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Nature of the oligomers formed by muscarinic m2 acetylcholine receptors in *Sf*9 cells

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Abstract

Wild-type, FLAG-tagged, and *c-myc*-tagged muscarinic m2 receptors extracted in digitonin–cholate from singly and co-infected *Sf*9 (*Spodoptera frugiperda*) cells were indistinguishable in their binding of [^{3}H]quinuclidinylbenzilate, either before or after purification. The FLAG epitope was found to coimmunoprecipitate with the *c-myc* epitope when co-infected cells were solubilised in digitonin–cholate, *n*-dodecyl-β-D-maltoside or Lubrol-PX. The degree of coprecipitation in digitonin–cholate was unaffected by preincubation of the extract for up to 60 min at 30°C, with or without muscarinic receptor ligands; no coimmunoprecipitation occurred in mixed extracts from singly infected cells. As measured by [^{3}H]quinuclidinylbenzilate, the efficiency of immunoprecipitation from co-infected cells was 87% of that from singly infected cells. The amount of receptor immunoprecipitated from the latter, as determined by densitometry, was 2.3-fold that expected from the loss of binding from the extract. The data suggest that at least some of the receptors were trimeric or larger and that oligomers neither formed nor dissociated under the conditions of the experiments. Also, some receptors appear to be non-functional or latent in digitonin-solubilised extracts.

Keywords: Muscarinic receptor; Oligomer; Oligomeric integrity; G protein-linked receptor; *Sf*9 cell

1. Introduction

It is commonly held that a G protein, or a subunit thereof, shuttles between its receptor on the one hand and the effector on the other (De Lean et al., 1980; Gilman, 1987; Conklin and Bourne, 1993). The receptor therefore is thought to act catalytically, visiting and activating multiple G proteins in succession. Central to this view is the notion that a reversible interaction between receptor and G protein accounts for the GTP-sensitive heterogeneity seen in the binding of agonists (De Lean et al., 1980; Ehlert, 1985). Mechanistic proposals along such lines typically embody several postulates, two of which are of interest here. First, heterogeneity is induced by the G protein in a population of mutually independent and otherwise identical sites; second, the transient receptor-G protein complex forms and dissociates spontaneously and rapidly on the time-scale of a binding assay.

Although attractive on many counts, the idea of a transient complex between receptor and G protein becomes problematic when applied in a quantitative and mechanistically consistent manner (e.g., Neubig et al., 1985; Lee et al., 1986; Wong et al., 1986; Wreggett, 1987; Graeser and Neubig, 1993; Neubig, 1994; Green et al., 1997; Wenzel-Seifert et al., 1999). Various lines of evidence suggest that the problem lies in the postulates noted above (Chidiac et al., 1997). In particular, a number of otherwise puzzling effects revealed in the binding properties can be explained in terms of cooperativity among interacting sites (e.g., Henis and Sokolovsky, 1983; Mattera et al., 1985; Boyer et al., 1986; Sinkins and Wells, 1993; Wreggett and Wells, 1995; Chidiac et al., 1997; Jordan and Devi, 1999). Cooperativity implies oligomers, however, and early biochemical studies on muscarinic receptors suggested that multimeric forms were a minor component at best (e.g., Peterson et al., 1986, 1988; Schimerlik et al., 1986; Berstein

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et al., 1988). In contrast, more recent data point increasingly to oligomers as a common feature of G protein-linked receptors under at least some conditions (e.g., Potter et al., 1991; Maggio et al., 1993; Wreggett and Wells, 1995; Hébert et al., 1996, 1998; Monnot et al., 1996; Jordan and Devi, 1999; Zeng and Wess, 1999; Angers et al., 2000; Lee et al., 2000; Rocheville et al., 2000).

Questions regarding the role of oligomers in G protein-mediated signalling relate in part to oligomeric size and stability. Dimers and larger oligomers have been identified on Western blots of several receptors, including muscarinic receptors (Wreggett and Wells, 1995; Zeng and Wess, 1999), and tetramers or larger aggregates have been inferred from functional studies (Wreggett and Wells, 1995; Chidiac et al., 1997). Oligomers also have been identified by coimmunoprecipitation of differently tagged receptors, but the data presently available do not permit a distinction among different oligomeric states. Data on oligomeric stability are similarly inconclusive. Agonists have been reported to favour dimerisation of β_2-adrenoceptors as revealed on Western blots (Hébert et al., 1996). Similarly, agonists and the level of expression were reported to affect the fluorescence resonance energy transfer between labelled somatostatin receptors in Chinese hamster ovary (CHO) cells (Rocheville et al., 2000). In both cases, it was suggested that agonists promote dimerisation within the membrane. In contrast, changes in bioluminescence resonance energy transfer between β-adrenoceptors have prompted the suggestion that agonist-dependent effects derive from conformational changes within the complex (Angers et al., 2000). Agonists were found to be without effect on the degree of dimerisation of muscarinic m3 receptors or κ-opioid receptors, as revealed on Western blots (Jordan and Devi, 1999; Zeng and Wess, 1999).

