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Distribution of m2 Muscarinic Receptors in Rat Brain Using Antisera Selective for m2 Receptors

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SUMMARY

The DNA fragment encoding the third intracellular loop of rat m2 muscarinic receptor was fused to the gene for staphylococcal Protein A. The resultant fusion protein, expressed in bacteria, was purified via IgG affinity chromatography and used as an antigen to raise a polyclonal antiserum. Chinese hamster ovary cells transfected with cDNA coding for a single muscarinic receptor subtype were used as tissue sources to screen antisera. The antiserum was shown to immunoprecipitate quantitatively (>90%) m2 receptors but not to precipitate m1, m3, m4, or m5 receptors. Additionally, immunoprecipitation by m2 antiserum could be inhibited by protein containing the third intracellular loop of the m2 receptor. This selective m2 antiserum was then used to study the distribution and density of m2 receptors in rat brain and heart. In agreement with previous studies, m2 receptors were found to be abundant in heart and comprise at least 92% of the total muscarinic receptor density. Hindbrain, brain stem, and midbrain regions such as cerebellum (75%), pons/medulla (70%), and thalamus/hypothalamus (43%) are also enriched in m2 receptors. In contrast, forebrain regions contain markedly lower percentages of m2 receptors, with cortex expressing 20%, hippocampus 19%, striatum 12%, and olfactory tubercle 20% of the total receptor density. Although the density of m2 receptors expressed as a percentage of total varied considerably from brain region to brain region, the absolute density of these receptors appeared relatively uniform throughout the brain. This study demonstrates that a gene fusion system can be used for efficient antibody production. The use of similar fusion protein antisera directed against other subtypes of muscarinic receptors should prove useful in future studies on regulation, function, and structure of muscarinic receptors.

The existence of muscarinic receptor subtypes has been postulated on the basis that the antagonist PZ exhibits high affinity for receptors (M1) in certain regions of the brain and low affinity for receptors (M2) in the heart (1). These two classes of receptor subtype appeared to mediate different biochemical effector systems (2). It became clear with the introduction of the selective antagonist AF-DX-116 that the M2 class of receptors should be subdivided into two categories, and the M3 class of receptors was defined as having low affinity for AF-DX-116 (3). However, it was not clear at the time whether these observations represented modifications of the same receptor or whether multiple proteins exist. Molecular cloning of the porcine cerebral (m1) and cardiac (m2) muscarinic receptors established that the receptor subtypes of brain and heart are indeed two different proteins encoded by distinct genes (4, 5). Further studies demonstrated that three more genes code for muscarinic receptors (6, 7). As a result, there are at least five muscarinic receptor subtypes (m1–m5) identified that have distinct primary structures, patterns of tissue-specific expression of mRNA, and ligand binding properties (7–10). For clarity, in this paper the terms M1 and M2 refer to pharmacologically defined subtypes, whereas the terms m1–m5 refer to genetically defined subtypes of muscarinic receptors.

From binding studies, it is now known that PZ, AF-DX116, and hexahydrosiladifenidol have highest affinity for m1, m2, and m3 receptors, respectively. Yet no antagonist exhibits greater than 5-fold selectivity for one subtype over all other subtypes and no antagonist examined to date is selective for m4 or m5 receptors (10). Thus, it is clear that current pharmacological tools are inadequate to selectively distinguish one subtype in the presence of the others. Oligonucleotide probes corresponding to the amino-terminal sequence of the muscarinic receptors have been constructed to localize the muscarinic receptor mRNA in the rat brain. Nevertheless, in some systems,

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ABBREVIATIONS: PZ, pirenzepine; AF-DX116, 11-[(2-[(dimethylamino)methyl]-1-piperidinyl)acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4] benzodiazepine-6-one; QNB, quinuclidinyl benzilate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; m2i3, the third intracellular loop of the m2 receptor subtype; B_{max}, maximum binding; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; CHO, Chinese hamster ovary; NMS, *N*-methylscopolamine; G protein, GTP-binding protein.

it has been shown that the existence of the message (mRNA) does not necessarily correlate with the expression of the protein itself (11). It was, therefore, the goal of this study to develop antibodies selective for the m2 receptor subtype and to utilize these antibodies to examine the distribution of m2 receptors in tissues that contain multiple subtypes.

The strategy used was to generate fusion proteins by subcloning of the DNA fragment coding for the third intracellular loop that is unique to the m2 receptor subtype into the vector containing the gene for staphylococcal Protein A (12). The resultant fusion proteins, expressed in bacteria, were purified with an IgG-Sepharose affinity column and used to raise antibodies in rabbits. We report here the successful production of antibodies against m2i3 and the utilization of these antibodies to determine quantitatively the distribution of m2 receptors in rat brain and heart.

Experimental Procedures

Materials. Linkers were obtained from OCS Laboratories. Restriction enzymes and other enzymes used in cloning reactions were obtained from New England Biolabs or Bethesda Research Laboratories. Vectors pRIT 23 and pET-3b were generous gifts of Dr. Mathias Uhlen (The Royal Institute of Technology, Stockholm, Sweden) and Dr. William Studier (State University of New York, Buffalo), respectively. The full length clone of the rat m2 receptor was obtained as a gift from Dr. Craig Venter (National Institute of Neurological Disease and Stroke). The fragment encoding m2i3 was subcloned into pRIT 23 and pET-3b to generate plasmids p23-rm2/226-380 and p3b-rm2/226-380, respectively, by using standard techniques (13).

