine whether efficiency was independent of the amount of added receptor. In each experiment, uniform precipitation was seen when 35–140 fmol of m1 receptors/tube were added. In most experiments, precipitation was uniform to >200 fmol/tube (data not shown). For the three experiments shown in Fig. 6, using m1 CHO membranes, the precipitation efficiency of the antiserum was 94 ± 7% (mean ± standard error).

Discussion

This report demonstrates that antisera raised against a fusion protein corresponding to a unique portion of the rat m1 muscarinic receptor will quantitatively and selectively immunoprecipitate m1 muscarinic receptors. Thus, a reagent has been developed that can measure a genetically defined subtype of muscarinic receptor in tissues containing multiple subtypes. Before the introduction of molecular cloning techniques to the study of muscarinic receptors, convincing information regarding distribution and density of muscarinic receptor subtypes was derived from studies conducted with the selective antagonist pirenzepine and the nonselective antagonist [^{3}H]QNB (19–22). Regional differences in apparent tissue receptor densities, as defined by these two ligands, supported the proposition that at least two classes of receptors existed. M1 binding sites, defined as possessing high affinity for pirenzepine, were found to be distributed in forebrain, and M2 binding sites, defined as possessing low affinity for pirenzepine, predominated in the

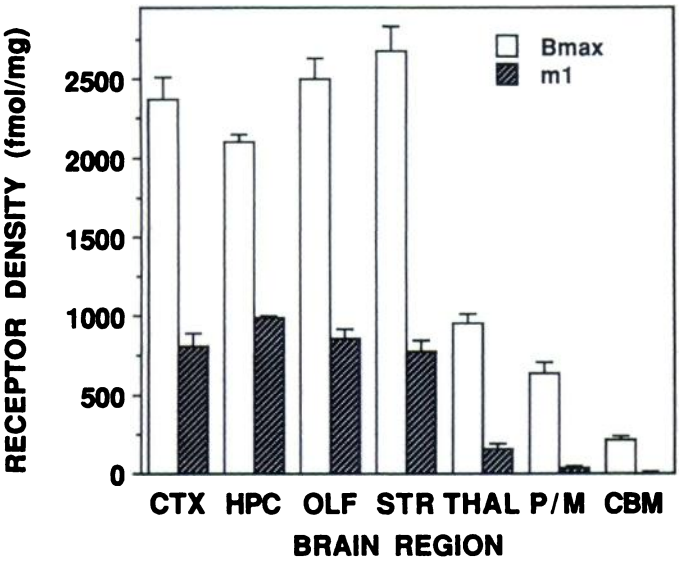

Fig. 6. Distribution and density of m1 muscarinic receptors in isolated regions of rat brain. Seven regions of rat brain were dissected and prepared as described in Materials and Methods. *CTX*, cortex; *HPC*, hippocampus; *OLF*, olfactory tubercule; *STR*, striatum; *THAL*, thalamus/hypothalamus; *P/M*, pons/medulla; *CBM*, cerebellum. Percentage of precipitation for each experiment was obtained from triplicate samples, with nonspecific precipitation (<2% of total) subtracted. Percentage of precipitation for the regions was cortex = 34%, hippocampus = 47%, olfactory tubercule = 35%, striatum = 29%, thalamus/hypothalamus = 16%, pons/medulla = 5%, and cerebellum = 2%. Total muscarinic receptor density (B_{max}) for each brain area was determined as described in Materials and Methods. The value for percentage of m1 receptor for a given region in each animal was multiplied by the corresponding B_{max} value for that animal (□), to give the m1 receptor density (▨). Data shown are the mean ± standard error of three experiments. In these experiments, the percentage of precipitation efficiency in m1 CHO cell membranes also was routinely determined. The mean ± standard error of these values was 94 ± 7%.

hindbrain. Additionally, functional studies provided evidence for distinct classes of muscarinic receptors. Pirenzepine was shown to potently block agonist-stimulated phosphoinositide turnover in rat forebrain, while weakly blocking agonist-mediated inhibition of forskolin-stimulated adenylate cyclase activity in heart (23). With the advent of cloning technology, elucidation of the properties of distinct muscarinic receptor subtypes has been made possible. Genetically defined m1 and m2 receptors were found to couple preferentially to effector systems mediating phosphoinositide turnover and cyclase inhibition, respectively (24). Binding studies using cells transfected with cDNA encoding individual muscarinic receptor subtypes revealed a range of affinities for pirenzepine (m1 > m4 = m5 > m3 > m2) (12). Together, these data indicated that m1 and m2 receptors fortuitously fell within the previously defined M1 and M2 classes of binding sites, respectively. It is, therefore, likely that a portion of the binding sites that have been defined as M1 include the m1 receptor, but it is unclear whether receptors expressing intermediate affinity for pirenzepine (m3, m4, and m5) fall into the M1 or the M2 class. Interpretations regarding subtype distributions are, thus, complicated by the tissue-dependent distribution of multiple subtypes of muscarinic receptors possessing differential affinity for radioligands. In the absence of selective ligands, studies on receptor distribution, receptor regulation, and identification of subtypes that are altered in disease states and aging are limited.

