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Purification and Functional Reconstitution with GTP-Binding Regulatory Proteins of Hexahistidine-Tagged Muscarinic Acetylcholine Receptors (m2 Subtype)[1]

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We have expressed human m2 muscarinic acetylcholine receptors tagged with six histidine residues at the carboxy-terminal region in insect cells (Sf9) and purified them using metal-immobilized Chelating Sepharose gels. Co^{2+}-immobilized gels were found to be much more efficient for purification of m2 receptors than gels containing Ni^{2+} or other metal ions. Twenty-fold purification was attained by a simple, single-step procedure, and approximately 40% of solubilized receptors were recovered as a partially purified preparation with a specific activity of 1.6 nmol/mg of protein. Purified receptors were functionally active in that carbamylcholine stimulated binding of [^{35}S]GTPγS to the G-protein G_{i2} reconstituted in lipid vesicles with purified m2 receptors. The extent of stimulation of [^{35}S]GTPγS binding to G_{i2} by hexahistidine-tagged m2 receptors was essentially the same as that observed for m2 receptors that lack histidine tags. In addition, palmitoylation at the carboxy-terminal region was not impaired by the hexahistidine-tag fusion. The method described in this study should be applicable to the purification of other G-protein-coupled receptors in functionally active form.

Key words: G-protein, histidine-tag, muscarinic receptor, purification.

Many receptors for hormones and neurotransmitters transduce extracellular signals to the cytoplasm through activation of heterotrimeric G-proteins by promoting GDP-GTP exchange (*1*). Many of these receptors have been identified by cDNA cloning. These receptors constitute a G-protein-coupled receptor superfamily, and have in common seven hydrophobic regions that are predicted to be membrane-spanning domains. Purification of each receptor is necessary for its biochemical and biophysical characterization. Purification of homogenous receptors, however, is usually difficult because most receptors are expressed in low levels and several receptor subtypes often coexist in a single tissue. Affinity chromatography has been practically the only method for efficient purification of receptors expressed in low levels (*2-6*).

We have attempted to develop a simple purification method that is generally applicable to G-protein-coupled receptors. For this purpose, we used the hexahistidine-tag method for purification of muscarinic m2 receptors and compared it to an established affinity chromatography method (*7, 8*). Oligohistidine tagging has been shown to be useful for purification of different kinds of proteins expressed in both prokaryotes and eukaryotes (*9-11*). The proteins tagged by hexahistidine bind to metal-immobilized gels and are eluted under mild conditions, such as low pH, addition of an excess amount of hexahistidine or imidazole, or removal of metal ions with EDTA.

The hexahistidine-tag method has already been used for purification of G-protein-coupled receptors such as bovine rhodopsin (*12*) and human β_2-adrenergic receptor (*13*). Such modifications did not affect spectral properties or ligand-binding activities of these receptors. It has not been shown, however, if the rhodpsin or β_2-adrenergic receptors purified with the hexahistidine-tag method are functionally intact and can interact with and activate G-proteins.

We have expressed hexahistidine-tagged m2 receptors in baculovirus-Sf9 protein expression system. The baculovirus-Sf9 system has been used for expression of many exogenous genes (*14, 15*) including G-protein-coupled receptors (*16-22*). We report here that hexahistidine-tagged m2 receptors can be purified 20-fold by a simple procedure using Co^{2+}-immobilized Chelating Sepharose, and that purified receptors can activate G-protein G_{i2} and can be palmitoylated at the carboxyl terminus.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Palmitic acid was purchased from American Radiolabeled Chemicals, Chelating Sepharose fast flow from Pharmacia, Centricon-10 from Amicon, digitonin, yeast extract, tryptose phosphate broth and pluronic F-68 solution from Sigma, fetal bovine serum from Cansera, and linearized baculovirus DNA BacPAK6 and Transformer™ site-directed mutagenesis kit from

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Abbreviations: G-protein, GTP-binding regulatory protein; [^{35}S]-GTPγS, [^{35}S]guanosine 5′-(α-thio)triphosphate; m2 receptor, muscarinic acetylcholine receptor m2 subtype; [^{3}H]QNB, L-[benzilic-4,4′-^{3}H(N)]-quinuclidinyl benzilate; Sf9, *Spodoptera frugiperda*.

