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Oligomeric potential of the M_2 muscarinic cholinergic receptor

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Abstract

G protein-coupled receptors are known to exist as oligomers. Although such aggregates often are referred to as dimers, there is little direct evidence regarding their oligomeric size. In the present investigation, c-Myc-, FLAG-, and influenza hemagglutinin (HA)-tagged forms of the M_2 muscarinic receptor have been coexpressed in *Sf*9 cells to probe for aggregates larger than a dimer. Immunochromatography, immunoprecipitation, and immunoblotting were carried out with various combinations of antibodies directed against the different epitopes to demonstrate that all three tagged forms of the receptor can be immunopurified within a single complex. Extracts of the M_2 muscarinic receptor from *Sf*9 cells therefore contain aggregates that are at least trimeric, and the levels detected point to the existence of larger complexes. The data also suggest that the oligomers coexist with a sizeable population of monomers.

Keywords: cooperativity, G protein-coupled receptor, immunoprecipitation, protein purification, receptor oligomerization, signal transduction.

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A growing body of evidence suggests that G protein-coupled receptors can form oligomers (Gomes *et al*. 2001; Milligan 2001; Angers *et al*. 2002; Gazi *et al*. 2002). The functional role of such structures may relate to the intriguing, nucleotide-sensitive dispersion of affinities revealed almost universally in the binding of agonists. The breadth of those dispersions is predictive of efficacy (Birdsall *et al*. 1977; Kent *et al*. 1980; Ehlert 1985), and the underlying effects therefore are likely to have a central role in the signaling process. It has been suggested that the multiple states of affinity are induced by the G protein in an otherwise homogeneous population of mutually independent sites (De Lean *et al*. 1980; Gilman 1987; Birnbaumer *et al*. 1990). This widely held view is problematic, however, and it is particularly difficult to sustain on quantitative grounds (Lee *et al*. 1986; Sinkins and Wells 1993; Green *et al*. 1997). An alternative view attributes the observed heterogeneity to nucleotide-sensitive cooperative effects between interacting sites (Chidiac and Wells 1992; Chidiac *et al*. 1997).

A similar but attenuated heterogeneity also has been detected in the binding of antagonists. Multiple states of affinity were reported early on for cardiac muscarinic receptors in assays with either *N*-[^{3}H]methylscopolamine (Hulme *et al*. 1981; Chidiac *et al*. 1997) or [^{3}H]quinuclidinylbenzilate (Burgisser *et al*. 1982), for D_2 dopamine receptors with [^{3}H]spiperone (Wreggett and De Lean 1984), and for adenosine receptors with [^{3}H]diethylphenylxanthine (Yeung and Green 1983). The binding patterns were found to be sensitive to guanylylimidodiphosphate in a manner that mimics the effect on agonists, but in the opposite sense: whereas guanyl nucleotides favor a state or states of lower affinity for agonists, a state of higher affinity is favored for antagonists. Since the dispersion of affinities is narrower with antagonists than with agonists, the heterogeneity is less apparent.

A distinct and more pronounced heterogeneity has been observed more recently in the binding of antagonists to the M_2 muscarinic receptor and the D_2 dopamine receptor (Wreggett and Wells 1995; Armstrong and Strange 2001; Park *et al*. 2002). In each case, a comparison of two radiolabeled antagonists revealed a difference in apparent capacity such that some receptors appeared to be of anomalously low affinity for one of the ligands. Also, the inhibitory effect of one antagonist on the binding of another was inconsistent with the notion of a competition for mutually independent sites. The effects could be accounted

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Abbreviations used: HA, influenza hemagglutinin; IB, immunoblot; IC, immunocolumn; IP, immunoprecipitation; QNB, (–)-quinuclidinylbenzilate.

for in terms of cooperativity between two or more interacting sites, presumably within an oligomer.

Cooperativity also has been detected within heteromeric complexes of pharmacologically distinct G protein-coupled receptors expressed in recombinant systems. With $GABA_B$ heterooligomers, allosteric interactions between the constituent subunits are critical for agonist-induced activation (Galvez *et al*. 2001). Similarly, cooperative effects have been described between subtype- or receptor-specific ligands to δ- and κ-opiate receptors (Jordan and Devi 1999), β_1 and β_2 adrenergic receptors (Lavoie and Hebert 2003), and D_2 dopamine and somatostatin SSTR5 receptors (Rocheville *et al*. 2000). In each case, ligands specific for one type or subtype of receptor appeared to affect the binding of those specific for another.

Cooperativity implies oligomers, since there appears to be one ligand-binding site per protein chain (Strader *et al*. 1994; Lu *et al*. 2002), but the oligomeric size is unknown. The analysis of cooperative effects in terms of explicit mechanistic models has suggested that the M_2 muscarinic receptor is at least tetrameric (Wreggett and Wells 1995; Chidiac *et al*. 1997; Park *et al*. 2002). Direct biochemical evidence for oligomers derives primarily from measurements of coimmunoprecipitation and energy transfer, which generally have not allowed dimers to be distinguished from larger aggregates. Images obtained by atomic force microscopy of native disk membranes from murine retina have shown that rhodopsin is arranged in large paracrystalline arrays (Liang *et al*. 2003). Similar data are not available for other G protein-coupled receptors, nor is it known whether or to what extent such ordered arrays are formed by other receptors in other tissues. To probe for higher order oligomers of the M_2 muscarinic receptor, we have used a combination of immunochromatography, immunoprecipitation, and immunoblotting to monitor the association of c-Myc-, FLAG-, and influenza hemagglutinin (HA)-tagged variants in extracts from *Sf*9 cells coexpressing all three isoforms.

Materials and methods

Antibodies, ligands and other materials

Agarose-conjugated and horseradish peroxidase-conjugated anti-c-Myc antibodies (9E10, mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Roche Diagnostics (Laval, QC, Canada), respectively. Agarose- and horseradish peroxidase-conjugated anti-FLAG antibodies (M2, mouse) were from Sigma-Aldrich (Oakville, ON, Canada). Agarose-conjugated anti-HA antibody (goat) for immunoprecipitation and horseradish peroxidase-conjugated anti-HA antibody (goat) for western blots were from Bethyl Laboratories (Montgomery, TX, USA). Agarose-conjugated anti-HA antibody (HA-7, mouse) used for immunochromatography was from Sigma-Aldrich. Ascites fluid (mouse) containing a monoclonal antibody directed against the porcine cardiac M_2 muscarinic receptor was purchased from Affinity Bioreagents (Golden, CA, USA). Peptides corresponding to the FLAG (DYKDDDDK) and HA epitopes (YPYDVPDYA) were from Sigma-Aldrich.

[^{3}H]Quinuclidinylbenzilate (lot 3467373, 39 Ci/mmol) was obtained from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Unlabeled (–)-quinuclidinylbenzilate and *N*-methylscopolamine bromide were purchased from Sigma-Aldrich. All other materials were from sources identified previously (Park *et al*. 2001; Park and Wells 2003).

