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Bacterial expression of human muscarinic receptor fusion proteins and generation of subtype-specific antisera

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A family of five muscarinic acetylcholine receptor genes (m1–m5) encode highly related proteins; however, for methodological reasons it has not been possible to detect the gene products individually. To develop antibody probes specific for the receptor subtypes, unique regions of m1–m5 cDNAs, corresponding to the third cytoplasmic (i3) loops, were subcloned into bacterial expression vectors and the fusion proteins expressed in *E. coli* were used to generate rabbit antisera. These antisera react specifically with the respective fusion proteins on immunoblots and selectively immunoprecipitate each of the native cloned receptors. Since the i3 loops are immunogenic and the epitopes in the cloned receptors are accessible to antibodies, this approach should be valuable for immunological studies of the native receptors.

Muscarinic receptor subtype; Fusion protein; Antibody

1. INTRODUCTION

A family of muscarinic receptors mediates a variety of cholinergic effects in the nervous system, heart, smooth muscle, and glands. Molecular cloning studies have identified five muscarinic receptor genes (m1–m5) [1–4], and each mRNA has been found to be transcribed selectively in certain brain regions and other tissues [5–7]. Investigations of the m1–m5 gene products, however, have been limited by the unavailability of reagents selective for each of the highly related proteins. Even the most 'specific' muscarinic drugs, such as pirenzipine, are not highly selective (i.e. have greater than 10-fold differences in affinities) for the cloned receptor subtypes [8,9]. To better understand the biology of the muscarinic receptor family, we describe a molecular/immunological strategy for discriminating the related proteins. The third cytoplasmic (i3) loops of the muscarinic receptor subtypes are well suited for raising subtype-specific antisera since they are divergent, large (157–203 amino acids), and hydrophilic [2,10], with the only homologies between subtypes at the extreme ends of the loops [11]. Recombinant bacterial expression plasmids were constructed that encode the unique regions of the i3 loops of the five human muscarinic receptors. The i3 fusion proteins were expressed in *E. coli* and used to produce rabbit antisera. We show that the antisera are highly specific for the fusion proteins and each cloned receptor subtype.

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2. MATERIALS AND METHODS

2.1. *Construction of recombinant plasmids and expression of the m1–m5 fusion proteins*

Portions of the i3 regions of the m1–5 receptor genes were subcloned into the bacterial expression vectors pET3a or pET3b [12] as shown in Table I. Because of difficulties expressing the m3i3 fusion protein, this loop was also subcloned into pGEX2T [13]. Deoxyoligonucleotide primers (48–51 bases) were used to amplify, by the polymerase chain reaction (PCR), the DNA regions encoding most of the i3 loops but excluding the conserved regions at the N- and C-terminal ends. Restriction sites for *Bam*HI, *Bgl*II, or *Eco*RI were incorporated into the primers at both ends of the DNA fragments. In addition, stop codons in all three reading frames were placed in the downstream primer to insure that no additional amino acids were translated. The sequences of the deoxyoligonucleotide primers were: m1 (5′), TGT CCA GGA TCC GAG ACG CCA GGC AAA GGG GGT GGC AGC AGC AGC AGC; m1 (3′), CGT AGC AGA TCT TAC TAC TTC CGC TTG GCC AGC TGC TCC TTT CCA CGG GG; m2 (5′), CGA TCG AGA TCT GTT GCC AAC CAA GAC CCC GTT TCT CCA AGT CTG GTA C; m2 (3′), CGT AGC AGA TCT TAT CAC TGC TTT TCA TCT CCA TTC TGA CCT GAA GAC CC; m3 (5′), AGG TCC GGA TCC AAC AGG AGG AAG TAT GGC CGC TGC CAC TTC TGG TTC ACA; m3 (3′), AGG TCC GAA TTC AAG TGA TCT GAC TTC TGG TCT TCA GAG CAA ACC TC; m4 (5′), AGG TCC GGA TCC GAA GGA GAA GAA AGC CAA GAC GCT GGC CTT CCT CAA GA; m4 (3′), CGT AGC AGA TCT TAC TAG CGC ACC TGG TTG CGA GCG ATG CTG GCG AAC TT; m5 (5′), AGG TCC GGA TCC AAA GCT GAG AAG AGA AAG CCA GCT CAT AGG GCT CTG TT; m5 (3′), CGT AGC AGA TCT TAC TAC ATT TGA TGG CTG GGG TTG GGA TTG AGG CCT TT. PCR amplification of the DNA, isolation and restriction digestion of the PCR fragments, and subcloning were all performed using standard procedures [14]. The i3 fragments were subcloned into the *Bam*HI site on pET3a and pET3b, and the *Bam*HI-*Eco*RI multiple cloning site on pGEX2T. Construction of the recombinant plasmids was verified by double-stranded