Clontech. Sf9 cells were obtained from Dr. E.M. Ross (University of Texas Southwestern Medical Center), cDNAs for human m2 subtype (Hm2/pSG5) and a m2 receptor mutant lacking 233-380 residues [des-(P233-S380)Hm2/pSG5] were from Dr. W. Sadée (University of California San Francisco) (*23*), and the baculovirus transfer vector pVL1393 was from Dr. M.D. Summers (Texas A&M University).

Fusion of a Hexahistidine-Tag to the Human Muscarinic m2 Receptor—The transfer vectors of hexahistidine-tagged m2 receptors were constructed as follows. Putative *N*-glycosylation sites were eliminated by substitution of Asn2, Asn3, Asn6, and Asn9 with aspartic acid residues. The substitutions were carried out by PCR-based mutagenesis. Two overlapping 5′-oligonucleotides were designed, because the region to be mutated is rather long. The 5′-oligonucleotide for the first PCR is 5′-GACTCCACGGACTCCTCTGACAATAGCCTG-3′ corresponding to 7-36 of m2 receptor-coding sequence, and that for the second PCR is 5′-GCTCTAGATCTGCCATGGATGACTCCACGGACTCCTC-3′, corresponding to 1-23 of the coding sequence and recognition sites for *Xba*I and *Bgl*II. Double-stranded cDNAs for human m2 subtype (Hm2/pSG5) and an m2 receptor mutant lacking 233-380 residues [des-(P233-S380)Hm2/pSG5] (*23*) were used as a template. These mutated cDNAs were digested with *Bgl*II and *Pst*I and inserted into the *Bam*HI and *Pst*I sites of pVL1393 [pVL m2(N-D), pVL m2(N-D)(I3del)].

Thrombin recognition and six histidine residues were also fused to the carboxyl terminus of m2 receptors by PCR. Two overlapping 3′-oligonucleotides were designed. The 3′-oligonucleotide used for the first PCR is 5′-GGTGGCTGCCACGGGGCACCAGCCTTGTAGCGCCTATGTTCT-3′, corresponding to 1388-1407 of m2 receptor coding sequence and thrombin recognition sequence, and that for the second PCR is 5′-CGCTGCAGACTAGTGATGGTGGTGATGGTGGCTGCCACGGGGC-3′, corresponding to 17 bases with the same sequence as a part of the first primer, six histidine residues and a recognition site for *Pst*I. Double-stranded cDNAs, pVL m2(N-D), and pVL m2(N-D)(I3del) were used as a template. These mutated cDNAs were digested with *Bgl*II and *Pst*I and inserted into the *Bam*HI and *Pst*I sites of pVL1393 [pVL m2(N-D)(6His), pVL m2(N-D)(6His)(I3del)].

Mutation of Cys-457 residue of m2 receptor to alanine was introduced to pVL m2(N-D)(6His) using the Transformer™ site-directed mutagenesis kit. 5′-CACCTTCTCATGGCTCATTATAAGAACA-3′ was used as the mutation primer.

Each transfer vector DNA (10 μg) was cotransfected with 0.5 μg of linearized baculovirus DNA BacPAK6 (*24*) to Sf9 cells by the calcium phosphate coprecipitation method as previously described (*14*). Resultant recombinant viruses were cloned by plaque isolation as described (*14, 15*).

Expression of m2 Receptors in Baculovirus-Sf9 System—The recombinant viruses were amplified and used for the large scale production of m2 receptor mutants as previously described (*25*). Expression levels of wild type m2, m2(N-D), m2(N-D)(6His), and m2(N-D)(6His)(I3del) receptors were, respectively, 5-7, 5-7, 5-7, and 8-12 nmol/liter of culture suspension containing approximately 2×10^9 cells as determined by [^{3}H]QNB binding (*26*).

Sf9 cells expressing m2 receptors were homogenized and centrifuged as previously described (*25*). Pellets were resuspended in 20 mM Hepes-KOH buffer (pH 8.0) containing a mixture of protease inhibitors (2.5 μg/ml pepstatin, 2 μg/ml phenylmethylsulfonyl fluoride, 0.02 mg/ml leupeptin, and 0.5 mM benzamidine), and stocked in aliquots at −80°C until use.