Owing to uncertainty over the interpretation of recent data, we have used coimmunoprecipitation to investigate the size and stability of oligomers formed by *c-myc*- and FLAG-tagged muscarinic m2 receptors extracted from *Sf*9 cells. The results suggest that the receptor retains its oligomeric integrity irrespective of agonists or antagonists, and that it exists at least in part as a trimer or larger oligomer. A preliminary report of this work has appeared elsewhere (Park et al., 2001).

2. Materials and methods

2.1. Ligands, detergents, antisera and other materials

[^{3}H]Quinuclidinylbenzilate was obtained from NEN Life Science Products (lots 3329907 and 3363333, 42 Ci/mmol), and *N*-methylscopolamine bromide was from Sigma-Aldrich. Digitonin used to solubilise and purify the receptor was purchased from Wako Bioproducts at a purity near 100%. Sephadex G-50 columns used in the binding assays were pre-equilibrated and eluted with buffer solutions containing digitonin obtained from Boehringer Mannheim (purity, > 75% or 93%) and Calbiochem (purity, > 75%). Cholic acid and Lubrol-PX (polyoxyethylene-9-lauryl ether, polidocanol) were purchased from Sigma-Aldrich, and *n*-dodecyl-β-D-maltoside was purchased from Calbiochem.

HEPES was obtained as the free acid from Roche Diagnostics. EDTA was obtained as the free acid from British Drug Houses and as the disodium salt from Bioshop, Canada. EGTA was obtained as the free acid from British Drug Houses. Bacitracin, all protease inhibitors, Tween-20, and Sephadex G-50 (fine) were from Sigma-Aldrich. Gentamycin, Fungizone and fetal bovine serum were from Life Technologies (Gibco-BRL), and dithiothreitol was from Bio-Rad Laboratories. Protein was estimated by the Lowry method using reagents and bovine serum albumin, taken as the standard, purchased from Pierce. Solubilised and purified receptor was concentrated as required by means of Centricon-10 and Centriprep-30 filters (Amicon) purchased from Millipore.

Murine antibodies to the *c-myc* epitope (9E10) were obtained as follows: unconjugated form, Sigma-Aldrich; a conjugate with horseradish peroxidase, Boehringer Mannheim; and conjugated to agarose beads, Santa Cruz Biotechnology. Murine antibodies to the FLAG epitope were obtained from Sigma-Aldrich, both in the unconjugated form and as a conjugate with horseradish peroxidase. Protein G PLUS-agarose was purchased from Santa Cruz Biotechnology.

2.2. Muscarinic receptors from Sf9 cells

Baculoviruses coding for the wild-type and *c-myc*-tagged human muscarinic m2 receptors were obtained from Biosignal (Montreal). Baculovirus coding for receptor containing the FLAG epitope (DYKDDDDA) was constructed from the cDNA for the human muscarinic m2 receptor, obtained from Dr. T.I. Bonner, Laboratory of Genetics, NIMH, Bethesda, MD 20892-4094. The FLAG tag was preceded by a cleavable signal sequence shown to enhance translocation of the receptor into the membrane of the endoplasmic reticulum and production of the functional protein (Guan et al., 1992). The *Nco*I and *Xba*I digestion sites were added to the amino-terminal and carboxyl-terminal, respectively, of the cDNA for the m2 receptor, which was fused to the FLAG coding sequence at the *Nco*I site. This construct was inserted into the pBlueBac4.5 baculovirus transfer vector (Invitrogen) at the *Xho*I and *Xba*I sites. The transfer vector was transfected into the baculovirus by means of the Bac-N-Blue transfection kit from Invitrogen. Baculovirus was titered using the plaque-assay method of Summers and Smith (1988).