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Table I

Construction of m1-m5i3 expression plasmids

Fusion protein	Vector	Nucleotides[a]	Amino acids[b]	N-terminus sequence[c] *Vector*-Linker-[i3 loop...]	M_r[d]
m1i3	pET3a	679–1059	126	*Met*[1]---*Arg*[12]-Gly-[Ser-Glu-Thr...]	13568
m2i3	pET3a	865–1268	135	*Met*[1]---*Arg*[12]-Gly-Ser-[Val-Ala-Asn...]	14369
m3i3	pGEX2T	907–1438	177	*Sj*[26]---Pro....Gly-[Ser-Asn-Arg...]	19117
m4i3	pET3b	1500–1955	152	*Met*[1]---*Arg*[12]-Asp-[Pro-Lys-Glu...]	16129
m5i3	pET3a	710–1278	190	*Met*[1]---*Arg*[12]-Gly-Ser-[Lys-Ala-Glu..]	21015

[a] The nucleotide positions correspond to the designations of the human sequences in GenBank (m2 and m4) and Bonner et al. (with the coding sequence starting at base 1 for m1, m3, m5; [3] and unpublished)
[b] The number of amino acids encoded in the subcloned regions of the i3 loops
[c] Shown are the amino acid sequences at the N-terminus of each fusion protein as encoded by the vector, linker, and i3 portion of the muscarinic genes. The first and last amino acid of the pET fusion protein from gene 10 of bacteriophage T7 are given followed by the linker and initial three amino acids of the i3 loops. For the m3i3 fusion, Sj26 is the glutathione *S*-transferase 26-kDa protein followed by the first and last amino acid of the linker and the initial three amino acids of the i3 loop
[d] The predicted molecular mass of only the i3 portions of each fusion protein

dideoxy sequencing. The recombinant pET vectors encode 12 amino acids from bacteriophage T7 gene 10 and one or two amino acids from the linkers fused at the N-terminus of each i3 loop (Table I). The m3i3pGEX2T fusion protein consists of the 26-kDa glutathione *S*-transferase (GST; EC 2.5.1.18, from *Schistosoma japonicum*) at the N-terminus of the m3i3 loop. The fusion proteins were expressed in strain BL21 (DE3)pLysS using isopropylthiogalactoside (IPTG) to induce transcription as previously described [12]. Culture samples were analyzed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) stained with Coomassie brilliant blue. The fusion proteins were recovered in the particulate fraction of lysed cultures, permitting easy partial purification by washing the pellets (spheroplasts) several times with 0.1% Triton X-100 in 10 mM Tris, 1 mM EDTA, pH 8.0. After this step, the fusion protein comprised greater than 75–80% of the total protein as determined by optical densitometer tracing. Further purification for immunization was achieved by preparative 10% SDS-PAGE, visualization of the proteins by immersion of the gel in cold 0.25 M KCl, and excision of the fusion protein bands. Purity was then assessed by repeating SDS-PAGE.

2.2. *Immunization of rabbits*

Two female New Zealand White rabbits were immunized with each fusion protein. Immunogens were prepared by trituration of the excised gel bands in equal volumes of phosphate-buffered saline through successively smaller bore needles. Gel suspensions were frozen at −20°C and mixed with an equal volume of Freund's adjuvant prior to immunization. Aninals received 10-25 μg of protein (in 0.75–1.0 ml) per primary injection followed by boosts at three weeks and then monthly. Venous blood was obtained at 3 and 4 weeks post-boosts, and the serum was stored at −70°C with aliquots kept at 4°C for testing. Since the immune responses of each pair of rabbits were variable, all studies described below used a single antiserum reactive with each fusion protein.