Purification of expressed proteins. *Escherichia coli* HB101 transformed with plasmid p23-rm2/226-380 were grown in 5 ml of medium (Luria broth supplemented with 0.1 mg/ml glucose and 0.25 mg/ml ampicillin) at 32°, at 300 rpm, for 4–6 hr. Bacteria were pelleted at 1200 × *g* for 10 min, resuspended in 300 ml of medium, and grown overnight at 32° at 300 rpm. The 300-ml culture was centrifuged at 1200 × *g* for 10 min, and the bacterial pellet was used to seed a 1500-ml culture, which was grown at 37° for 3 hr at 300 rpm. Proteins expressed in *E. coli* were released from the periplasmic space of the cells by osmotic shock, as described by Nilsson and Abrahmsen (12). Protein A-m2 fusion proteins were purified using a 10-ml IgG-Sepharose Fast Flow column (Pharmacia). After washing, fusion proteins were eluted with ammonium acetate (0.5 M, pH 3.3), lyophilized, and resuspended in H_2O. An aliquot was removed for SDS-PAGE and protein determination. The remaining eluted proteins were loaded onto a 100-ml Sephacryl S-200 column (Pharmacia) that had been preequilibrated with ammonium bicarbonate (100 mM, pH 7.9). An aliquot (12 μl) of each fraction (1.2 ml) was removed for SDS-PAGE. Fractions identified as containing fusion proteins were pooled and lyophilized.

One liter of M9ZB medium was inoculated with *E. coli* BL21 (DE3) harboring plasmids p3b-rm2/226-380 and pLysS and was incubated at 37° to an A_{600} of 0.6 O.D. units (14). Expression of m2i3 protein was induced by the addition of IPTG (1 mM). The incubation was continued for another 3 hr. The cells were harvested by centrifugation at 1200 × *g* for 20 min, and the resulting bacterial pellet was resuspended in buffer (50 mM Tris, 2 mM EDTA, pH 8) and frozen at −20°. The bacterial suspension was thawed, to lyse the cells, and sonicated. The bacterial slurry was centrifuged at 10,000 × *g* for 20 min, and the supernatant was dialyzed and lyophilized. To isolate m2i3 protein from other bacterial proteins, lyophilized protein was dissolved in 10 mM Tris (pH 8.0), 2% ampholyte (pH 3–10; Bio-Lyte; Bio-Rad), and loaded onto an isoelectrical focusing apparatus (Rotofor; Bio-Rad). An aliquot (12 μl) of each fraction (1–3 ml) was removed for SDS-PAGE. Fractions containing induced m2i3 protein were pooled, dialyzed against 1 M NaCl to remove ampholyte and then against 100 mM ammonium bicarbonate, and lyophilized.

Antisera preparation. Two rabbits (New Zealand females, 2 kg) were each injected (10–15 sites, subcutaneously) with approximately 0.5–1.5 mg of purified Protein A-m2 fusion proteins suspended in 1 ml of H_2O, mixed with an equal volume of Freund's complete (first injection) or Freund's incomplete (booster injections) adjuvant. Booster injections were done at 1-month intervals. The first bleed was collected 2 weeks after the first boost. Subsequent bleeds were collected 12–14 days after injection. Blood was allowed to clot at room temperature, followed by centrifugation at 10,000 × *g* for 15 min at 4°. Antibodies were partially purified from serum by precipitation with a 50% solution of ammonium sulfate at 4° for 30 min, followed by centrifugation at 10,000 × *g* for 20 min at 4°. Pellets were resuspended in buffer (10 mM $NaPO_4$, pH 7.4) and the solution was dialyzed (10-kDa cutoff) against phosphate buffer (10 mM, pH 7.4) 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 preparation. Pansorbin (Calbiochem) was centrifuged 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 washed in Tris-buffered saline (50 mM Tris, pH 7.4, 500 mM NaCl) five times. The final Pansorbin suspension in Tris-buffered saline was identical to the starting volume. The Pansorbin slurry was stored at 4° and used within 2 days, although IgG binding activity appears to be stable for at least 1 week.

Membrane preparation. CHO cells transfected with cDNA encoding m1 and m2 receptors were a generous gift of Dr. Daniel Capon (Genetech). CHO cells transfected with cDNA encoding m3, m4, or m5 subtypes of muscarinic receptors were generously provided by Dr. Mark Brann (National Institutes of Health). Cells were harvested in ice-cold TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA). Membranes were homogenized (Tekmar Tissuemizer, setting 6, 7 sec) and centrifuged for 15 min at 30,000 × *g* at 4°. Membranes were resuspended in TE buffer, aliquoted into microfuge tubes, and stored at −70°. Protein in stored samples was determined by the method of Lowry *et al.* (15). Brain regions (cortex, hippocampus, striatum, olfactory tubercle, thalamus, pons/medulla, and cerebellum) and hearts were dissected on ice from adult male rats (Sprague-Dawley, 200 g), weighed, and immediately frozen at −70°. At the time of experiment, tissues were thawed on ice and transferred to TE 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 30,000 × *g* for 15 min at 4°, and the membrane pellet was resuspended in ice-cold TE buffer. To determine receptor density, 100–250 mg of membrane protein were incubated with [^{3}H]QNB (1 nM) for 30 min at 32° in TE buffer (5 ml final volume). Nonspecific binding was determined in the presence of atropine (5 μM). Ice-cold wash buffer (5 ml of 10 mM Tris, 0.9% NaCl, pH 7.4) was added to each tube, followed by filtration through glass fiber filters (Schleicher and Schuell no. 30). Filters were washed with 2 × 5 ml of ice-cold wash buffer, dried, and transferred to scintillation vials. Protein content was determined by the method of Lowry *et al.* (15).