*Sf*9 cells were cultured at 27°C in Ex-Cell 400 insect media (JRH Biosciences) containing 2% fetal bovine serum, 1% Fungizone, and 0.1% gentamycin. Confluent cells growing at a density of about 2×10^6 cells/ml were

infected with baculovirus at a multiplicity of infection of 5. Between 48 and 72 h after infection, the cells were collected by centrifugation at $1000 \times g$. The pellet then was suspended in buffer A (20 mM KH_2PO_4, 20 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 200 μg/ml bacitracin, 2 μg/ml leupeptin, 20 μg/ml pepstatin A, and 156 μg/ml benzamidine to pH 7.40 with NaOH) with three bursts of a Brinkman Polytron (setting 6, 10 s), and the resulting homogenate was centrifuged for 45 min at 4°C and $100{,}000 \times g$. The membranes were washed once by resuspension and centrifugation in the same manner, and the pellet was stored at −75°C.

Solubilisation of muscarinic receptors in digitonin–cholate, *n*-dodecyl-β-D-maltoside, or Lubrol-PX was carried out in a single extraction as described by Peterson et al. (1988). Washed *Sf*9 membranes were thawed on ice and suspended in buffer B (20 mM KH_2PO_4, 20 mM NaCl, 1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride to pH 7.60 with NaOH; 5.5 mg of protein/ml) by means of the Polytron (setting 6, 10 s). Detergent was then added from a stock solution prepared in deionised water (digitonin plus cholate, 4% and 0.8%, respectively; *n*-dodecyl-β-D-maltoside and Lubrol-PX, 12%). The suspension was shaken on a horizontal shaker for 10 min at room temperature and then centrifuged for 45 min at 4°C and $100{,}000 \times g$. The supernatant fraction was stored on ice until required subsequently for electrophoresis or binding assays. The final concentrations of digitonin and cholate were 0.86% and 0.17%, respectively; that of *n*-dodecyl-β-D-maltoside and Lubrol-PX was 1.1%.

Solubilisation in cholate plus NaCl was carried out according to a procedure modified from that described by Carson (1982). Washed membranes were thawed on ice and suspended in buffer C (40 mM NaH_2PO_4, 1 M NaCl, 1 mM EGTA, 0.16% sodium cholate, and 0.1 mM phenylmethylsulfonylfluoride to pH 7.40 with NaOH; 2 mg of protein/ml), and the mixture was shaken for 1 h at room temperature. The suspension then was diluted 1:1 with a solution of 40 mM NaH_2PO_4 and 1 mM EGTA adjusted to pH 7.40 with NaOH and was centrifuged for 40 min at 4°C and $100{,}000 \times g$. The final concentrations of cholate and NaCl were 0.08% and 0.5 M, respectively, and the supernatant fraction was stored on ice until required.

Receptors extracted in digitonin–cholate were purified via affinity chromatography on 3-(2′-aminobenzhydryloxy)tropane-Sepharose (ABT-Sepharose) as described previously (Wreggett and Wells, 1995), and the final concentrations of digitonin and cholate were 0.1% and 0.02%, respectively. Purified receptor was stored at −75°C until required for subsequent assays.

2.3. Immunoprecipitation, electrophoresis, and Western blotting

To immunoprecipitate the *c-myc*-tagged receptor, samples (500–650 μl) containing solubilised or purified receptor (9.7–23 pmol receptor or 370–750 μg protein) plus the agarose-conjugated anti-*c-myc* antibody (40 μg) were shaken overnight at 4°C. To immunoprecipitate the FLAG-tagged receptor, samples (500 μl) containing the receptor (9.7–19 pmol) plus the anti-FLAG antibody (0.09 μg/μl) were first shaken for 1 h at 4°C; the mixture was then supplemented with a slurry of protein G PLUS-agarose (20 μl) and shaken overnight at 4°C. Immunoadsorbed receptor was collected by centrifugation for 5 min at 4°C and $1000 \times g$, and the precipitated beads were washed four times by resuspension in 1 ml of a washing buffer (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, pH 7.40 with NaOH) and subsequent centrifugation (5 min, 4°C, $1000 \times g$). The pellet from the last wash was suspended in 40 μl of buffer D (sample buffer; 65 mM Tris, 10% glycerol, 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 0.001% bromophenol blue, pH 6.80 with HCl) and boiled for 5 min, and an aliquot of the mixture or the supernatant fraction (20 μl) was applied to the top of the polyacrylamide gel.