2.3. *Immunoblotting studies*

Samples of bacterial cultures were electrophoresed on 10% SDS-PAGE, electroblotted onto Immobilon (Whatman) membranes overnight at 150 mA constant current, blocked in 5% nonfat dried milk for 30 min at room temperature, and incubated overnight at 4°C with antisera diluted 1:250. All sera were diluted in Tris-buffered saline (TBS), pH 8.0, containing 1% milk and 3% normal goat serum. Antisera were preadsorbed with *E. coli* lysates 30 min to reduce background staining due to natural and induced antibodies reactive with bacterial components. After washing several times with TBS containing 0.1% Tween, the blots were incubated with 1 μg/ml affinity-purified horseradish peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Inc.) for 2–4 h at room temperature. Blots were washed and immunoreactivity was visualized with 0.05% 3,3′-diaminobenzidine (Sigma Chemicals) and 0.01% hydrogen peroxide.

2.4. *Immunoprecipitation studies*

The cloned m1–m5 receptors used for these experiments were expressed individually in stable CHO-K1 cells lines and the receptor binding properties have been previously described [8,9]. The total number of receptors/mg membrane protein for each subtype is: m1, 2518 fmol; m2, 747 fmol; m3, 1831 fmol; m4, 1778 fmol; m5, 954 fmol [8,9]. After polytron homogenization in 50 mM sodium phosphate buffer, pH 7.0, containing a protease inhibitor cocktail (0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μM pepstatin A, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor) membranes were collected by centrifugation at 30000×g for 20 min at 4°C, washed, and resuspended in the same buffer to a protein concentration of 0.5 mg/ml, and frozen at −70°C. Muscarinic receptors were labeled with either 2.0 nM [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB, 32.9 Ci/mmol, New England Nuclear) for 1 h at 30°C or 5 nM [^{3}H]propylbenzilylcholine mustard ([^{3}H]PrBCM, 40.0 Ci/mmol, New England Nuclear) for 30 min at 30°C in the presence or absence of 1.0 μM atropine to determine nonspecific binding. The membranes were washed once in 10 mM Tris buffer, 1.0 mM EDTA, pH 7.4 (TE) and recentrifuged. The [^{3}H]QNB-labeled receptors were solubilized by resuspending the membranes (to 1.0 mg/ml protein) in TE buffer containing 1.0% digitonin, 0.1% cholic acid as previously described [15] and [^{3}H]PrBCM-labeled receptors were solubilized with 0.5% Lubrol. To determine antibody binding, sera were diluted 1:50 into 96 well microtiter plate wells containing 15–30 fmol of solubilized receptor and incubated for 4 h at 4°C. Goat anti-rabbit IgG (Pel-Freez) was then added to a final dilution of 1:10. After overnight incubation, plates were centrifuged at 1000×*g* for 10 min, the immunoprecipitates washed twice with TE containing 0.1% digitonin and 0.01% cholic acid, resuspended in 1% SDS, and radioactivity determined. Controls employing antisera raised against an irrelevant pET fusion protein (consisting of the rat neuronal nicotinic N-terminus β_2 subunit) were used to determine nonspecific trapping of receptor in immunoprecipitates and were subtracted from experimental values (average of 13% of the total radioactivity added, $n = 10$). Concentrations of rabbit and goat sera were optimized for complete precipitation of immunoglobulins.

3. RESULTS AND DISCUSSION

Bacteria transformed with each of the recombinant m1–m5i3pET vectors, except m3i3, expressed high levels (5–10 μg/ml) of a single protein (Fig. 1). Since