M_2 muscarinic receptor from S*f*9 cells

Baculovirus for the c-Myc-tagged human M_2 muscarinic receptor was obtained from Biosignal (Montreal, QC, Canada), and that for the FLAG-tagged human M_2 muscarinic receptor was prepared as described previously (Park *et al*. 2001). Baculovirus for the M_2 muscarinic receptor tagged with the HA epitope (YPYDVPDYA) was prepared as described previously for the FLAG-tagged M_2 muscarinic receptor (Park *et al*. 2001). All tags were located at the amino terminus of the receptor.

Expression and processing of receptor from *Sf*9 cells was carried out essentially as described previously (Park *et al*. 2001). Cells growing at a density of about 2×10^6 cells/mL were supplemented with 10 μM quinuclidinylbenzilate and then infected with baculovirus. The total multiplicity of infection was five regardless of whether the cells were infected with a single virus or coinfected with two or three viruses added in equal amounts. The baculoviruses were titered by plaque assay. Cells were harvested 2 days after infection, and the membranes were solubilized in digitonin-cholate (0.86% digitonin, 0.17% cholate). The level of receptor in the soluble extract was estimated at a saturating concentration of [^{3}H]quinuclidinylbenzilate (~100 nM) (Park and Wells 2003).

Extracts of receptor in digitonin-cholate (8 mL) were immunopurified on columns packed with agarose-conjugated anti-FLAG or anti-HA antibody to a bed volume of 1 mL. Immunopurification was carried out essentially as described previously (Park and Wells 2003). The anti-HA resin was regenerated with 15 mL glycine-HCl (pH 2.5) and pre-equilibrated with 15 mL buffer A (20 mM KH_2PO_4, 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% digitonin, and 0.02% cholate, pH 7.4 with NaOH). Adsorbed receptor was recovered by competition with the appropriate peptide dissolved in 5 mL of buffer A (100 μg/mL).

Immunoprecipitation and western blotting

Immunoprecipitation, electrophoresis, western blotting, and densitometry were carried out essentially as described previously (Park *et al*. 2001; Park and Wells 2003). Aliquots of solubilized tagged receptor (400–500 μL) were supplemented with a 50% slurry of the appropriate agarose-conjugated antibody (20 μL) and shaken overnight at 4°C. Samples for electrophoresis were heated at 65°C for 5 min prior to loading on pre-cast polyacrylamide gels from Bio-Rad [Ready Gel Tris-HCl, 10% (Figs 1–5) or 7.5% (Fig. 6)]. The conditions of electrophoresis were selected to avoid heat-induced aggregation of the receptor (Park and Wells 2003).

Results

M_2 muscarinic receptors bearing the c-Myc, FLAG, and HA epitopes were expressed separately in *Sf*9 cells and extracted in digitonin-cholate. Such extracts have been shown to

contain functional oligomers of the receptor if the antagonist quinuclidinylbenzilate was present either at the time of infection or when the cells were harvested; oligomers extracted from untreated cells are largely inactive (Park and Wells 2003). The cells therefore were treated with quinuclidinylbenzilate at the time of infection. The tags have been shown not to affect function, at least in binding assays (Park *et al*. 2001; Park and Wells 2003). Western blots prepared with antibodies specific for each of the three epitopes revealed bands that correspond to the monomeric form of the receptor (Fig. 1). Each antibody appeared to be specific for its respective epitope, as there was no cross-reactivity with either of the other two epitopes. The receptor sometimes migrated as a doublet (e.g. Fig. 1b), which may correspond to glycosylated and non-glycosylated forms.

All possible pairs of epitope-tagged receptor were coexpressed in *Sf*9 cells, which then were solubilized and examined for coimmunoprecipitation using the corresponding, complementary antibodies (Fig. 2). For example, the extract from cells coexpressing the c-Myc- and FLAG-tagged receptors was treated with the immobilized anti-c-Myc antibody, and the precipitate was blotted with the anti-FLAG antibody (Fig. 2a); conversely, the precipitate obtained with the anti-FLAG antibody was blotted with the anti-c-Myc antibody (Fig. 2d). In each case, the two epitopes were found to coimmunoprecipitate, indicating that the receptor can be at least dimeric (Figs 2a–f, lane 1). The occurrence of bands migrating as monomers indicates that the conditions of electrophoresis caused the disaggregation of some of the oligomers present in the solubilized extracts.

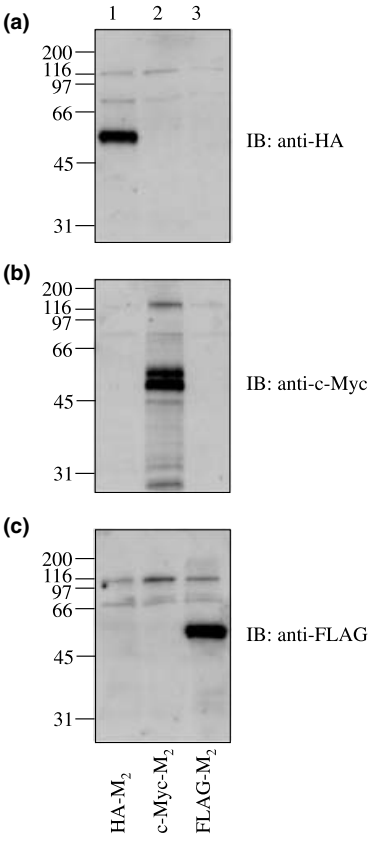

Fig. 1 Epitope-tagged M_2 muscarinic receptors. Cells singly expressing HA- (lane 1), c-Myc- (lane 2), or FLAG-tagged M_2 muscarinic receptor (lane 3) were extracted in digitonin-cholate. Aliquots containing 2.5 ng of receptor, as determined by [^{3}H]QNB, were resolved on polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane and then blotted with the antibodies indicated in the figure. IB, immunoblot.