Electrophoresis was performed according to the method of Laemmli (1970) in the Mini-Protean II cell from Bio-Rad. Polyacrylamide gels either were prepared in situ (7.5% resolving gel, 4% stacking gel, 0.1% SDS) or were purchased precast from Bio-Rad (Tris–HCl Ready Gel; 7.5% resolving gel, 4% stacking gel). Samples were mixed with buffer D, boiled for 5 min and applied to the gel. Resolved proteins were transferred to nitrocellulose membranes (Bio-Rad, 0.45 μm) in a Mini Trans-Blot Transfer Cell (Bio-Rad). The membranes were incubated with the appropriate antibodies and visualised by chemiluminescence using reagents and film purchased from Amersham (ECL™, Hyperfilm MP). Densitometric scanning was performed at a resolution of 600 dpi, and the intensity was determined using 1D Image Analysis software (Kodak Digital Science).

2.4. Binding assays

Muscarinic receptor ligands were dissolved in buffer E (assay buffer; 20 mM HEPES, 20 mM NaCl, 1 mM Na_2EDTA, 5 mM $MgSO_4$, 0.1 mM phenylmethylsulfonylfluoride, pH 7.4 with NaOH) supplemented with the detergent used to solubilise the receptor. The concentration of digitonin, *n*-dodecyl-β-D-maltoside, Lubrol-PX or cholate was 0.1%, and digitonin was accompanied by 0.02% cholate. An aliquot of the ligand-containing solution (33–50 μl) was added to the solubilised receptor (10 or 20 μl in cholate–NaCl, 3 μl in other detergents) in polypropylene microcentrifuge tubes. The total volume was 53 μl throughout. The reaction mixture was incubated for 2 h at 30°C, and bound [^{3}H]quinuclidinylbenzilate was then separated by applying an aliquot of the sample (50 μl) to a column of Sephadex G-50 fine (0.3×6.5 cm). The column was pre-equilibrated and eluted with buffer F (column buffer; 20 mM HEPES, 1 mM EDTA, 20 mM NaCl, 5

mM $MgSO_4$, pH 7.40 with NaOH) supplemented with digitonin, *n*-dodecyl-β-D-maltoside, Lubrol-PX or cholate, as appropriate, at a concentration of 0.017%. All of the eluant up to and including the void volume was collected (1.45 ml) and assayed for radioactivity. Non-specific binding was taken throughout as total binding in the presence of 1 mM unlabeled *N*-methylscopolamine. Other details were as described previously (Wreggett and Wells, 1995).

The binding of [^{3}H]quinuclidinylbenzilate was characterised at graded concentrations of the radioligand as described in the Results. Routine estimates of capacity were performed with [^{3}H]quinuclidinylbenzilate at a concentration of about 0.3 μM. Under those conditions, non-specific binding represented less than 10% of total binding in all detergents.

2.5. Analysis of data

All data were analysed according to the expression $B_{obsd} = B_{sp} + NS([P]_t - B_{sp})$, where B_{obsd} and B_{sp} represent total observed binding and specific binding, respectively. The variable $[P]_t$ represents the total concentration of [^{3}H]quinuclidinylbenzilate, and the parameter NS is the fraction of unbound radioligand that appears as non-specific binding. The value of B_{sp} was calculated according to the Hill equation, formulated as shown in Eq. (1). The parameter n_H represents the Hill coefficient, and EC_{50} is the concentration of unbound radioligand when B_{sp} equals $1/2\,B_{max}$. Eq. (1) was solved numerically as described previously [cf. Eq. 204 in Wells, 1992]. Further details regarding the analyses and related statistical procedures have been described elsewhere (Wells, 1992; Chidiac et al., 1997).

$$B_{sp} = B_{max}\frac{\left([P]_t - B_{sp}\right)^{n_H}}{EC_{50}^{n_H} + \left([P]_t - B_{sp}\right)^{n_H}}. \quad (1)$$

3. Results

Muscarinic m2 receptors tagged with the *c-myc* and FLAG epitopes were identified by their respective antibodies on Western blots prepared from singly infected and co-infected *Sf*9 cells. Each epitope was detected as an immunoreactive band at the position expected for the monomeric form of the receptor (i.e., 54,600 ± 700 Da, $N = 13$) (Fig. 1), based on the amino acid sequence (i.e., 51,715 Da). There was no reaction between the anti-*c-myc* antibody and the FLAG-tagged receptor or between the anti-FLAG antibody and the *c-myc*-tagged receptor. Most preparations also contained a second reactive species of lower molecular size (39,800 ± 500 Da, $N = 13$). Immunoreactive bands corresponding to dimers and larger aggregates generally were absent, although some gels revealed comparatively weak bands at those locations. In