Purification of Hexahistidine-Tagged m2 Receptors Using Chelating Sepharose—The Chelating Sepharose gel (Fast Flow, 0.5 ml) was washed with H_2O, and a solution (0.5 ml) of either 0.2 M $CuCl_2$, 0.2 M $NiCl_2$, 0.2 M $CoCl_2$, 0.2 M $ZnSO_4$, 0.4 M $MgCl_2$, or 0.4 M $CaCl_2$ was loaded for immobilization. The Chelating Sepharose gel was equilibrated with a column-washing buffer containing 20 mM Hepes-KOH (pH 7.0), 0.5 M NaCl, and 0.1% digitonin. Sf9 membranes containing 90 mg of proteins were suspended in 6 ml of a buffer solution containing 50 mM Hepes-KOH (pH 7.0), 50 mM NaCl, and a mixture of protease inhibitors. Digitonin and sodium cholate were added to the membrane suspension to levels of 1 and 0.5%, respectively, and the suspension was stirred for 1 h for solubilization. After centrifugation at 200,000 × *g* for 45 min, the supernatant was diluted 5-fold with 20 mM Hepes-KOH buffer (pH 7.0) containing 0.5 M NaCl and loaded onto the metal-immobilized Chelating Sepharose at a rate of 10-20 ml/h. After washing the column with 8 ml of the column-washing buffer, the bound receptor was eluted with 1.5 ml of the column-washing buffer with 100 mM imidazole-Cl (pH 7.0). In some experiments, the remaining proteins were further eluted with 1.5 ml of a metal-stripping buffer containing 50 mM Hepes-KOH (pH 7.0), 0.5 M NaCl, 0.1% digitonin, and 50 mM EDTA.

Reconstitution of m2 Receptors and G-Protein G_{i2}—Recombinant m2 receptors expressed in Sf9 cells were purified using an affinity chromatography with a muscarinic ligand, aminobenztropine, and reconstituted with G-protein G_{i2}, as described previously (*7, 17, 25, 27*). G_{i2} was purified from bovine lung as described previously (*26*).

[^{3}H]Palmitic Acid Labeling of m2 Receptors Expressed in Sf9 Cells—Palmitoylation of m2 receptors expressed in Sf9 cells was detected by metabolic labeling with [^{3}H]-palmitic acid. Sf9 cells from 20 ml of culture (5×10^7 cells) were infected with recombinant virus for expression of m2(N-D)(His6). After 48 h, 185 Mbq of [^{3}H]palmitic acid was added, as previously described for palmitoylation of β_2-adrenergic receptor (*28, 29*). The cells were collected and purified using Co^{2+}-immobilized gel as described in the previous section. The purified receptor was concentrated from 1.5 ml to 100 μl with Centricon-10 and subjected to SDS-PAGE. The receptor labeled with [^{3}H]palmitic acid was detected by fluorographic methods using 2,5-diphenyloxazole dissolved in DMSO as previously described (*30*).

Miscellaneous Procedures—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using 12% (w/v) acrylamide. The reducing reagent β-mercaptoethanol was not included in the sample buffer to avoid reduction and precipitation of metal ions stripped from gels. Protein concentration was determined using Folin phenol agent as described (*31*).

RESULTS AND DISCUSSION

Hexahistidine-Tagged m2 Receptor Mutants—Three

mutations were introduced into human m2 receptors (Fig. 1). First, in "6His," six histidine residues and a thrombin cleavage site were added to the carboxyl terminus of m2 receptors for purification using metal-immobilized gels. Second, in "N-D," putative *N*-glycosylation sites near the amino terminus were eliminated by converting asparagine residues (Asn2, 3, 6, and 9) in the consensus sequence Asn-X-Ser to aspartic acid residues. Glycosylation is undesirable because structural variability is expected to occur due to heterogenous glycosylation. Third, in "I3del," the central part of the third intracellular loop (235-380) of m2 receptors was deleted. This region is susceptible to proteolysis, and its elimination prevents m2 receptors from being degraded during the preparation from Sf9 cells. This domain is involved in phosphorylation by G-protein-coupled receptor kinase and the subsequent sequestration of receptors (*23, 32, 33*). It is not necessary, however, for activation of G-proteins (*26*).