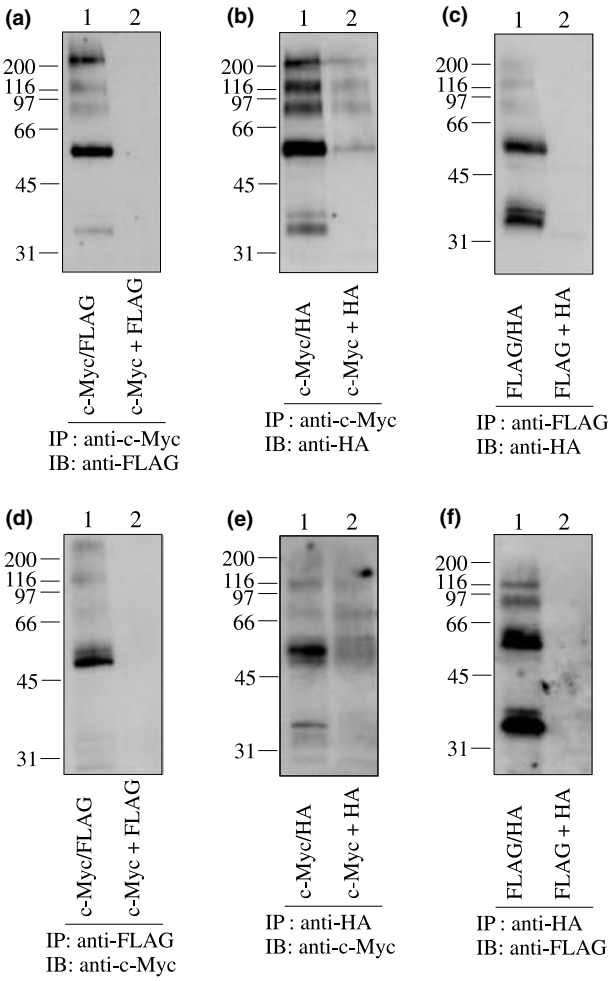

Fig. 2 Coimmunoprecipitation of dually expressed, tagged M_2 muscarinic receptors. All possible pairs of c-Myc-, FLAG-, and HA-tagged M_2 muscarinic receptors were coexpressed in *Sf*9 cells, and the membranes were solubilized in digitonin-cholate (lanes 1; c-Myc/FLAG, c-Myc/HA, or FLAG/HA). Membranes from cells singly expressing one or the other of two mutants were mixed prior to solubilization (lanes 2; c-Myc + FLAG, c-Myc + HA, or FLAG + HA). The soluble extracts were immunoprecipitated and immunoblotted using the combinations of antibodies indicated in the figure. The concentration of receptor used for immunoprecipitation was 5 nM, as determined by [^{3}H]QNB. IB, immunoblot; IP, immunoprecipitation.

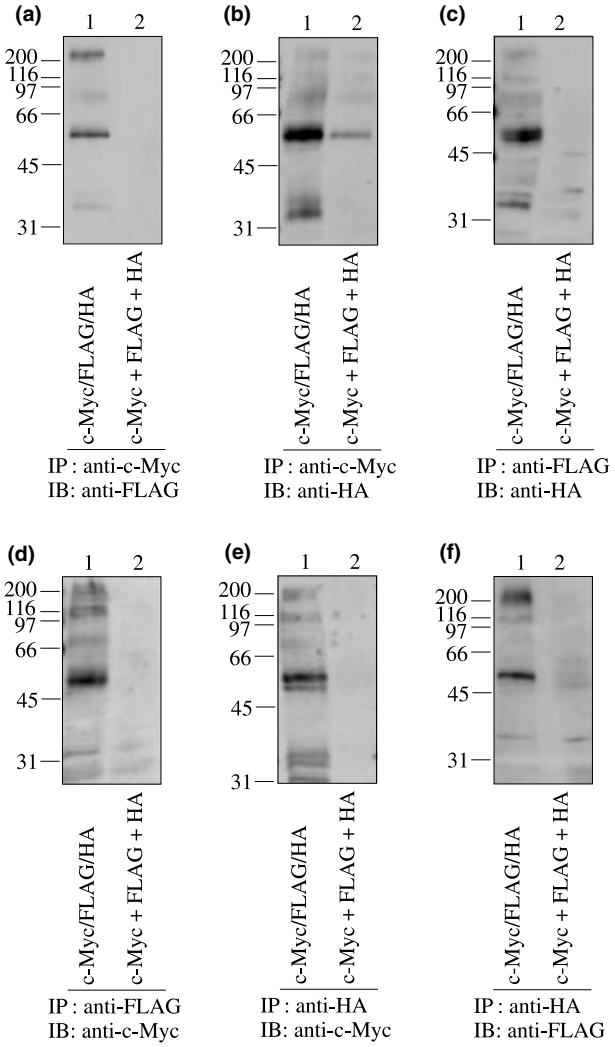

Fig. 3 Coimmunoprecipitation of triply expressed, tagged receptors. c-Myc-, FLAG-, and HA-tagged M_2 muscarinic receptors were expressed together in *Sf*9 cells and extracted into digitonin-cholate (lanes 1, c-Myc/FLAG/HA). Membranes from cells singly expressing the tagged receptors were mixed prior to solubilization (lanes 2, c-Myc + FLAG + HA). The soluble extracts were immunoprecipitated and immunoblotted using the combinations of antibodies indicated in the figure. The concentration of receptor used for immunoprecipitation was 5 nM, as determined by [^{3}H]QNB. IB, immunoblot; IP, immunoprecipitation.

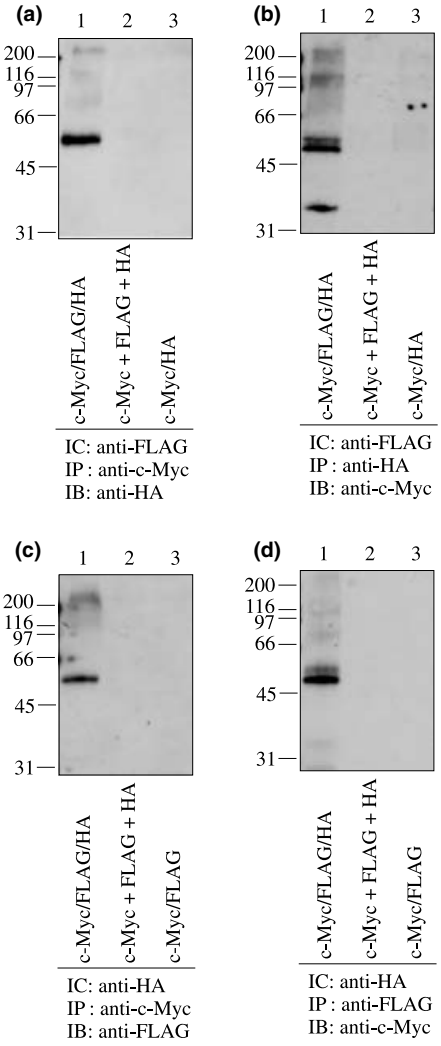

Fig. 4 Detection of an oligomer containing the c-Myc-, FLAG-, and HA-tagged M_2 muscarinic receptors. Tagged receptors were triply coexpressed (lanes 1, c-Myc/FLAG/HA) or dually coexpressed (lanes 3, c-Myc/HA or c-Myc/FLAG) in *Sf*9 cells, and the membranes were solubilized in digitonin-cholate. Membranes from cells singly expressing one or another of the three different receptors were mixed prior to solubilization (lanes 2, c-Myc + FLAG + HA). Solubilized receptor was purified on anti-FLAG-agarose (a and b) or on anti-HA-agarose (c and d), and the immunopurified material was immunoprecipitated and immunoblotted using the combinations of antibodies indicated in the figure. The concentration of receptor subjected to immunoprecipitation was as follows, as determined by [^{3}H]QNB: (a) and (b) lane 1, 4.1 nM; lane 2, 3.2 nM; lane 3, 0.05 nM; (c) and (d) lane 1, 2.0 nM; lane 2, 2.2 nM; lane 3, 0.08 nM. IB, immunoblot; IC, immunocolumn; IP, immunoprecipitation.