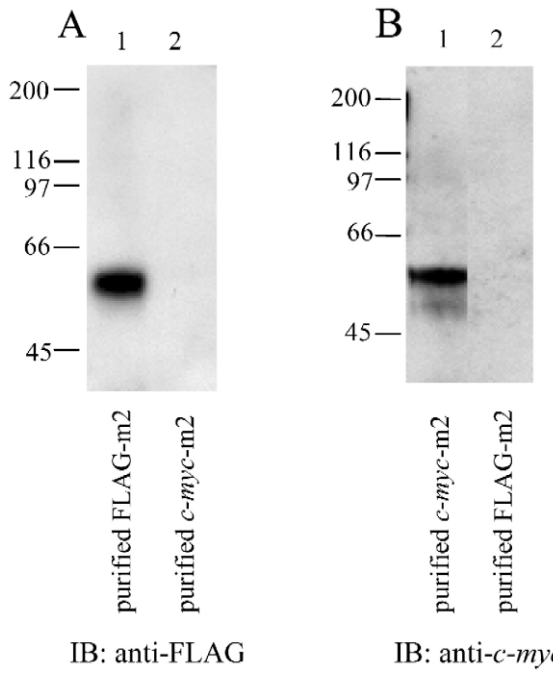

Fig. 1. Immunodetection of epitope-tagged human muscarinic m2 receptors purified from *Sf*9 cells. Tagged receptor was purified from cells infected with either the FLAG-tagged or the *c-myc*-tagged m2 baculovirus. Samples containing 10 ng of receptor were subjected to electrophoresis on polyacrylamide gels (7.5%), blotted onto nitrocellulose and detected with the anti-FLAG (A) and anti-*c-myc* (B) antibodies.

such cases, the slower-moving bands appeared to be more prevalent with antibodies from some manufacturers and were absent from controls lacking the relevant epitope. Oligomeric species have been less abundant with extracts from *Sf*9 cells than with extracts and purified receptors from porcine heart (e.g., Wreggett and Wells, 1995), where their prevalence seems to derive in part from the use of chloroform and methanol to extract the receptor from digitonin–cholate prior to electrophoresis. When that extraction is omitted, the density of bands corresponding to oligomers is less. Similarly, monomers appear to outnumber dimers on blots of the β_2-adrenoceptor extracted and purified from *Sf*9 cells (Hébert et al., 1996).

The functionality of m2 receptors expressed in *Sf*9 cells was established in binding studies with [^{3}H]quinuclidinylbenzilate. The affinity of the radioligand was the same for the wild-type receptor and for each of the tagged products, both in unprocessed extracts and after purification; also, the Hill coefficient was near or indistinguishable from 1 throughout (Table 1). Such tags therefore appear to be without effect on function, in accord with previous reports (e.g., Hébert et al., 1996; Mouillac et al., 1992; Parker et al., 1991; Schöneberg et al., 1995; Zeng and Wess, 1999). Similarly, the binding properties were the same when the *c-myc*- and FLAG-tagged receptors were co-expressed. There was no discernible effect of either tag on the stability of binding or on the yield obtained after solubilisation or purification, at least in digitonin–cholate (Table 2).

Table 1
Specific binding of [^{3}H]quinuclidinylbenzilate to solubilised and purified muscarinic receptors from *Sf*9 cells
Muscarinic receptors were extracted from *Sf*9 cells in digitonin–cholate and purified, as described under Materials and methods. The binding of [^{3}H]quinuclidinylbenzilate to the unprocessed extract and the purified receptor was measured over a range of concentrations (0.03–562 nM) and the data were analysed in terms of the Hill equation. The values of log EC_{50} and n_H from individual experiments were averaged to obtain the means (±S.E.M.) listed in the table; the individual values of B_{max} are within the range shown. The number of experiments is shown in parentheses (unprocessed extract, purified receptor).