Receptor labeling and solubilization. Membranes were incubated with [^{3}H]QNB (1.5 nM) at 32° for 20 min, in the presence of a protease inhibitor cocktail (1 μg/ml leupeptin, 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), followed by centrifugation at 30,000 × *g* for 15 min at 4°. The supernatant was discarded, and the pellet was washed twice in ice-cold TE/protease inhibitor cocktail buffer. For receptor solubilization, a volume of ice-cold TEDC buffer (1% digitonin, 0.2% cholic acid, 10 mM Tris, pH 7.4, 1 mM EDTA) 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, 3 sec) and transferred to an

ice bath for 30 min. 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. Antisera were reconstituted in TE buffer 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 40–55 hr. At the end of incubation, samples were loaded onto Sephadex G-50 columns (3 ml) that had been preequilibrated with column buffer (0.1% digitonin, 0.02% cholic acid, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.9% NaCl). After the sample entered the resin, 0.9 ml of column buffer was applied, followed by a 1.6-ml aliquot of column buffer. The 1.6-ml fraction was collected and was found to elute approximately 90% 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 (1.2 ml) was incubated with Pansorbin for 2 hr at 4°, in 1.5-ml microfuge tubes, with constant rotation at 30 rpm. 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 complex, were surface washed with 1 ml of ice-cold TE buffer. The wash was discarded, and the pellet was centrifuged. Wash volume remaining above the pellet was removed. The pellet was incubated with 0.2 ml of NaOH/deoxycholate (0.1 N NaOH, 3% sodium deoxycholate) for 30 min at room temperature and resuspended with 0.3 ml of H_2O, and the radioactivity was quantified. NaOH/deoxycholate (0.2 ml) was added to fraction B (0.3 ml of the 1.6-ml eluate), and the radioactivity was quantified. The 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 antisera from animals injected with truncated Protein A (i.e., Protein A isolated from *E. coli* transformed with expression vector pRIT 23) represented 0.5–2% 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 <2% of the counts found in samples labeled in the absence of atropine. It should be noted that, although two rabbits were immunized, only one rabbit produced antibodies detected by our assay.

Results

Expression and purification of fusion proteins. The construction of the Protein A gene fusion vector (pRIT 23) used in this study has been described earlier (12). Briefly, this vector contains the coding sequence for the IgG binding domains of bacterial Protein A under control of the staphylococcal Protein A promoter. It also contains a signal sequence to allow expressed proteins to be secreted through the cell membrane into the periplasmic space of Gram-negative bacteria.

The full length clone of the rat m2 receptor (16) was digested with the restriction enzymes *BalI* and *SmaI* simultaneously, to generate a 463-base pair blunt-ended fragment that included the coding region from Ala-226 to Arg-381 (m2i3). The first 20 amino acids of m2i3 were avoided, because they are conserved between m2 and m4 receptors. A synthetic linker (5′-CGGAATTCCG) was synthesized and ligated to the m2i3 DNA fragment to add *Eco*RI sites and to put the m2i3 DNA fragment in frame with the Protein A sequence. This recombinant plasmid was partially sequenced to verify that the fragment was in the correct orientation and reading frame. The resulting plasmid is shown diagrammatically in Fig. 1.

Fusion proteins were expressed in *E. coli* and purified using an IgG-Sepharose affinity column, as described in Experimental Procedures (12). The products were then analyzed by SDS-

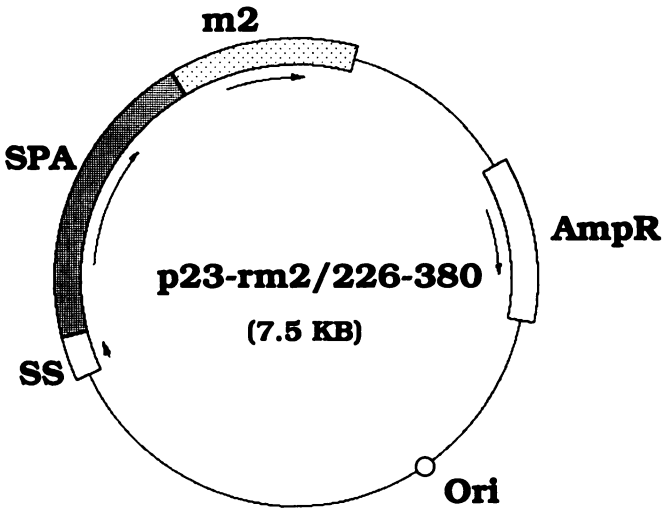


Fig. 1. Schematic representation of the plasmid constructed for expression of protein A-m2 fusion proteins in *E. coli*. The m2i3 DNA fragment was inserted into the unique *Eco*RI cloning site of pRIT 23. *SS*, Signal sequence for Protein A; *SPA*, gene encoding staphylococcal Protein A; *m2*, m2i3 DNA fragment; *AmpR*, gene encoding β-lactamase; *Ori*, origin of replication in *E. coli*.