To confirm that solubilization does not cause the aggregation of monomers, membranes from cells singly expressing one epitope-tagged form or the other were mixed prior to extraction in digitonin-cholate. Most combinations showed no coimmunoprecipitation, suggesting that solubilization does not promote aggregation and that the oligomers detected in solution had been present in the membrane (Fig. 2, lane 2). Comparatively faint bands were observed with the combination of antibodies used to probe the mixture of c-Myc- and HA-tagged receptors (Figs 2b and e, lane 2). A weak signal also was detected when extracts of singly expressed HA-tagged receptor were treated with the immobilized anti-c-Myc antibody and the precipitate was probed with the anti-HA antibody. This suggests that the agarose-conjugated anti-c-Myc and anti-HA antibodies exhibit some

cross-reactivity and precipitate a small but detectable amount of HA- and c-Myc-tagged receptor.

To test for oligomers larger than a dimer, extracts were prepared from *Sf*9 cells concurrently expressing all three epitope-tagged forms of the receptor. Each of the three possible pairs was found to coprecipitate when immunoprecipitation and blotting were carried out with the corresponding pairs of antibodies (Fig. 3), as described above for the extracts from doubly infected cells. To demonstrate that all three forms of the receptor can occur within the same complex, extracts of triply infected cells were processed on either an anti-FLAG or an anti-HA immunoaffinity column. The yield of receptor eluted specifically by the appropriate peptide was 17% and 14% of that applied to the anti-FLAG and anti-HA column, respectively, as determined by [^{3}H]quinuclidinylbenzilate (Table 1).

The purified receptor from the anti-FLAG immunocolumn was treated with the immobilized anti-c-Myc antibody, and the precipitate was probed with the anti-HA antibody on western blots; similarly, the immunopurified receptor was treated with the immobilized anti-HA antibody and blotted with the anti-c-Myc antibody. The c-Myc- and HA-tagged receptors were found to coimmunoprecipitate in each case (Figs 4a and b). Since the product from the anti-FLAG immunocolumn is expected to contain only FLAG-tagged receptor plus any associated proteins, all three epitopes must be present within a single complex.

The same result was obtained when purified receptor recovered from the anti-HA column was immunoprecipitated with the anti-c-Myc antibody and blotted with the anti-FLAG antibodies, and *vice versa* (Figs 4c and d). Both the c-Myc and the FLAG epitopes were found to coimmunoprecipitate, thereby confirming that at least some of the receptor exists as a trimer or larger oligomer. The immobilized anti-c-Myc antibody was not used for chromatography owing to the low recovery of receptor from that column (Park and Wells 2003).

The specificity of the two immunocolumns was tested by immunopurifying extracts from cells coexpressing either the c-Myc- and HA-tagged receptors or the c-Myc- and FLAG-tagged receptors. As monitored by [^{3}H]quinuclidinylbenzilate, about 95% of the c-Myc- and HA-tagged receptor applied to anti-FLAG-agarose emerged in the flow-through or the wash; similarly, about 96% of the c-Myc- and FLAG-tagged receptor was not retained by anti-HA-agarose (Table 1). Little or no receptor was detected by [^{3}H]quinuclidinylbenzilate in the specific eluate from either immunocolumn (Table 1). Likewise, no receptor was detected when the immunopurified material from either column was probed on western blots with the anti-M_2, anti-FLAG, anti-HA, or anti-c-Myc antibody (Fig. 5, lanes 3 and 6). The immunocolumns therefore appear to be specific for their respective epitopes, adsorbing only those receptors bearing the correct tag. Also, the separation on either column appears to be complete in that the levels of contaminating receptor are below the limits of detection on western blots.

To confirm that the two immunocolumns are without effect on oligomeric status, membranes from singly infected cells expressing the c-Myc-, FLAG-, or HA-tagged receptor were mixed prior to solubilization and subsequent immunopurification. The recovery of receptor upon specific elution, as determined by [^{3}H]quinuclidinylbenzilate, was 15% and 9% of that applied to the anti-FLAG and anti-HA columns, respectively (Table 1). The composition of the purified

Table 1 Efficiency of purification on immunocolumns

			Receptor at different stages of purification (%)			
Immunocolumn	Receptor	Initial (pmol) (A)	Flow-through (B)	Wash (C)	Bound (D)	Specific elution (E)
anti-FLAG-agarose (5)	c-Myc-M_2/FLAG-M_2/HA-M_2	130–139	57 ± 4	12 ± 1	31 ± 4	17 ± 1
anti-FLAG-agarose (2)	c-Myc-M_2 + FLAG-M_2 + HA-M_2	86–107	58 ± 1	16 ± 2	26 ± 3	15 ± 0.03
anti-FLAG-agarose (2)	c-Myc-M_2/HA-M_2	81–91	87 ± 0.3	8 ± 3	4 ± 3	1 ± 0.7
anti-HA-agarose (7)	c-Myc-M_2/FLAG-M_2/HA-M_2	89–143	56 ± 2	10 ± 1	34 ± 2	14 ± 1
anti-HA-agarose (2)	c-Myc-M_2 + FLAG-M_2 + HA-M_2	86–102	69 ± 2	12 ± 1	19 ± 3	9 ± 3
anti-HA-agarose (2)	c-Myc-M_2/FLAG-M_2	118–123	83 ± 7	13 ± 2	4 ± 9	− 0.1 ± 0.4

Solubilized extracts prepared from membranes containing coexpressed receptor (c-Myc-M_2/FLAG-M_2/HA-M_2, c-Myc-M_2/FLAG-M_2, or c-Myc-M_2/HA-M_2) or from membranes containing singly expressed receptor mixed in equal amounts (c-Myc-M_2 + FLAG-M_2 + HA-M_2) were purified on an immunoaffinity resin as shown in the table. The amount of receptor was estimated throughout from the level of binding at a saturating concentration of [^{3}H]QNB (~ 100 nM). That applied to each column is shown in absolute terms (A, initial); that recovered in various fractions or adsorbed is shown as a percentage of the amount applied initially, as follows: receptor not adsorbed to the column (B, flow-through), receptor leached from the column during washing (C, wash), receptor adsorbed to the column (D, bound), and purified receptor eluted specifically with the appropriate peptide (E, specific elution). Receptor adsorbed to the column was taken as the difference between that applied and that recovered in both the flow-through and during washing. Each type of purification was carried out two to seven times, as shown in parentheses, and the values from individual runs were averaged to obtained the means (± SEM) listed in the table (B–E).