Purification of Hexahistidine-Tagged m2 Receptors with Metal-Immobilized Gels—Hexahistidine-tagged m2 receptors were purified by Chelating Sepharose chromatography. First, we compared various metal ions for their ability to bind hexahistidine-tagged m2 receptors [m2(N-D)(6His)(I3del)] (Fig. 2A). Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+} were immobilized on Chelating Sepharose gels, and hexahistidine-tagged m2 receptors solubilized with digitonin from Sf9 membrane were loaded. Bound receptors were eluted with a solution containing 100 mM imidazole-Cl buffer (pH 7.0), because m2 receptors were found to be more stable in this buffer compared with other eluting solutions containing sodium phosphate (pH 5.0) or 50 mM EDTA.

Cu^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+}-immobilized Chelating Sepharose bound hexahistidine-tagged m2 receptors but Mg^{2+}- or Ca^{2+}-immobilized gel did not. Approximately 50% of m2(N-D)(6His)(I3del) receptors, as assessed by the [^{3}H]-QNB-binding activity, were eluted from Co^{2+}- or Zn^{2+}-immobilized Chelating Sepharose with the 100 mM imidazole solution. In contrast, only 7 and 15% of receptors were eluted from Cu^{2+} and Ni^{2+}-immobilized gels, respectively. Loss of the [^{3}H]QNB-binding activity during the purification procedure amounted to 30-50% using Ni^{2+}, Co^{2+}, or Zn^{2+}-immobilized Chelating Sepharose, and approximately 90% using Cu^{2+}-immobilized gel.

Figure 2B shows silver-stained bands after SDS-PAGE of fractions eluted with the imidazole buffer from various metal-immobilized gels. In control experiments, m2(N-D)-(6His)(I3del) receptors were purified by affinity chromatography with ABT-agarose (lane 1, Fig. 2B). Two bands with apparent molecular mass of 30 and 27 kDa were observed for preparations purified with ABT-agarose. Two major bands with essentially the same mobilities were observed for preparations purified with Co^{2+}- or Zn^{2+}-immobilized gels (lanes 4 and 5 in Fig. 2B). It is reasonable to

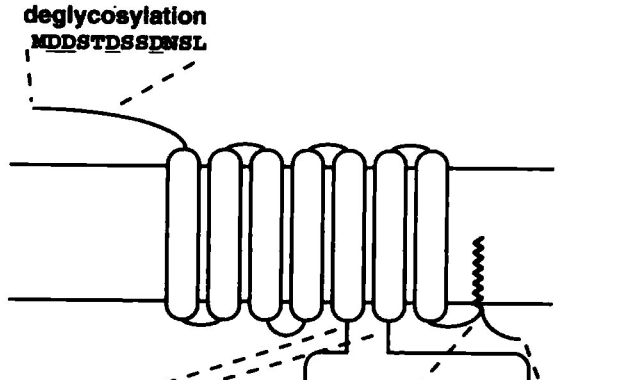

Fig. 1. **Schematic diagram of histidine-tagged m2 receptor mutants expressed in baculovirus-Sf9 system.** The expressed m2 receptor mutants lack *N*-glycosylation sites near the amino terminus and have a thrombin recognition site and six histidine residues fused to the carboxyl terminus. A mutant which lacks the sequence 233-380 was also constructed. The mutants are named as follows: m2(N-D), mutants lacking *N*-glycosylation sites; m2(I3del), mutants with a deletion of the sequence 233-380; m2(6His), mutants with six histidine residues.