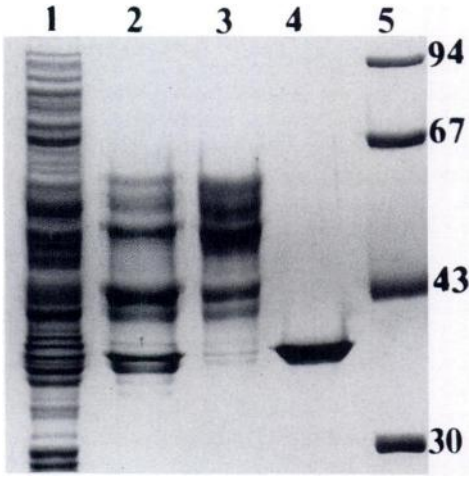


Fig. 2. SDS-PAGE (12.5%) of Protein A-m2 fusion proteins. *Lane 1*, crude proteins from periplasmic lysates of *E. coli*; *lane 2*, fusion proteins purified with IgG-Sepharose affinity chromatography; *lane 3*, fusion proteins further purified with S200 size-exclusion chromatography; *lane 4*, Protein A expressed by the gene fusion vector pRIT23; *lane 5*, standard proteins, with the molecular size shown in kDa.

PAGE (Fig. 2). The most slowly migrating band corresponds to a full-length fusion product having the anticipated molecular size of 58 kDa (Fig. 2, *lane 2*). Two major faster migrating bands (53 and 43 kDa) probably represent breakdown products of the full-length fusion protein. The most quickly migrating band (39 kDa) is presumably truncated Protein A itself. To remove the truncated proteins and to enrich Protein A-m2 fusion proteins, the IgG purified proteins were further purified using a Sephacryl S200 size-exclusion column (Fig. 2, *lane 3*). The overall yield of the fusion products after IgG and S200 column purification was 30 mg/liter of original culture, which provided sufficient material for immunization.

Production and characterization of m2 antisera. To immunize rabbits, 0.5–1.5 mg of Protein A-m2 fusion proteins were used for multiple-site injections at monthly intervals. Sera were collected approximately 2 weeks after each boost, precip-

itated using $(NH_4)_2SO_4$, lyophilized, and stored as described above.

Immunoprecipitation of radiolabeled and solubilized m2 receptors was used as a method to screen antisera. Membranes prepared from CHO cells transfected with cDNA coding for the m2 receptor were incubated with a saturating concentration of [^{3}H]QNB. Labeled receptors were then solubilized and incubated with various amounts of m2 antisera, in 0.5-ml volume, at 4° for at least 40 hr, the minimum time required for antisera to reach equilibrium (data not shown).

As can be seen in Fig. 3, no detectable precipitation was measured for the first four bleeds. But all subsequent bleeds were able to immunoprecipitate m2 receptors. The delay of antibody production is probably due to the use of a lower dose of antigen (0.5 mg) to immunize rabbits for the first four bleeds. After the injection dose was increased to 1–1.5 mg, titers of the m2 antisera improved with each bleed. It is worth pointing out that the antisera were able to precipitate >90% of labeled m2 receptors by the seventh bleed. Thus, the m2 antisera generated appear to quantitatively immunoprecipitate the m2 receptor subtype.

An experiment often used to demonstrate specificity of an antiserum is preincubation of antigen with antibody to show the blocking effect of antigen. However, the antigen we used to immunize rabbits contains a portion of Protein A, which has high affinity for the Fc portion of IgG, and blockade of immunoprecipitation by this protein may not be a convincing control. Thus, we constructed another plasmid using the expression vector pET-3b (14). This vector contains a bacteriophage T7 promoter for T7 RNA polymerase, a fragment encoding the first 11 amino acids of the gene 10 protein (the major capsid protein of T7), and transcription and translation termination signals. The plasmid p23-rm2/226-380 was digested with *Eco*RI to regenerate m2i3 DNA. A linker (5′-AATTCGGATCCG) was synthesized and ligated to the m2i3 DNA to convert *Eco*RI sites into BamHI sites and to put m2i3 DNA in frame with the T7 gene 10 protein initiation site. The m2i3 DNA fragment was inserted into the unique *Bam*HI cloning site of pET-3b vector. The resultant plasmid was partially sequenced to verify that the orientation and reading frame were correct. The final construction of the plasmid (p3b-rm2/226-380) is shown schematically in Fig. 4A.