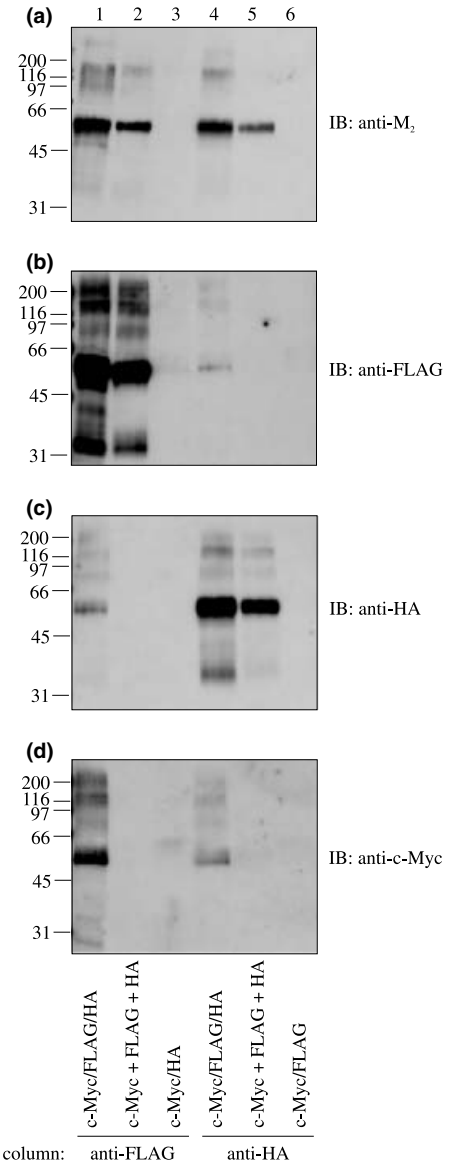

Fig. 5 Specificity of detection and relative abundance of different epitopes in immunopurified M_2 muscarinic receptor. The immunopurified samples used for the immunoprecipitation represented in Fig. 4 were blotted directly with the antibodies indicated in the figure. An equal volume of each specifically eluted sample was loaded on the gels; the amount of receptor was as follows, as determined by [^{3}H]QNB: lane 1, 1.7 ng; lane 2, 1.3 ng; lane 3, 0.02 ng; lane 4, 0.8 ng; lane 5, 0.9 ng; lane 6, 0.03 ng. The corresponding lanes in all four gels (a–d) were loaded with aliquots of the same sample. IB, immunoblot.

fraction was probed on western blots, and the product from both columns reacted positively with the anti-M_2 antibody (Fig. 5a, lanes 2 and 5). Only the FLAG-tagged receptor was detected in the purified product from the anti-FLAG immunocolumn (Figs 5b–d, lane 2). Likewise, the HA-tagged receptor was the only form to emerge from the anti-HA immunocolumn (Figs 5b–d, lane 5). There was no detectable coimmunoprecipitation of the c-Myc- and HA-tagged receptors in the purified fraction from the anti-FLAG column or of the c-Myc- and FLAG-tagged receptors in the purified fraction from the anti-HA column (Fig. 4, lane 2). These results suggest that neither immunoaffinity column promotes the aggregation of receptors or the exchange of monomers among pre-existing oligomers.

Discussion

Direct evidence that G protein-coupled receptors form homo- and heterooligomers has come largely from two approaches: the coimmunoprecipition of immunologically non-uniform pairs and the transfer of resonance energy from a fluorescent or bioluminescent donor to a suitable acceptor (reviewed in Gomes *et al*. 2001; Milligan 2001; Angers *et al*. 2002). Whereas such strategies demonstrate that the receptor can be at least dimeric, they have contributed only indirectly to the determination of an explicit oligomeric size. The approach has been to compare the index of aggregation, measured at known levels of expression, with predictions based on the assumption that oligomers form randomly from the constituent monomers (Ayoub *et al*. 2002; Park *et al*. 2001; Mercier *et al*. 2002). In the current study, three differently tagged forms of the M_2 muscarinic receptor were coexpressed in *Sf*9 cells in order to test directly for trimers or larger aggregates.

A combination of immunochromatography, immunoprecipitation, and immunoblotting has demonstrated that the c-Myc-, FLAG-, and HA-tagged M_2 muscarinic receptors coexist within a complex that can be immunopurified from extracts of cells coexpressing all three proteins. Neither solubilization in digitonin-cholate nor purification on the antibody columns was found to promote aggregation. Also, the antibodies used for immunoprecipitation and detection were specific for their respective epitopes, and non-specific adsorption of the receptor to either anti-FLAG- or anti-HA-agarose was negligible. The detection of a heteromeric complex containing all three tags is therefore indicative of a trimer or larger oligomer that appears to exist in the membrane prior to extraction.

Oligomers of G protein-coupled receptors most likely are formed prior to their transport to the plasma membrane, presumably at the level of protein biogenesis. This is suggested by evidence that the trafficking of wild-type receptors to the cell surface can be inhibited by mutants (e.g. Le Gouill *et al*. 1999; Zhu and Wess 1998); also, the transfer of resonance energy between tagged receptors can be detected in membrane fractions enriched in endoplasmic reticulum (Terrillon *et al*. 2003). It follows that the oligomeric status of the receptor is established early on and therefore is expected to be the same regardless of whether the receptor is localized intracellularly or at the plasma membrane.

Table 2 Relative levels of tagged receptor after purification on anti-FLAG agarose

Status of individual sites within the extract (%)				Predicted recovery of tagged receptor (%)			
Monomer	Dimer	Trimer	Tetramer	c-Myc	FLAG[a]	HA	Total
100	0	0	0	0	33.33	0	33.33
0	100	0	0	11.11	33.33 (3.00)	11.11	55.56
0	0	100	0	18.52	33.33 (1.80)	18.52	70.37
0	0	0	100	23.46	33.33 (1.42)	23.46	80.25
25	75	0	0	8.33	33.33 (4.00)	8.33	50.00
50	50	0	0	5.56	33.33 (6.00)	5.56	44.44
75	25	0	0	2.78	33.33 (12.00)	2.78	38.89
25	0	75	0	13.89	33.33 (2.40)	13.89	61.11
50	0	50	0	9.26	33.33 (3.60)	9.26	51.85
75	0	25	0	4.63	33.33 (7.20)	4.63	42.59
25	0	0	75	17.59	33.33 (1.89)	17.59	68.52
50	0	0	50	11.73	33.33 (2.84)	11.73	56.79
75	0	0	25	5.86	33.33 (5.68)	5.86	45.06
33 1/3	33 1/3	33 1/3	0	9.88	33.33 (3.38)	9.88	53.09
25	25	25	25	13.27	33.33 (2.51)	13.27	59.88

The relative levels of c-Myc-, FLAG-, and HA-tagged receptor expected after purification on the anti-FLAG immunocolumn were calculated for various states of aggregation. It was assumed that all three forms were coexpressed at equal levels, that the status of receptor in solution is static, that oligomers are assembled in a random manner from a sufficiently large pool of monomers, and that purification on the immunocolumn was complete for receptor bearing the FLAG tag. The levels of tagged receptor were calculated from the multinomial expansion (Park *et al*. 2001) and are expressed as a percentage of the total amount of receptor loaded onto the immunocolumn.

[a]The values in parentheses are the ratio of recovered FLAG-tagged receptor to recovered c-Myc- or HA-tagged receptor.

In *Sf*9 cells, an additional step or factor appears to be required if the oligomers formed during biogenesis are to retain function after extraction in detergent. Solubilized oligomers of the M_2 muscarinic receptor were functional, insofar as they bound ligands with characteristic muscarinic affinity, only if the cells were pre-treated with an antagonist prior to solubilization (Park and Wells 2003). With the hydrophobic antagonist quinuclidinylbenzilate, the protective effect was obtained regardless of whether the ligand was added at the time of infection or when the cells were harvested, suggesting that it occurs subsequent to biogenesis. The hydrophilic antagonist *N*-methylscopolamine afforded little or no protection when added at the time of infection. It therefore appears that the functional oligomers obtained upon treatment with quinuclidinylbenzilate derived largely from intracellular compartments, in accord with the observation that the localization of muscarinic receptors in *Sf*9 cells is largely intracellular (Vasudevan *et al*. 1995).