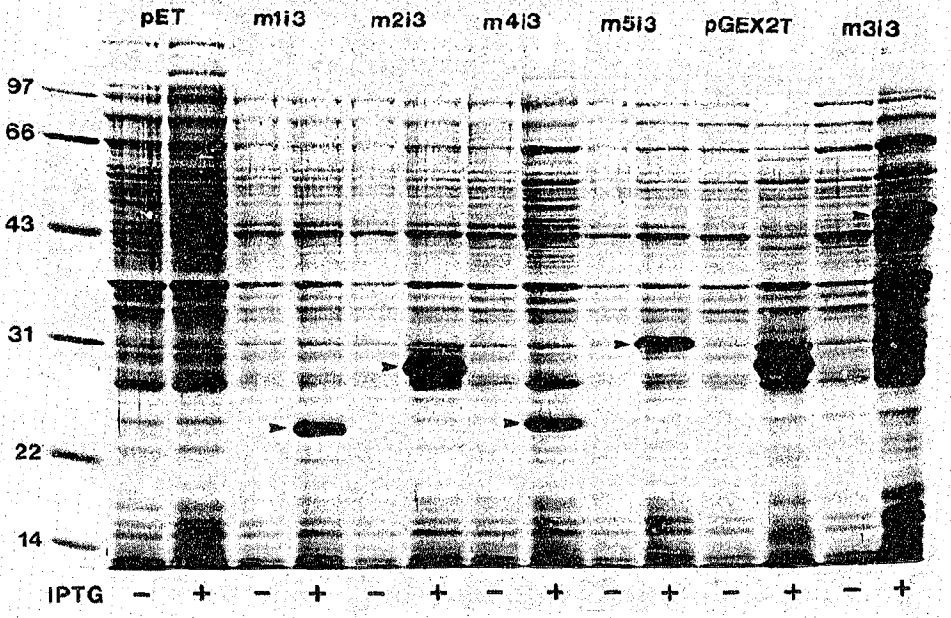

Fig. 1. Bacterial expression of the third cytoplasmic loops of muscarinic receptors m1–m5. 10% SDS-PAGE was performed on samples of *E. coli* cultures transfected with the parental expression vectors pET or pGEX2T, or the recombinant expression vectors containing the m1–m5 third cytoplasmic loop (i3) inserts as indicated at the top of each pair of lanes, m1i3, m2i3, m4i3, and m5i3 were subcloned into the pET vector and m3i3 was subcloned into pGEX2T. Culture samples were taken before (−) and after (+) three hours of induction with 1.0 mM IPTG. Each lane was loaded with 200 μl of culture medium, and the pGEX2T samples had a higher density of bacteria (and protein) than the pET cultures. The arrowheads mark the positions of the five muscarinic fusion proteins. Molecular weights of the markers (far left lane) are noted on the left. The gel was stained with Coomassie brilliant blue.

these proteins were not expressed prior to induction of transcription with IPTG, they were clearly encoded by the recombinant plasmids, and therefore, represent the i3 loop fusion proteins. Since we were unable to observe induction of an m3i3 fusion protein with the pET vector, this subtype was inserted into pGEX2T [13], and abundant fusion protein expression was achieved. The fusion proteins all migrated at apparent molecular weights somewhat larger than predicted from their primary sequences (Table I).

Rabbit antisera were raised against each of the five purified muscarinic receptor i3 loop fusion proteins. On immunoblots, each antiserum was highly specific for the single fusion protein to which it was raised (Fig. 2). These studies demonstrated that the i3 loops of m1–m5 were immunogenic and that under denaturing conditions the five antisera did not cross-react with other subtypes.

The ability of the antisera to bind individually to the five native muscarinic receptors was tested using the immunoprecipitation assay. Each antiserum reacted highly specifically to the appropriate receptor subtype labeled with [^{3}H]QNB (Fig. 3) and with the irreversible ligand [^{3}H]PrBCM mustard (data not shown), even at high concentrations (1/50 final dilution of antisera). The assay with [^{3}H]QNB was more sensitive than with [^{3}H]PrBCM because of more nonspecific binding with the latter compound. Almost complete receptor binding was observed with the m3 and m4 antisera, but significant fractions of the other receptors were not bound. The many possible explanations for the lack of complete binding (e.g. dissociation of ligand, denaturation or degradation of receptors, post-translational modifications) are presently being investigated. However, no further binding was observed with fewer receptors per assay (data not shown), suggesting that antibody concentrations were not limiting. These results demonstrate that the antisera raised against genetically engineered and SDS-denatured i3 fusion proteins bind to the undenatured muscarinic receptors, and most importantly, are specific for each of the m1-m5 subtypes. Moreover, the binding of the antibodies to the proposed inner cytoplasmic loops did not abolish the ligand binding abilities of the receptors. These properties should make feasible the immunological detection and quantitation of the native receptor subtypes in animal tissues.