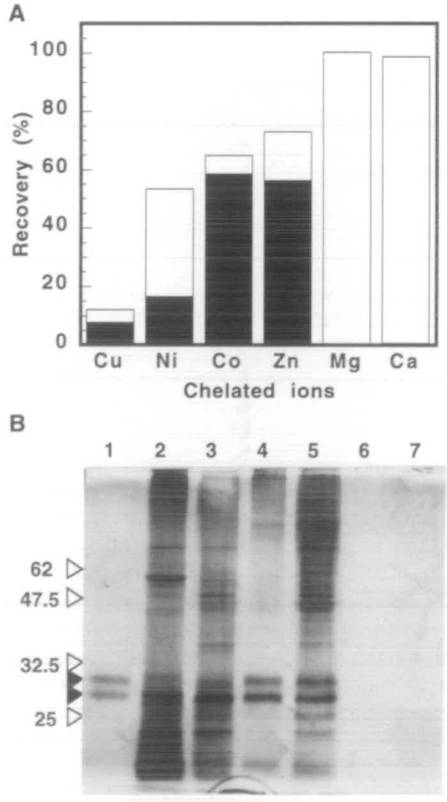

Fig. 2. **Purification of m2(N-D)(6His)(I3del) receptors using metal ion-immobilized Chelating Sepharose.** (A) The recovery of hexahistidine-tagged m2 receptors at each step of purification with each metal ion-immobilized Chelating Sepharose. The [^{3}H]QNB-binding activity of solubilized preparations was taken as 100%. The activity of flow-through fractions is shown by open bars, and that of imidazole eluates by closed bars. (B) Purified receptors were subjected to SDS-PAGE followed by silver staining. Lane 1, receptors purified with ABT-agarose. Lanes 2-7, aliquots (5 μl) of imidazole eluates from each metal ion-immobilized Chelating Sepharose: lane 2, Cu^{2+}-immobilized gel; lane 3, Ni^{2+}-immobilized gel; lane 4, Co^{2+}-immobilized gel; lane 5, Zn^{2+}-immobilized gel; lane 6, Mg^{2+}-immobilized gel; lane 7, Ca^{2+}-immobilized gel. Open arrowheads, molecular weight markers; closed arrowheads, receptors.

assume that the two bands represent m2(N-D)(6His)-(I3del), because these bands were specifically bound to and eluted from ABT-agarose and metal-immobilized gels. The lower band with an apparent molecular size of 27 kDa is thought to represent a degradation product lacking an amino-terminal portion. Similarly, two bands with slightly smaller molecular mass, 29 and 26 kDa, were observed for hexahistidine-free m2(N-D)(I3del) receptors purified with ABT-agarose (data not shown). A difference in the apparent molecular mass of 1 kDa may be due to the fusion of 12 amino acid residues.

The preparation eluted from Co^{2+}-immobilized gels had much less contaminating protein than those from other metal ion-immobilized gels. These results indicate that the Co^{2+}-immobilized Chelating Sepharose is the most effective among those examined for purification of hexahistidine-tagged m2 receptors. Hexahistidine-tagged m2 receptors of full length, m2(N-D)(6His), were also purified most efficiently with Co^{2+}-immobilized Chelating Sepharose. However, the purity of the final preparation was not as apparent as that of m2(N-D)(I3del) because of the presence of degradation products (data not shown).

Figure 3 shows silver-stained patterns after SDS-PAGE of samples at each step of purification. When m2(N-D)-(6His)(I3del) receptors were purified from 150 ml of culture medium of Sf9 cells, 1.63 nmol of receptors was solubilized and 0.66 nmol of purified receptors was obtained. The yield and specific activity of purified receptors were 38.2% and 1.6 nmol/mg protein, respectively. Thus, an approximately 20-fold purification was attained in a single step. The specific activity of receptors purified with Chelating Sepharose was lower by a factor of 3 compared with receptors purified with ABT-agarose. On the other hand, the purification takes only 6 h with the Chelating Sepharose, much less than 48 h required with ABT-agarose.

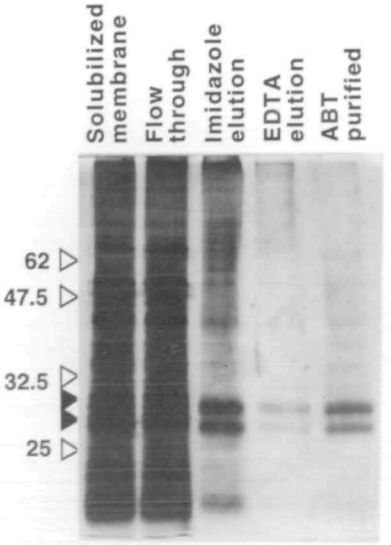

Fig. 3. **Purification of m2(N-D)(6His)(I3del) receptors using Co^{2+}-immobilized Chelating Sepharose.** Aliquots (5 μl) at each step of the purification using Co^{2+}-immobilized Chelating Sepharose were subjected to SDS-PAGE followed by silver staining. Open arrowheads, molecular weight markers; closed arrowheads, receptors.