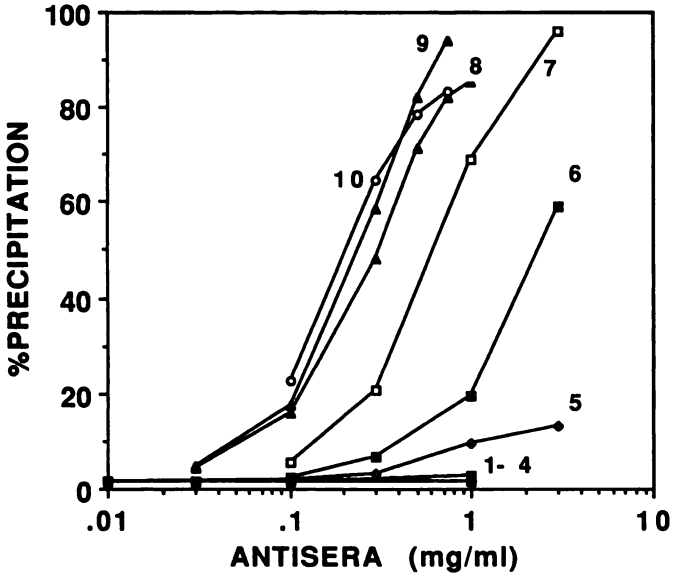


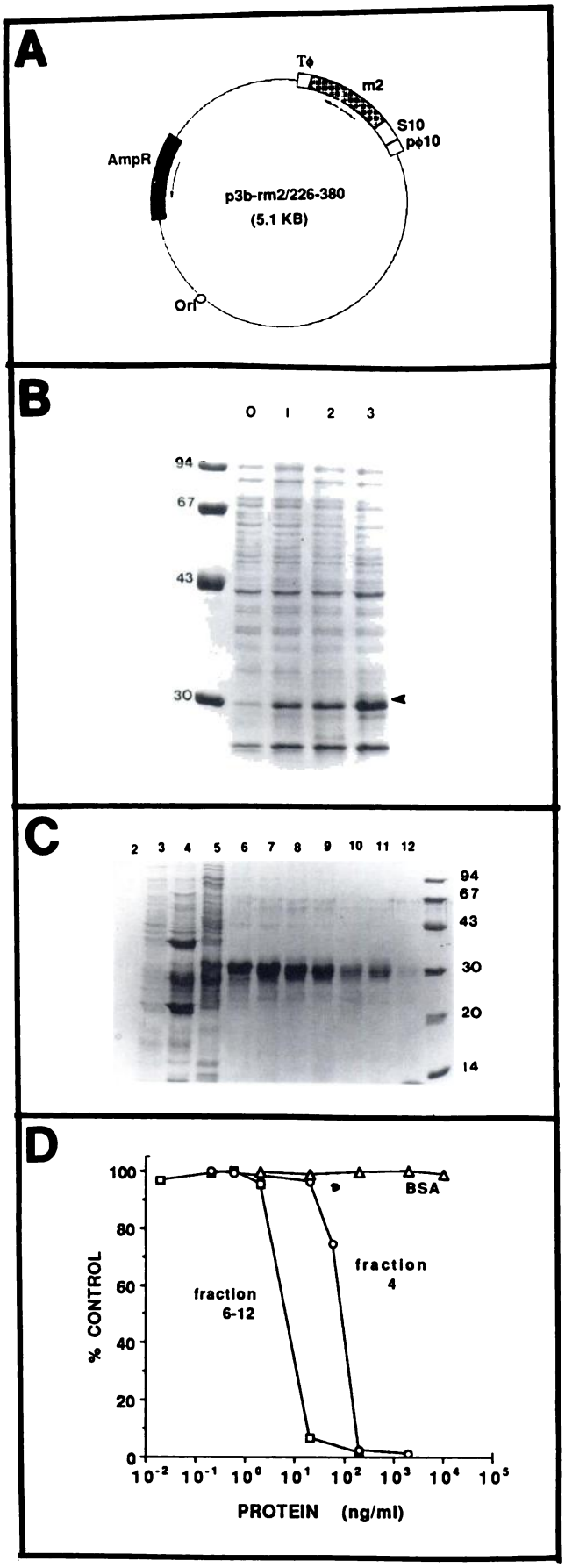
Fig. 3. Titer curves of m2 antisera obtained from different bleeds. Rabbits were injected with 0.5–1.5 mg of purified fusion proteins once every month and were bled 14 days after each injection, to collect sera. Membranes prepared from m2 CHO cells were used to screen the ability of m2 antisera to immunoprecipitate m2 receptors. The final concentration of lyophilized antisera is indicated on the *abscissa*. The percentage of total m2 receptors added that were precipitated is indicated on the *ordinate*. Data shown represent the average of triplicate determinations at each concentration. Nonspecific precipitation, assessed with antisera obtained from rabbits injected with truncated Protein A (Fig. 2, *lane 4*), was <2% of total and was subtracted. *Numbers*, order of different bleeds.

To express m2i3 protein, *E. coli* BL21 (DE3) containing pLysS was transformed with p3b-rm2/226-380. BL21 (DE3) has a copy of the gene encoding T7 polymerase under control of the IPTG-inducible *lac* UV5 promoter. Expression was done essentially as described by Studier and Moffatt (17). As shown in Fig. 4B, m2i3 protein running at 30 kDa accumulated upon IPTG induction and was found in the soluble fraction of bacterial lysates. To purify the induced m2i3 protein on a large scale, 1 liter of transformed BL21 (DE3) bacteria was grown in M9ZB medium, induced by addition of IPTG (1 mM) for 3 hr, and processed as described in Experimental Procedures. The m2i3 protein was separated from other bacterial proteins utilizing isoelectric focusing. The soluble fraction from the lysed bacteria was separated using a Rotofor (Bio-Rad) apparatus. The protein profile of collected fractions (fractions 2–12) is shown in Fig. 4C (fractions not shown contained no protein). Most other bacterial proteins eluted in fractions 2–5 (pH 3–6), whereas m2i3 protein eluted mainly between fraction 6 and fraction 12 (pH 6.5–7.5). These latter fractions were, therefore, pooled and used in blocking experiments.