Antibodies specifically reactive with muscarinic receptors m1–m5 are essential to study the turnover, regulation, and localization of receptor subtypes. Previous attempts to produce such reagents have met with limited success. For instance, monoclonal antibodies against brain muscarinic receptors were developed prior to knowledge of the subtypes [15,16],

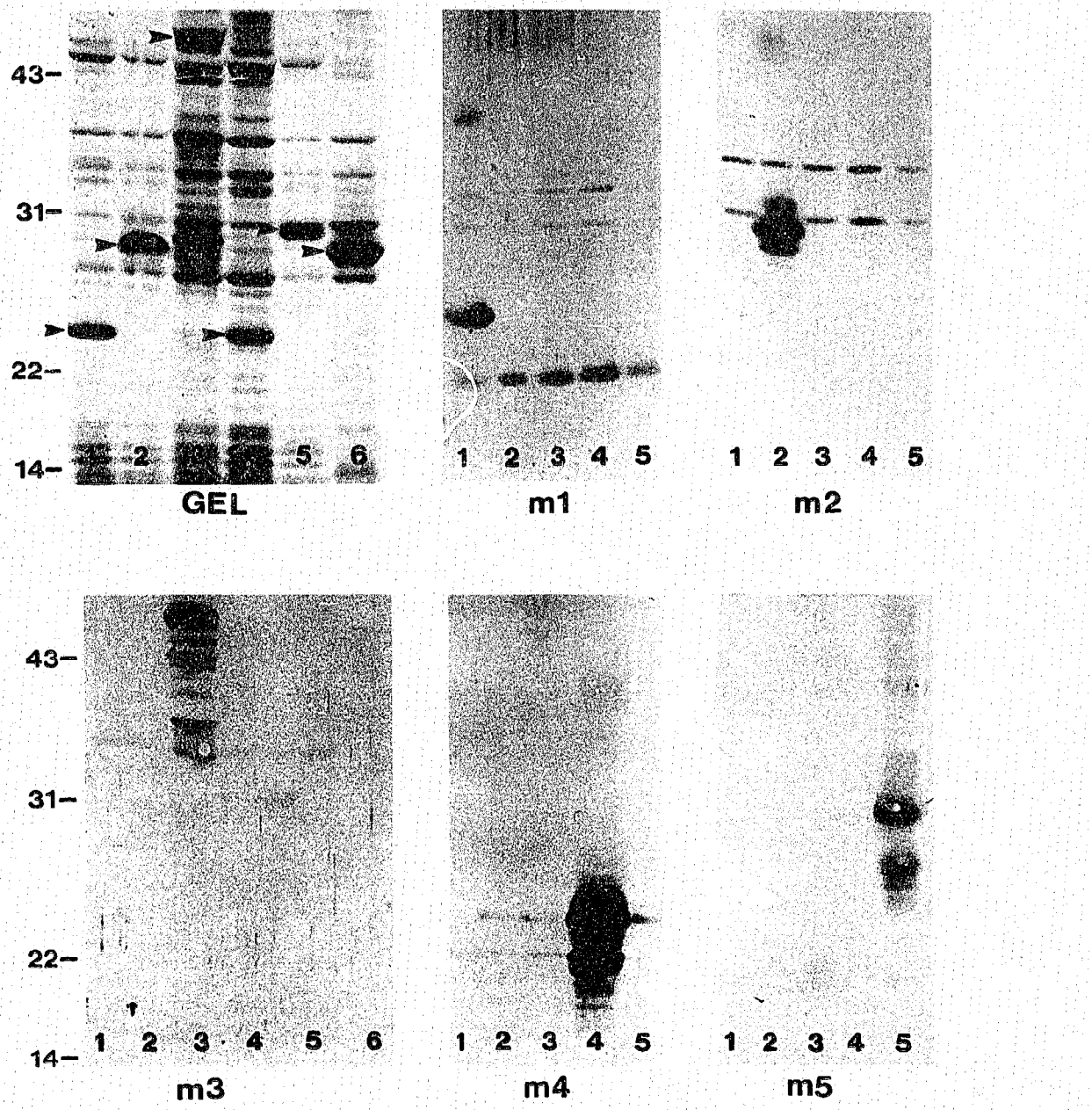

Fig. 2. Immunoblotting of the muscarinic receptor i3 fusion proteins. Total protein from 150 μl samples of bacterial cultures transfected with either m1i3pET (lane 1), m2i3pET (lane 2), m3i3pGEX2T (lane 3), m4i3pET (lane 4), m5i3pET (lane 5), or pGEX2T (lane 6) were analyzed by 10% SDS-PAGE and stained with Coomassie brilliant blue as shown in the upper left panel (GEL). In the other panels are shown immunoblots from replicate gels reacted with antisera (diluted 1:250) to each of the five muscarinic fusion proteins (indicated under each blot, m1–m5). Antisera were preadsorbed with total protein in lysates from the equivalent of 750 μl of bacterial cultures in order to reduce background staining of *E. coli* proteins (those proteins evident in all lanes of a given blot). Immunoreactivity was localized on the blots using 1 μg/ml affinity-purified horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized with 0.05% 3,3'-diaminobenzidine/0.01% hydrogen peroxide. Each antiserum showed strong reactivity specifically with the numerically matched fusion protein. The m3i3 antiserum showed no immunoreactivity with the glutathione *S*-transferase moiety (lane 6), demonstrating that reactivity with the fusion protein antiserum (lane 3) resulted from antibodies reactive with the i3 insert.