Ni^{2+}-immobilized nitrilotriacetic acid (Ni^{2+}-NTA) agarose is the first gel proven to be useful and is currently the most frequently used gel for purification of hexahistidine-tagged proteins. Commercially available Ni^{2+}-NTA agarose is an agarose gel crosslinked with Ni^{2+}-immobilized nitrilotriacetic acid (NTA). The present results indicate that Ni^{2+} was not the best ion for purification of m2 receptors, and that Co^{2+}-immobilized gel was much better than Ni^{2+}-immobilized gel. Whatever the reason for this or the mechanism involved may be, our results suggest that metal ions other than Ni^{2+} should be tested for their efficiency for purification of proteins. Among G-protein coupled receptors, rhodopsin (*12*) and β_2-adrenergic receptors (*13*) were reported to be hexahistidine-tagged at the carboxyl termini, expressed in baculovirus-Sf9 system, and purified with Ni^{2+}-NTA agarose gel and Ni^{2+}-immobilized Chelating Sepharose, respectively. Significant purification was attained in a single step in both cases. Whether the Co^{2+}-immobilized gel would also be more efficient than the Ni^{2+}-immobilized gel for purification of rhodopsin and β_2-adrenergic receptors remains to be seen. Our results, together with those on rhodopsin and β_2-adrenergic receptors, showed that the hexahistidine-tagging at the carboxyl terminus is a useful method for purification of G-protein-coupled receptors.

Palmitoylation of Hexahistidine-Tagged m2 Receptor— Many G-protein-coupled receptors have cysteine residues in their carboxy-terminal region, which is assumed to contain palmitoylation sites. Each subtype of muscarinic receptor has a putative palmitoylation site, but no evidence for palmitoylation is available. We examined whether palmitoylation occurs in m2 receptors produced by Sf9 cells and is affected by hexahistidine-tag fusion at the carboxyl terminus.

Sf9 cells expressing hexahistidine-tagged m2 receptors were incubated with [^{3}H]palmitic acid, then the receptors were purified and analyzed by SDS-PAGE and fluorography. As shown in Fig. 4, the receptor was radiolabeled. When cysteine residue 457, which is a putative palmitoyla-

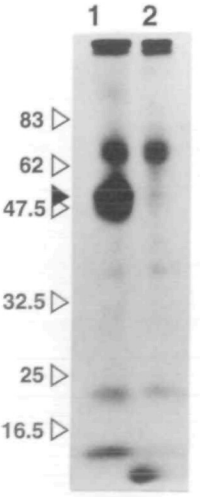

Fig. 4. **Palmitoylation of hexahistidine-tagged m2 receptor.** Fluorography of m2 receptors purified from Sf9 cells which had been incubated with [^{3}H]palmitic acid. Lane 1, m2(N-D)(6His) receptors; lane 2, m2(N-D)(C-A)(6His) receptors with a substitution from Cys457 to alanine.

tion site, was replaced by alanine, no palmitoylation was detected. These results indicate that m2 receptors produced in Sf9 cells are palmitoylated at Cys-457, and that hexahistidine-tag fusion at the carboxyl terminus does not interfere with the palmitoylation.

Reconstitution of Hexahistidine-Tagged m2 Receptors with G-Protein G_{i2}—We examined whether the addition of six histidine residues to the carboxyl terminus affects the ability of muscarinic receptors to activate G-proteins. The m2 receptor mutants, m2(N-D) and m2(N-D)(6His), were purified using ABT-agarose, then reconstituted with G_{i2} purified from bovine lung into phospholipid vesicles. The reconstituted vesicles were incubated with 100 nM [^{35}S]-GTPγS in the presence of 5 μM GDP and a muscarinic agonist, carbamylcholine, or an antagonist, atropine. The time courses of [^{35}S]GTPγS binding to G_{i2} are shown in Fig. 5A. Carbamylcholine stimulated [^{35}S]GTPγS binding to G_{i2} to the same extent for both m2(N-D) and m2(N-D)-(6His) receptors. Figure 5B shows the displacement curves by carbamylcholine of the [^{3}H]QNB binding to m2(N-D) and m2(N-D)(6His) receptors in the presence or absence of 0.1 mM GTP. The curves were essentially the same for m2(N-D) and m2(N-D)(6His) receptors. At lower concentrations of carbamylcholine, [^{3}H]QNB binding to both receptors was displaced more readily in the absence of GTP than in its presence. This reflects a higher affinity of carbamylcholine for the m2-G_{i2} complex formed in the absence of guanine nucleotides than for the free receptor (*34*).