With the development of selective and quantitative m1 antisera, previous experiments examining the distribution of high affinity [^{3}H]pirenzepine binding sites can be reevaluated. M1 binding sites, estimated as the density of [^{3}H]pirenzepine binding sites, were reported to comprise 50–55% of cortical, striatal, and hippocampal receptors but less than 10% of muscarinic receptors in hindbrain regions (22). The values obtained from the immunoprecipitation experiments presented in Fig. 6 thus closely approach percentages defined with [^{3}H]pirenzepine. It should be noted, however, that the ratio of [^{3}H]pirenzepine to [^{3}H]QNB binding sites has been reported to vary with assay conditions (25), which is likely to be due to an alteration of the K_d values of [^{3}H]pirenzepine for some or all of the subtypes of muscarinic receptors. Thus, the density of [^{3}H]pirenzepine binding sites agrees with the immunoprecipitation data presented here only under certain conditions of [^{3}H]pirenzepine binding. In a recent study, a series of antagonists was used to generate competition curves against 1-[*N-methyl*-^{3}H]scopolamine in homogenates of rat forebrain (26). These data were used to estimate the percentages of four muscarinic receptor subtypes in three areas of rat brain. The proportions of m1 receptors calculated (cortex = 35%, hippocampus = 55%, and striatum = 27%) are also in close agreement with the values presented in Fig. 6 (cortex = 34%, hippocampus = 47%, and striatum = 29%).

In situ hybridization histochemistry analysis of rat brain with an oligonucleotide corresponding to the sequence of the m1 cDNA was reported to show a diffuse distribution of m1 mRNA in cortex and striatum, a particularly strong signal in the hippocampal formation and olfactory tubercule, and a lack of signal in thalamic nuclei and hindbrain structures (11). As shown here, prevalence of m1 immunoreactivity in forebrain structures appears to coincide well with the distribution of mRNA.

Although the immunoprecipitation assay we have developed here is useful in terms of quantifying m1 receptors in various tissues, the sensitivity of the assay is such that at least 10 fmol of receptor/tube are required for accurate determinations. Typically, immunocytochemical or Western blot analyses are more sensitive than this and, additionally, with immunocytochemistry one can address questions at the cellular level. However, at present, our attempts at specific immunocytochemical staining or Western blot analysis using either cultures of transfected m1 CHO cells or rat brain have not been successful.

It is important to note that muscarinic receptors are particularly amenable to study using the immunoprecipitation assay described here, because the radioligand used, [^{3}H]QNB, has a slow rate of dissociation. This is important given the time required (i.e., 45 hr) to achieve quantitative immunoprecipitation. The avidity with which [^{3}H]QNB binds to muscarinic receptors (K_d = 5–15 pM) contributes to high signal to noise ratios in these experiments. However, it was necessary to remove free ligand with several washes of membrane pellet and passage of the receptor-antibody complex over a G-50 column before addition of Pansorbin, because our data indicate that Pansorbin avidly binds free [^{3}H]QNB. As a consequence, if experiments are carried out in which the free ligand is not removed before the addition of Pansorbin, background levels increase significantly. This observation could have an impact on the general applicability of this method for other receptors, if the radioligands available do not have the extremely slow

dissociation rate of [^{3}H]QNB and if the ligand binds to Pansorbin. If free ligand has a more rapid rate of dissociation but does not bind significantly to Pansorbin or Protein A-agarose, then incubations can be carried out in the presence of excess free ligand to maintain receptor saturation. Thus, the two basic requirements for the methods outlined in this report are 1) that nonselective, high affinity ligands that are specific for the receptor family exist and 2) that the proteins possess a unique domain that can be targeted for use as an antigen.

In summary, a portion of the cDNA encoding the rat m1 muscarinic receptor was cloned and used to express a fusion protein with which m1-selective antibodies were raised. These antibodies were used to evaluate, for the first time, the distribution and density of a specific subtype of muscarinic receptor. This approach should be applicable to a large number of receptor subtypes whose cDNA has been cloned.

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