and no further studies of their specificity have been reported. More recently, monoclonal antibodies were produced against the heart receptors that appear to be m2-selective [17]. Synthetic peptides have been used to develop antibodies against the m1 subtype [18] and more recently, the other subtypes as well [19]. However, the few epitopes available on the small peptides may limit the diversity and intensity of the antibody

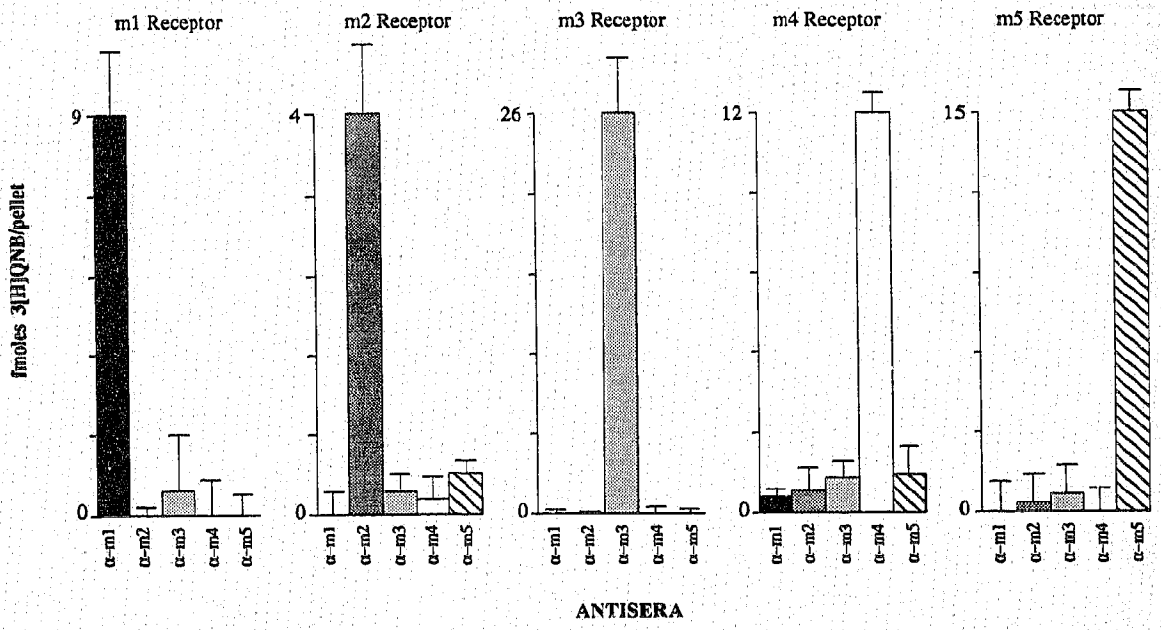

Fig. 3. Immunoprecipitation of [^{3}H]QNB-labeled muscarinic receptors m1–m5. Cloned human muscarinic receptor subtypes were labeled with [^{3}H]QNB, solubilized, incubated with rabbit antisera to the fusion proteins (α-m1–α-m5), coprecipitated with goat anti-rabbit immunoglobulin, and radioactivity in the immunoprecipitates was determined as described in the text. In order to facilitate comparisons between receptors, the ordinates were scaled differently for each receptor. The total number of fmol of [^{3}H]QNB per assay was: m1, 33; m2, 14; m3, 31; m4, 14; and m5, 28. Values are the mean of triplicate samples and error bars represent SD.

response and sometimes their ability to recognize the native proteins. This study has demonstrated the bacterial expression of the i3 loops of each of the five human muscarinic receptor subtypes and their use for production of rabbit antisera. The antisera, characterized by immunoblotting and immunoprecipitation, are highly specific for each of the five fusion proteins and undenatured receptor subtypes m1–m5. The fusion proteins and the antisera will be useful for future studies of the structure, function, and localization of the family of muscarinic receptors.

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