Although at least some of the receptors extracted from ligand-treated *Sf*9 cells are trimeric or larger, the patterns of detection on western blots suggest that there also is a sizeable population of monomers (Fig. 5). The predicted complement of an immunoaffinity-purified complex can be calculated from the multinomial expansion if the oligomers are assembled randomly from a sufficient pool of differently tagged receptors (Park *et al*. 2001). The values listed in Table 2 indicate the amounts of c-Myc-, FLAG-, and HA-tagged receptor that would be recovered from an anti-FLAG immunocolumn loaded with an extract of cells coexpressing the three proteins in equal amounts. The calculation was performed for monomers and different sizes of oligomer, taken alone and in various combinations, and the results are presented as a percentage of the total receptor applied to the column. It was assumed that the separation was complete and without loss: that is, species lacking the FLAG epitope were excluded, whereas all FLAG-containing species were adsorbed and subsequently recovered in full.

When only one form of the receptor is present, the total predicted recovery is 33% with monomers and approaches 100% with larger oligomers. In a population of immunopurified dimers, the FLAG-tagged receptor is three-fold more abundant than either the c-Myc- or the HA-tagged form. In a population of trimers, the FLAG-tagged receptor is 1.8-fold more abundant. With larger oligomers, each tagged form of the receptor approaches one third of the total amount recovered. With an oligomer of any given size, coexisting monomers will reduce the abundance of c-Myc- and HA-tagged receptors relative to that of the FLAG-tagged form. In a mixture of monomers and trimers, for example, the FLAG-tagged receptor is expected to be 2.4-fold more abundant than either the c-Myc- or the HA-tagged form when 25% of the receptors are monomeric; it will be 7.2-fold more abundant when 75% of the receptors are monomeric (Table 2).

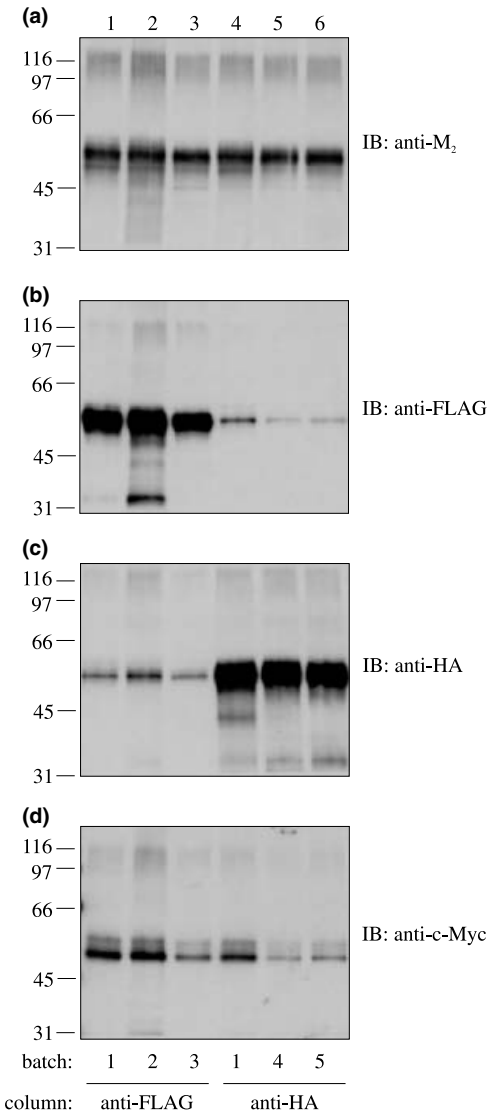

Fig. 6 Immunoreactivity of immunopurified receptor from different batches of triply infected cells. Extracts of cells coexpressing the c-Myc-, FLAG-, and HA-tagged receptors were processed on either anti-FLAG-agarose (lanes 1–3) or anti-HA-agarose (lanes 4–6). The purified receptor was applied to the gel and blotted with the antibodies indicated in the figure. Lanes 1 and 4 were loaded with material from the same batch of coinfected cells (batch 1), and the remaining lanes were loaded with material from four different batches (2–5). The amount of receptor applied to each lane was adjusted to yield bands of similar density with the anti-M_2 antibody (a), and the mean densitometric ratios (± SEM) for all possible combinations of anti-FLAG-purified receptor (lanes 1–3) and anti-HA-purified receptor (lanes 4–6) are as follows ($n = 9$): 1.09 ± 0.08 (anti-FLAG : anti-HA) or 0.95 ± 0.07 (anti-HA : anti-FLAG). The ratios obtained for individual pairs of purified receptor with the anti-M_2 antibody (a) were used to normalize the corresponding values obtained with the other antibodies (b–d). The mean normalized ratios (± SEM) for all possible combinations of purified receptor are as follows (anti-FLAG- : anti-HA-purified receptor or anti HA- : anti-FLAG-purified receptor) ($n = 9$): (b) 39 ± 7 or 0.04 ± 0.01; (c) 0.07 ± 0.01 or 16 ± 2; (d) 2.7 ± 0.5 or 0.56 ± 0.15. Densitometric analyses were performed on the images shown in the figure for panels (a) and (d); those for panels (b) and (c) were performed on images obtained at a lower exposure. The corresponding lanes in all four gels (a–d) were loaded with aliquots of the same sample. IB, immunoblot.

The predictions summarized in Table 2 can be compared with the results in Fig. 5, where the composition of immunopurified receptor from triply infected cells has been examined on western blots. The anti-FLAG antibody gave a strong signal with receptor purified on anti-FLAG agarose (Fig. 5b, lane 1), whereas the density was much less (< 10%) with receptor purified on anti-HA agarose (Fig. 5b, lane 4). Likewise, the anti-HA antibody gave a strong signal with receptor purified on anti-HA agarose (Fig. 5c, lane 4), whereas the density was much less (< 10%) with receptor purified on anti-FLAG agarose (Fig. 5c, lane 1). Although different preparations are compared in the different lanes of Fig. 5, similar quantities of receptor were applied to the gel: the densities were almost the same when blots of the anti-FLAG- and anti-HA-purified receptor were probed with the anti-M_2 antibody (Fig. 5a, lane 1 : lane 4 = 1.1 : 1.0).

To confirm the differences in intensity illustrated in Fig. 5 (cf. lanes 1 and 4), immunopurified receptors from several batches of triply infected cells were examined in parallel (Fig. 6). The amount of receptor applied to the gel was similar in all lanes, as determined by the densities of the bands obtained with the anti-M_2 antibody (Fig. 6a). Blots with the anti-FLAG, anti-HA, and anti-c-Myc antibodies exhibited the pattern established in Fig. 5. The normalized densities of the bands obtained with the anti-FLAG antibody were 39-fold less with the anti-HA-purified receptor than with the anti-FLAG-purified receptor; conversely, the densities obtained with the anti-HA antibody were 16-fold less with the anti-FLAG-purified receptor than with the anti-HA-purified receptor.