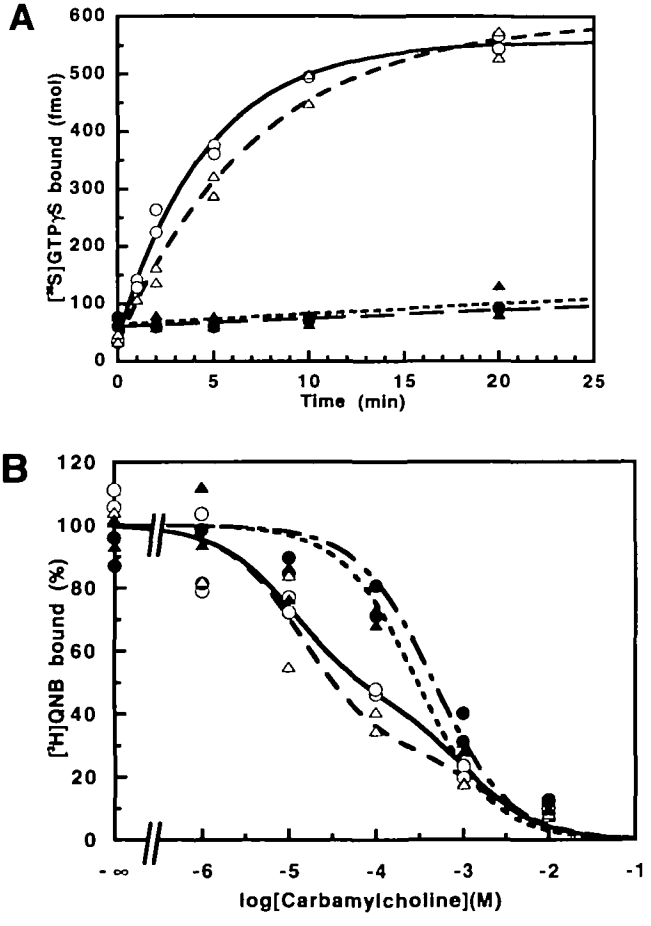

Fig. 5. **Interaction between G protein G_{i2} and m2 receptors with or without a hexahistidine tag.** (A) The m2(N-D) and m2(N-D)(6His) receptors were purified using ABT-agarose, then reconstituted with G protein G_{i2} in lipid vesicles. Reconstituted vesicles were incubated with [^{35}S]GTPγS for the indicated times, and bound [^{35}S]GTPγS was measured. Reaction mixture contained 1.4 pmol of G_{i2}, 36 fmol of m2(N-D) (○ and ●) or 50 fmol of m2(N-D)-(6His) (△ and ▲), 1 mM carbamylcholine (○ and △) or 10 μM atropine (● and ▲), 100 nM [^{35}S]GTPγS and 5 μM GDP in 200 μl. Data shown are representative ones from three experiments that yielded similar results. (B) Displacement by carbamylcholine of [^{3}H]QNB binding to m2(N-D) (36 fmol/tube, ○ and ●) or m2(N-D)-(6His) (50 fmol/tube, △ and ▲) receptors reconstituted with G_{i2} (1.3 pmol/tube) in the presence (● and ▲) or absence (○ and △) of 0.1 mM GTP. Data shown are a representative ones from three experiments that yielded similar results.