The m2 antisera were preincubated with various amounts of protein, either collected fraction 4, pooled fractions 6–12 (m2i3 protein), or BSA, for 1 hr, followed by standard immunoprecipitation protocol. As shown in Fig. 4D, m2i3 protein was able to block totally immunoprecipitation of m2 receptors by m2 antisera at 20 ng/ml. Fraction 4, which still contained some m2i3 protein (Fig. 4C), also affected immunoprecipitation but was approximately 10-fold less potent. In contrast, BSA did not have any effect on the ability of m2 antisera to immunoprecipitate m2 receptors, even when experiments were carried out in the presence of 10 μg/ml BSA.

To verify further the specificity of m2 antisera, membranes from five CHO cell lines, each expressing one receptor subtype,

Fig. 4. Preabsorption of antisera with m2i3 protein. A, m2i3 DNA was inserted into the unique *Bam*HI cloning site of the pET-3b vector. The T7 promoter (*pϕ10*), β-lactamase (*AmpR*), the origin of replication (*Ori*) of the plasmid, gene 10 (S10), and the termination signal (*Tϕ*) are indicated. B, Time course of expression of m2i3 protein. BL21 (DE3) containing both pLysS and the plasmid p3b-rm2/226-380 were grown in M9ZB medium in the presence of chloramphenicol (25 μg/ml) and ampicillin (200 μg/ml). Cultures were induced by 1 mM IPTG when the A_{600} reached about 0.6 O.D. units. Aliquots (50 μl) were removed before (0) and 1, 2, and 3 h after induction (noted above each lane). *Arrowhead*, position of the induced protein. *Left lane*, standard proteins, with sizes (kDa) indicated. C, Protein profile for the soluble fraction of bacterial lysate after isoelectric focusing purification using a Rotofor apparatus. Aliquots (12 μl) from fractions 2–12 (from *left* to *right*) are shown. Fraction sizes varied from 1 to 3 ml. *Right lane*, standard proteins, with sizes (kDa) indicated. D, Inhibition of immunoprecipitation by m2i3 protein. m2 antisera were preincubated with various amounts of competing proteins (△, BSA; ○, proteins in fraction 4; □, m2i3 protein pooled from fractions 6–12). Samples were processed for immunoprecipitation as described in Experimental Procedures.



were prepared. Receptors were labeled with [^{3}H]QNB, solubilized, and incubated with m2 antisera (0.75 mg/ml), as described. As shown in Fig. 5, antisera were able to immunoprecipitate m2 receptor quantitatively (90%), with no significant precipitation of m1, m3, m4, or m5 receptors. Thus, antisera generated against Protein A-m2 fusion proteins were highly selective and quantitative for immunoprecipitating the m2 receptor subtype.

Determination of m2 receptor distribution in rat brain and heart. Rat brains were dissected into seven regions, as follows: cortex, hippocampus, striatum, olfactory tubercle, thalamus/hypothalamus, pons/medulla, and cerebellum. Membranes prepared from each brain region and rat heart (ventricle and atria) were labeled with [^{3}H]QNB, solubilized, and incubated with m2 antisera, as described in Experimental Procedures. As expected, m2 receptors were abundant in the heart and comprised almost all the receptor density (92%) (Fig. 6A). In the brain, m2 receptors predominated in pons/medulla (70%) and cerebellum (75%), with significant but lower percentages present in thalamus/hypothalamus (42%). In contrast, m2 receptors as a fraction of the total muscarinic receptors decreased dramatically in the forebrain, with cortex expressing approximately 19%, hippocampus 17%, striatum 12%, and olfactory tubercle 19% of the receptor density. Because the total density of muscarinic receptors is higher in the forebrain region but lower in the hindbrain region, the absolute density of m2 receptors was distributed relatively uniformly throughout the brain (150–350 fmol/mg) (Fig. 6B).

A standard curve of precipitation efficiency versus labeled m2 receptor added (fmol/tube) was also determined in parallel experiments. The purpose was to show that efficiency of immunoprecipitation was independent of the amount of added receptor. Consistent precipitation was seen when 25–200 fmol

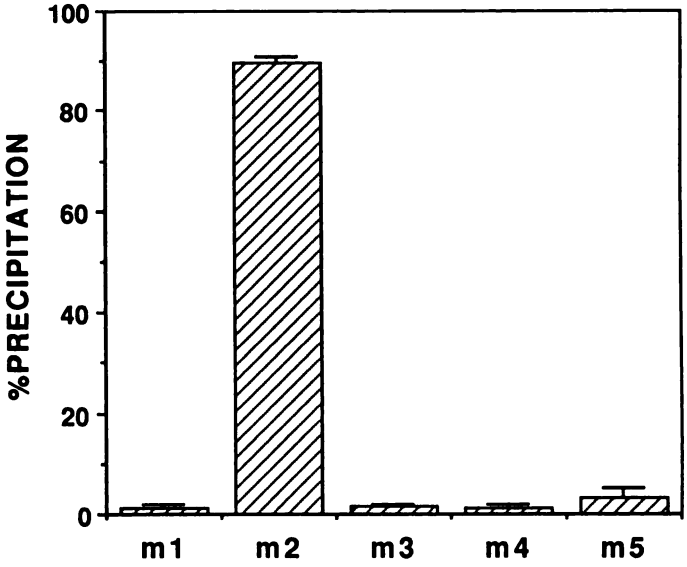


Fig. 5. Specificity of m2 antisera. Membranes prepared from m1–m5 CHO cells were labeled, solubilized, and incubated with m2 antisera (0.75 mg/ml), as described in Experimental Procedures. Control antisera (see legend to Fig. 3) were used in parallel to determine nonspecific precipitation. Data are shown as average percentage of precipitation ± standard error of three separate experiments (bleeds), with nonspecific precipitation (<2%) for each subtype subtracted. The values of average percentage of precipitation for each receptor subtype are: m1, 1.4 ± 0.6%; m2, 89.7 ± 1%; m3, 1.4 ± 0.7%; m4, 1.3 ± 0.6%; m5, 3.1 ± 1.9%.