The large differences in intensity illustrated in Figs 5 and 6 (panels b and c) suggest that the receptor occurs as a heterogeneous population of monomers and oligomers. If receptors purified on the anti-FLAG and anti-HA columns were exclusively tri- or tetrameric, the ratio of densities obtained with the anti-FLAG and anti-HA antibodies would be expected not to exceed 1.8 : 1 (Table 2). Dimers alone would yield a ratio of only 3 : 1. Values approaching the measured ratio of at least 16 : 1 are expected only if receptors in a trimeric or larger oligomer are outnumbered by monomers or, perhaps, by a mixture of monomers and dimers.

Whereas the complement of c-Myc-tagged receptor is expected to be the same in the product from each immunocolumn, the ratio of normalized densities obtained with the anti-c-Myc antibody reveals a higher level of that epitope in the

preparation from the anti-FLAG column (Fig. 6d, anti-FLAG column : anti-HA column = 2.7 ± 0.5). Calculations analogous to those represented in Table 2 indicate that such a difference can be accounted for by comparatively small differences in the levels of expression: for example, a twofold excess of FLAG-tagged receptor over either of the other two forms, or a twofold excess of c-Myc- and FLAG-tagged receptor over HA-tagged receptor. Variations of this magnitude are consistent with the ratios of intensities obtained from blots with the anti-FLAG and anti-HA antibodies.

Calculations based on the multinomial expansion assume a stochastic process, but differences in intensity such as those seen in Fig. 6 also could arise from non-random effects. Receptors bearing the same tag might cluster preferentially, thereby leading to an excess of homomeric over heteromeric forms. If so, the FLAG and the HA tags appear to be similar in their tendency to favor homooligomers. It has been suggested that G protein-coupled receptors associate at least in part through interactions involving helices 4 and 5 (Guo *et al*. 2003; Liang *et al*. 2003). If the primary contacts are between helical domains, it seems unlikely that a preferential interaction could be achieved by an epitope of 8–10 amino acids located at the amino terminus. An alternative possibility is that an immunocolumn may exhibit a degree of selectivity for oligomers enriched in the complementary epitope. A higher density of complementary tags on the surface of the oligomer may increase its overall affinity for the column and thereby result in a higher level of retention.

The inferred coexistence of monomers and oligomers is consistent with the observation that the two forms of the receptor can be separated and purified under conditions that do not appear to promote interconversion from one form to the other (Park and Wells 2003). Although the distribution of receptors among different states of aggregation is static in solution, and oligomers are not an artifact of solubilization, the origin of monomers and their prevalence in the membrane remains unclear (Park and Wells 2003). It has been suggested on the basis of resonance energy transfer and biotinylation that most if not all β_2 adrenergic and A_{2A} adenosine receptors are oligomeric in the plasma membrane (Mercier *et al*. 2002; Canals *et al*. 2004). If that is so for the M_2 muscarinic receptor, the monomers identified in crude extracts and after purification may arise in part from the disaggregation of oligomers during solubilization. Such an effect may be prevented by quinuclidinylbenzilate when it acts to preserve the functionality of oligomers in purified preparations (Park and Wells 2003).

If the monomers detected in solution also exist in the membrane, they may arise as a by-product of the expression system. G protein-coupled receptors are known to interact with various proteins in addition to the G protein. Examples include chaperones that aid in biogenesis, accessory proteins

Table 3 Effect of unequal expression on the level of tagged receptor found in heteromeric complexes

		Tagged receptor within complexes containing all three tags (%)											
		c-Myc : FLAG : HA = 1 : 1 : 1				c-Myc : FLAG : HA = 1 : 1 : 5				c-Myc : FLAG : HA = 5 : 1 : 5			
Oligomeric status	Combinations of tagged receptor	Total M_2	c-Myc-M_2	FLAG-M_2	HA-M_2	Total M_2	c-Myc-M_2	FLAG-M_2	HA-M_2	Total M_2	c-Myc-M_2	FLAG-M_2	HA-M_2
Trimer	CFH	22.2	7.4	7.4	7.4	8.8	2.9	2.9	2.9	11.3	3.8	3.8	3.8
Tetramer	CCFH	14.8	7.4	3.7	3.7	2.5	1.2	0.6	0.6	10.2	5.1	2.6	2.6
	CFFH	14.8	3.7	7.4	3.7	2.5	0.6	1.2	0.6	2.0	0.5	1.0	0.5
	CFHH	14.8	3.7	3.7	7.4	12.5	3.1	3.1	6.2	10.2	2.6	2.6	5.1
	Total	44.4	14.8	14.8	14.8	17.5	5.0	5.0	7.5	22.5	8.2	6.1	8.2
Pentamer	CCCFH	8.2	4.9	1.6	1.6	0.6	0.4	0.1	0.1	7.8	4.7	1.6	1.6
	CCFFH	12.3	4.9	4.9	2.5	0.9	0.4	0.4	0.2	2.3	0.9	0.9	0.5
	CCFHH	12.3	4.9	2.5	4.9	4.5	1.8	0.9	1.8	11.6	4.7	2.3	4.7
	CFFFH	8.2	1.6	4.9	1.6	0.6	0.1	0.4	0.1	0.3	0.1	0.2	0.1
	CFFHH	12.3	2.5	4.9	4.9	4.5	0.9	1.8	1.8	2.3	0.5	0.9	0.9
	CFHHH	8.2	1.6	1.6	4.9	14.9	3.0	3.0	8.9	7.8	1.6	1.6	4.7
	Total	61.7	20.6	20.6	20.6	25.9	6.5	6.5	12.9	32.1	12.3	7.5	12.3

The complement of c-Myc- (C), FLAG- (F), and HA-tagged (H) receptors within a population of trimeric, tetrameric, or pentameric complexes was determined for three different relative levels of expression (i.e. c-Myc : FLAG : HA = 1 : 1 : 1, 1 : 1 : 5, or 5 : 1 : 5). Only complexes containing at least one equivalent of each tagged form were considered. With a tetramer, for example, the possible combinations are CCFH, CFFH, and CFHH. The probability of forming each combination was calculated according to the multinomial expansion, and the individual values were summed to obtain the total levels of heteromeric trimer, tetramer, or pentamer and the complement of c-Myc, FLAG, and HA tags within each oligomeric state. The values listed in the table are expressed relative to the total amount of receptor taken as 100%. As the size of the oligomer increases, the likelihood of forming a species lacking one or more of the tags decreases.

involved in trafficking, and scaffolds that link the receptor to the elements of other signalling pathways (Brady and Limbird 2002; Hall and Lefkowitz 2002; Bockaert *et al*. 2003). G proteins that couple to the M_2 muscarinic receptor are absent from *Sf*9 cells (Heitz *et al*. 1995; Parker *et al*. 1991); similarly, at least some chaperones are absent altogether or scarce relative to the levels of protein obtained upon baculoviral infection (Higgins *et al*. 2003). The incidence of other receptor-associated proteins is unknown, but amounts native to the insect cell may be limiting relative to the amount of M_2 muscarinic receptor. The absence or deficiency of attendant proteins may hamper the assembly of muscarinic receptors into oligomers, thereby resulting in an accumulation of unassembled monomers.