These results show that the ability of m2 receptors to interact with and activate G-protein G_{i2} was not affected by the fusion of hexahistidine tag at the carboxyl terminus. The domains in muscarinic receptors that are involved in interaction with G-proteins include the second intracellular loop and the amino and carboxyl terminus of the third intracellular loop (*35-39*). It is not known if the carboxy-terminal tail of muscarinic receptors is involved in the interaction with G-proteins. The involvement of the carboxy-terminal tail in the interaction with G-proteins has been shown for rhodopsin (*40*), EP3 receptors (*41*), and somatostatin receptor 2 subtypes (*42*). The present result is consistent with, although does not prove, the assumption that the carboxy-terminal tail of m2 receptors is not involved in the interaction with G-proteins.

Results shown in Fig. 5 provide direct evidence that the sugar moiety in the m2 receptor is not necessary for interaction of the receptor with G-proteins. This is consistent with the results that the *N*-glycosylation-deleted m2 receptors expressed in Chinese hamster ovary cells retain

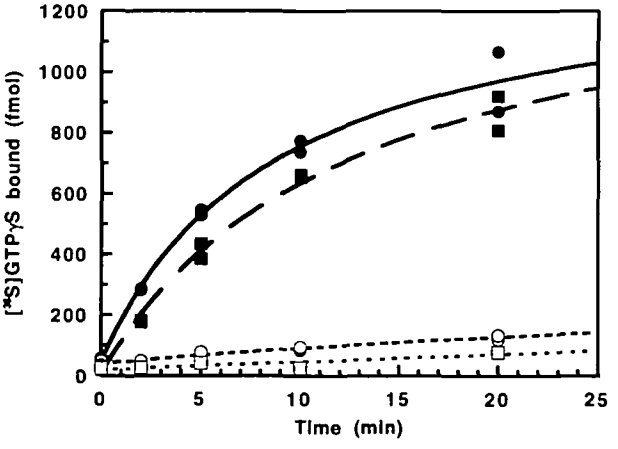

Fig. 6. **Activation of G protein G_{i2} by m2(N-D)(6His) receptors purified with Co^{2+}-immobilized Chelating Sepharose.** The m2(N-D) and m2(N-D)(6His) receptors were purified using Co^{2+}-immobilized Chelating Sepharose, then reconstituted with G protein G_{i2} in lipid vesicles. Reconstituted vesicles were incubated with [^{35}S]-GTPγS binding for the indicated times, and bound [^{35}S]GTPγS was measured. Reaction mixture contained 2.4 pmol of G_{i2}, 12 fmol m2(N-D)(6His) or 17 fmol of m2(N-D)(6His)(I3del), 1 mM carbamylcholine or 10 μM atropine, 100 nM [^{35}S]GTPγS and 5 μM GDP in 200 μl. Data shown are a representative ones from two experiments that yielded similar results.

the ability to inhibit adenylate cyclase (*43*). The interaction of brain muscarinic receptors with G-proteins is also known not to be affected by elimination of sugars by treatment with endoglycosidase F (*44*). The m2 receptor mutant lacking *N*-glycosylation sites does not have structural variability due to heterogenic glycosylation and will be useful for three-dimensional structural analysis.

Hexahistidine-tagged muscarinic receptors purified by use of Co^{2+}-immobilized gels were reconstituted with G_{i2} to confirm that purified receptors are intact in activating G-proteins. As shown in Fig. 6, carbamylcholine stimulated [^{35}S]GTPγS binding to G_{i2} reconstituted with either m2(N-D)(6His) or m2(N-D)(6His)(I3del) receptors purified with the Co^{2+}-immobilized gels. These results provide direct evidence that Co^{2+}-immobilized gels can be used for purification of functionally intact muscarinic receptors. Histidine-tagged rhodopsin and β_2-adrenergic receptors have been purified, but it has not been shown whether histidine-tagging affects their interaction with G-proteins G_t and G_s, respectively. Thus, this is the first demonstration that the hexahistidine-tagged receptors purified with metal-immobilized gels can interact with and activate G-proteins, and it suggests that other G-protein-coupled receptors could be purified with Chelating Sepharose in functionally intact forms.

In conclusion, we have shown that the expression in the baculovirus-Sf9 system and the purification with the Co^{2+}-bound gels of histidine-tagged muscarinic m2 receptors provide a simple means to prepare a large amount of purified receptors with intact function to activate G-proteins. The method described here should be useful for the purification of other G-protein-coupled receptors.

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