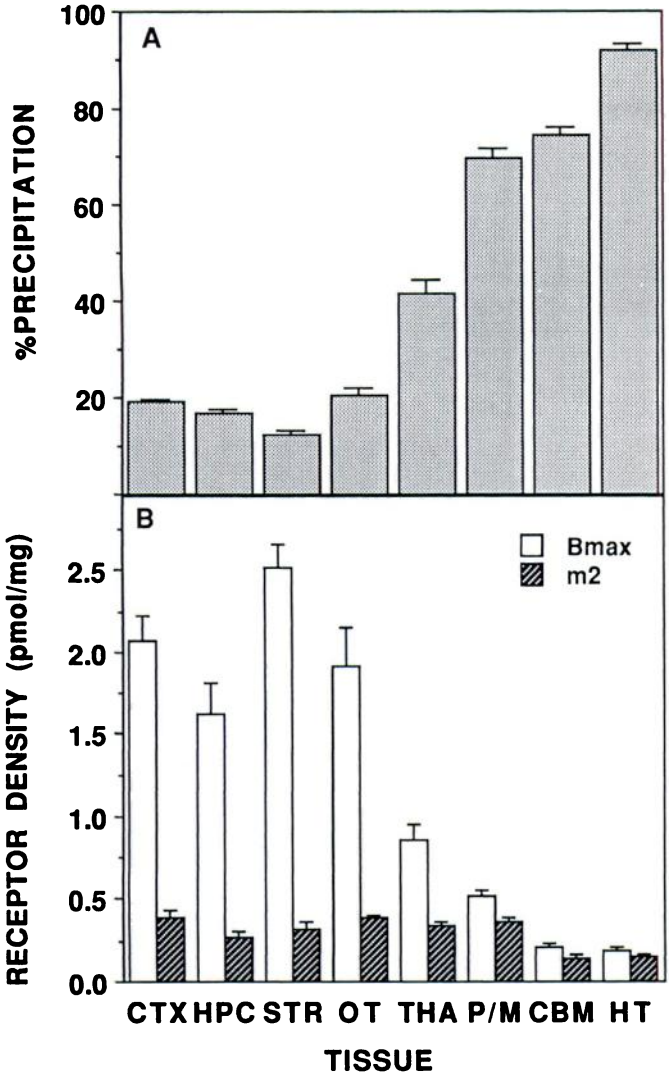


Fig. 6. Distribution and density of m2 receptors in rat heart and brain. A, Membranes prepared from rat heart (*HT*) and seven regions of rat brain (*CTX*, cortex; *HPC*, hippocampus; *STR*, striatum; *OT*, olfactory tubercle; *THA*, thalamus/hypothalamus; *P/M*, pons/medulla; *CBM*, cerebellum) were labeled, solubilized, and incubated with m2 antisera (0.75 mg/ml), as described in Experimental Procedures. Data are shown as average percentage of precipitation ± standard error of five separate experiments. The values of average percentage of precipitation for each brain region and heart are given in the text. B, Aliquots of membranes from rat heart and brain used in A were used to determine the B_{max} (pmol/mg of membrane protein), using a saturating concentration of [^{3}H]QNB (1 nM). The B_{max} values (□) obtained for each tissue are: cortex, 2.07; hippocampus, 1.62; striatum, 2.51; olfactory tubercle, 1.92; thalamus/hypothalamus, 0.86; pons/medulla, 0.52; cerebellum, 0.21; heart, 0.19 pmol/mg. The value for percentage of m2 receptors (A) for a given region in each animal was multiplied by the corresponding B_{max} value (□) for that animal to obtain the m2 receptor density (▨). Data shown are the mean ± standard error of five experiments.

of m2 receptors were added per tube (data not shown). These values encompass the range of m2 receptor concentrations utilized in the experiments shown in Fig. 6.

Discussion

The pharmacology and binding properties of muscarinic receptor subtypes have been studied extensively. M1 receptors, characterized as exhibiting high affinity for PZ, in brain mediate phosphoinositide breakdown, whereas M2 receptors, characterized as possessing low affinity for PZ, in heart mediate inhibition of adenylyl cyclase (2). Recent molecular cloning studies have identified the existence of a family of muscarinic receptor genes (m1–m5) (6–8). Several cell lines that lack endogenous muscarinic receptors, such as A9 L cells and CHO cells, were used to express each individual receptor subtype, to study their binding and functional properties (10, 18, 19). It is now known that m1 and m4 receptors have high affinities for PZ, whereas others have intermediate (m3 and m5) or low (m2) affinities for PZ (10). Functionally, it was found that m1, m3, and m5 receptor subtypes couple to stimulation of phosphoinositide breakdown and m2 and m4 receptor subtypes couple to inhibition of adenylyl cyclase (20). Therefore, M1 and M2 receptors, previously defined pharmacologically and biochemically, are each likely to represent more than one genetically defined receptor subtype.