In a reversal of traditional thinking, the functional relevance of monomers now seems questionable. Oligomerization has been implicated in various aspects of signalling, including trafficking, desensitization, signalling *per se*, and pharmacological diversity (e.g. Chidiac *et al*. 1997; Terrillon and Bouvier 2004). The full complement of oligomeric states is unknown, however, and it remains possible that different stages or branches of the signalling process involve different forms of the receptor, including monomers. Purified monomers of the M_2 muscarinic receptor are active in binding assays (Park and Wells 2003), and monomers may yet be found to play a functional role. Single equivalents of G protein-coupled receptors also may occur in heteromeric complexes with proteins of other classes.

The co-localization of three tagged muscarinic receptors recalls the example of bacteriorhodopsin, an unrelated but structurally similar transmembrane protein that appears to exist as a trimer (Müller *et al*. 1999). Other evidence suggests, however, that G protein-coupled receptors are tetrameric or larger. The potential for extended oligomeric complexes has been demonstrated by atomic force microscopy performed on rhodopsin in disk membranes from the rod outer segments of murine retina, where the receptor was found to exist in lengthy arrays comprising 10–30 pairs (Liang *et al*. 2003). The basic structural unit appears to be a tetramer, but the basic functional unit is unknown. The oligomeric size of the melatonin receptor and the β_2 adrenergic receptor has been inferred from studies in which the association was monitored by measuring the transfer of energy from a bioluminescent tag (Ayoub *et al*. 2002; Mercier *et al*. 2002). The effects of varying the ratio of donor and acceptor or of an unlabeled competitor and the donor-acceptor pair led in each case to the conclusion that the receptor exists as a dimer. In contrast, the β_2 adrenergic receptor has been shown by chemical cross-linking to exist as larger oligomers in the membrane (Salahpour *et al*. 2003). Likewise, higher order oligomers have been required to rationalize the data obtained from disulfide trapping studies on the C5a receptor (Klco *et al*. 2003).

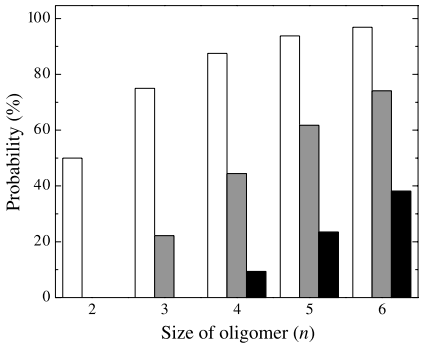

Fig. 7 Effect of oligomeric size and the number of unique tagged species on the likelihood of forming a heteromer containing all available tags. The multinomial expansion was used to calculate the probability of obtaining a heteromeric complex comprising *n* equivalents of receptor and containing at least one equivalent of each unique tag when two (white bars), three (gray bars), or four (black bars) differently tagged proteins are coexpressed in equal amounts.

If the M_2 muscarinic receptor forms contacts similar to those found in the paracrystalline arrays of paired rhodopsin, the detection of a heteromer containing all three tags suggests that the receptor is at least tetrameric. The possibility of larger oligomers also emerges from the apparent abundance of such heteromeric species under different conditions. When receptor from triply infected cells was purified on either the anti-FLAG or the anti-HA immunocolumn, structures containing the other two tags were readily detected in both products (Fig. 4). The cells in those experiments were infected with roughly equal quantities of the three baculoviruses. In contrast, similar assays on receptor from cells in which the different forms were expressed at different levels led to the detection of all three tags with some combinations of antibodies but not others. This dependence on the relative levels of expression suggests that oligomers of the M_2 muscarinic receptor are larger than a trimer.

The probability of obtaining all three tags in the same complex is shown in Table 3 for a trimer, a tetramer, and a pentamer when the levels of expression are the same (c-Myc : FLAG : HA = 1 : 1 : 1) or different (1 : 1 : 5, 5 : 1 : 5). If the receptor is trimeric, the number of subunits equals the number of tags. It therefore is equally likely that any one tag will occur within a complex containing all three, regardless of the relative levels of expression. In that case, immunopurification followed by immunoprecipitation and immunoblotting is expected to yield a positive result with all combinations of antibodies. If the receptor is a tetramer or a pentamer, however, the likelihood of finding any one tag in association with the other two will depend upon the tag and the relative level of expression. It follows that the likelihood of detecting all three tags in the same complex will be greater with some combinations of antibodies than with others, as observed in the present investigation.

When two or more differently tagged receptors are expressed at equal levels, and when the number of unique tags equals the number of subunits within the complex, heteromers containing each of the tagged forms decrease in quantity with the size of the complex (Fig. 7). In the case of a trimer, complexes containing all three tags will account for 22% of the total receptor; in the case of a tetramer, complexes containing all four tags will account for only 9% of the total receptor. This statistical disadvantage, together with the probable yield of sequential biochemical procedures, seems likely to preclude the direct identification of larger oligomers. In contrast, the likelihood of finding a full complement of tags in the same complex increases with the size of the oligomer as the number of subunits exceeds the number of tags. In the present example of three different tags, the probability of detecting all three forms of the receptor increases from 22% in a trimer to 74% for the same three tags in a hexamer (Fig. 7). If the M_2 muscarinic receptor forms comparatively large oligomers, a complex containing four unique tags ought to be detectable in studies analogous to those described here.

The suggestion that the M_2 muscarinic receptor forms large aggregates has emerged previously from the quantitative assessment of binding properties in terms of mechanistic models. Whereas the nucleotide-sensitive binding of agonists to myocardial membranes is inconsistent with the notion of a transient 1 : 1 complex between receptor and G protein (Lee *et al*. 1986; Green *et al*. 1997), the data can be described in terms of cooperativity within a receptor that is at least tetravalent (Chidiac *et al*. 1997). Cooperativity within a comparatively large aggregate also can account for otherwise paradoxical effects identified in the binding of radiolabeled antagonists to muscarinic receptor extracted from porcine atria in cholate–NaCl (Park *et al*. 2002). A two-fold difference in the apparent capacity for *N*-[^{3}H]methylscopolamine and [^{3}H]quinuclidinylbenzilate and an accompanying non-competitive effect of unlabeled *N*-methylscopolamine on the binding of [^{3}H]quinuclidinylbenzilate can be described in terms of cooperative effects among four or more interacting sites (Park *et al*. 2002). Similar behaviour has been observed for the M_2 muscarinic receptor extracted from cholesterol-enriched *Sf*9 cells, also in cholate–NaCl. In that case, however, at least eight interacting sites are required for cooperativity to reconcile the difference in apparent capacity for the two radioligands with the inhibitory effect of unlabeled *N*-methylscopolamine on the binding of [^{3}H]quinuclidinylbenzilate (A. T. Colozo, P. S.-H. Park, C. S. Sum, and J. W. Wells, unpublished observations). The cooperative effects that emerge under some conditions not only imply oligomers but also suggest a functional role, in that such effects can account for the allosteric interactions between agonists and guanyl nucleotides (Chidiac and Wells 1992; Chidiac *et al*. 1997).

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