Numerous workers have tried to examine the distribution of muscarinic receptors using radioligand binding studies and *in vitro* autoradiography. Thus, M2 receptors estimated as low affinity PZ binding sites were observed in tissues such as heart, cerebellum, and pons/medulla (21, 22). Using quantitative autoradiography, Cortes and Palacios (23) further measured the density of muscarinic receptors using [^{3}H]NMS and examined the inhibition of [^{3}H]NMS binding by PZ in the rat brain. Low affinity sites for PZ, when expressed as a percentage of total [^{3}H]NMS binding sites, were found mainly in the brain stem and parts of the thalamus. With the development of quantitative and selective m2 antisera, previous studies examining M2 binding sites were reevaluated.

It was found that m2 receptors comprise almost all the muscarinic receptors in heart. This correlates well with results using a monoclonal antibody raised against muscarinic receptors purified from porcine heart (24). In support of binding and autoradiography studies, we found that thalamus/hypothalamus, pons/medulla, and cerebellum expressed a predominant m2 receptor population. This is consistent with the idea that many vegetative functions of cardiovascular and respiratory systems regulated in brainstem centers may be modulated by m2 receptors. Although high affinity [^{3}H]PZ M1 binding sites comprise at least 50–55% of forebrain regions, a subpopulation of binding sites with low affinity for PZ was also present (21–23). This study confirmed the existence of the low but detectable level of m2 receptors in forebrain regions (10–20%). Our results are in good agreement with estimates of m2 receptors in the central nervous system made by others, using radioligand binding methods (25, 26).

Because the third intracellular loop of muscarinic receptors has been implicated in functional coupling to G proteins (27), we examined the ability of our antiserum to precipitate m2 receptors labeled with [^{3}H]oxotremorine-M. The binding of this ligand to m2 receptors is completely GTP sensitive,[1] and it has been shown that the binding of an agonist to the m2 receptor results in an association of the receptor with a G protein (28). The hypothesis being tested was that, if a G protein was already bound to the third intracellular loop at the m2 receptor, epitopes for the antiserum may be blocked, resulting in a decreased ability of the antiserum to precipitate the receptor. However, the result of this experiment was that

[1] M. Li and B. B. Wolfe, unpublished observations.

immunoprecipitation of m2 receptors was just as efficient with [^{3}H]oxotremorine-M bound as it was when [^{3}H]QNB was bound (data not shown). The fact that this result was obtained was probably related to the fact that, when we designed our fusion protein vector, the first 20 amino acids of the third intracellular loop were purposely excluded, due to homology with the m4 receptor in this region. Wess *et al.* (27) have shown that it is just this region that appears to be the determinant of coupling to G proteins. The fact that our antiserum was not affected by agonist occupancy might suggest that the portion of the protein targeted by our serum is not involved in G protein coupling.

More recently, *in situ* hybridization histochemistry analysis of rat brain with an oligonucleotide corresponding to the amino-terminal sequences of the m2 cDNA showed that m2 mRNA existed in the medial septum, diagonal band, olfactory bulb, and pontine nuclei (9), but low or no detectable m2 mRNA was found in thalamus, cerebral cortex, striatum, or cerebellum. Because mRNA is only present in cell bodies and proximal dendrites, locations of mRNA may or may not reflect the status of the receptor itself. Thus, the present study demonstrates the existence of m2 receptor protein in several areas of brain where mRNA is undetectable. In this regard, it would be useful to have a method with higher anatomical resolution to compare the distribution of protein with the distribution of mRNA. In preliminary experiments, efforts to utilize the m2 antiserum in immunocytochemical, as well as Western blot, protocols have not yet been successful.

It should be noted that the values in Fig. 6 may slightly underestimate m2 receptor number, because even in a tissue known to have 100% m2 receptors (m2 CHO cells) not all of the receptors are precipitable (e.g., Figs. 3 and 5). Thus, for example, the fact that 92% of the receptors in heart were precipitable may indicate that 100% of receptors are of the m2 receptor subtype. At the least, we consider the values shown in Fig. 6 to be the lower limits of m2 receptor density.

This study demonstrates that the fusion of a cloned DNA to the Protein A gene can be used for the successful production of a specific antibody. This Protein A fusion system provided large amounts of fusion protein, which were easily purified and used as an antigen without chemical coupling to a carrier protein. In addition, it has been shown that the repetitive globular units in Protein A enhance immune response. Thus, a normally poor antigen, Insulin-like growth factor-I, elicited good immune response after fusion to Protein A (29). Together, these are reasons why the Protein A-m2 fusion protein was chosen as an antigen to immunize rabbits, rather than the m2i3 protein expressed by the pET-3b vector. However, the m2i3 protein may be useful in future immunocytochemical studies as a reagent for blocking or for affinity purification of the antisera.

In conclusion, we have developed selective m2 antisera raised against a unique portion of the m2 receptor. In combination with radioligand binding techniques, these antibodies were used to evaluate the distribution and density of the m2 receptor subtype in rat brain. These antibodies should prove useful in studies regarding regulation of muscarinic receptors and also identification of subtypes that are altered in disease states.

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