Receptor	Unprocessed extract			Purified receptor		
	log EC_{50}	n_H	B_{max} (pM)	log EC_{50}	n_H	B_{max} (pM)
Wild-type-m2 (14, 5)	−9.27 ± 0.05	1.29 ± 0.10	148–1054	−8.89 ± 0.04	1.17 ± 0.15	214–1003
c-myc-m2 (5, 4)	−9.25 ± 0.11	0.89 ± 0.06	318–440	−9.00 ± 0.02	0.96 ± 0.05	524–1606
FLAG-m2 (3, 5)	−9.05 ± 0.08	0.83 ± 0.15	517–772	−8.99 ± 0.09	0.76 ± 0.02	672–831
Coexpressed *c-myc*- and FLAG-m2 (3, 2)	−9.16 ± 0.03	1.01 ± 0.03	656–721	−8.95 ± 0.00	0.87 ± 0.06	214–927

Accordingly, it is assumed below that the ratio of FLAG to *c-myc*-tagged receptor in preparations from co-infected cells was the same prior to solubilisation and after subsequent processing. No specific binding of [^{3}H]quinuclidinylbenzilate was detected in preparations from *Sf*9 cells that were not infected with the baculovirus. Receptors from *Sf*9 cells resembled m2 receptors purified from porcine atria, both in their affinity for the radioligand and in the overall pattern of binding as reflected in the Hill coefficient (Wreggett and Wells, 1995).

For cells expressing both epitope-tagged receptors, the multiplicity of infection of each baculovirus was adjusted to achieve equal levels of expression. This was confirmed by Western blotting and densitometry for one batch of purified receptor, which yielded a value of 1.1 for the molar ratio of FLAG to *c-myc* epitopes (i.e., FLAG-m2, 32 ng; *c-myc*-m2, 29 ng). The level of expression of each tagged product was determined from assays in which the receptor from co-infected cells was run in parallel with purified receptor from singly infected cells. The latter was applied in four different amounts over the range from 5 to 60 ng, as determined from the specific binding at 300 nM [^{3}H]quinuclidinylbenzilate. For each epitope, the density of the band increased linearly with the amount of receptor over the range examined in the assays. The ratio of FLAG- to *c-myc*-tagged receptor was not determined for unpurified extracts or immunoadsorbed material. With unpurified extracts, quantitative assessments were precluded by high backgrounds. Studies in which uninfected and singly infected cells were tested for the missing epitope indicated that the anti-*c-myc* and anti-FLAG antibodies both interacted with unidentified constituents of the *Sf*9 cells.

Extracts from cells co-expressing the *c-myc*- and the FLAG-tagged receptors were prepared in different detergents and tested for coimmunoprecipitation of the two epitopes. In the case of digitonin–cholate, *n*-dodecyl-β-D-maltoside and Lubrol-PX, material immunoprecipitated by the anti-*c-myc* antibody reacted positively to the anti-FLAG antibody; conversely, material extracted in digitonin–cholate and immunoprecipitated by the anti-FLAG antibody reacted positively to the anti-*c-myc* antibody (Fig. 2). Receptors from *Sf*9 cells therefore appear to exist at least partly as dimers or larger oligomers, notwithstanding the weakness or lack of corresponding bands on polyacrylamide gels. There seems to be little if any correlation between the occurrence of multimeric species on the gels and their detection via coimmunoprecipitation. Such multimeric forms presumably are labile under the conditions of electrophoresis, and the oligomers observed on Western blots are probably only a remnant of the native state. No

Table 2
Yields obtained upon solubilisation and purification of wild-type and epitope-tagged muscarinic receptors from *Sf*9 cells
Yields are given relative to the total cellular protein and as a percentage of receptor in the initial soluble extract for solubilised and purified receptors, respectively. The means (±S.E.M.) are shown in parentheses. The quantity of receptor was estimated at a saturating concentration of [^{3}H]quinuclidinylbenzilate (0.3 μM), and total cellular protein was estimated using the Lowry method. Each preparation of receptor was from a different batch of *Sf*9 cells except in the case of solubilised *c-myc*-m2 (five batches) and solubilised *c-myc*-m2 plus FLAG-m2 (six batches). There is no significant difference between the means for *c-myc*-m2 and FLAG-m2 in either the solubilised or the purified preparation ($P > 0.05$).

Expression	Solubilised receptor		Purified receptor	
	Preparations	Yield, nmol/g protein	Preparations	Yield, %
Wild-type-m2	3	13–17 (15 ± 1)	1	15
c-myc-m2	6	4.6–14 (10 ± 2)	3	10–29 (20 ± 5)
FLAG-m2	6	6.4–16 (12 ± 1)	3	11–26 (19 ± 4)
c-myc-m2 plus FLAG-m2	8	5.4–18 (11 ± 2)	2	14–16

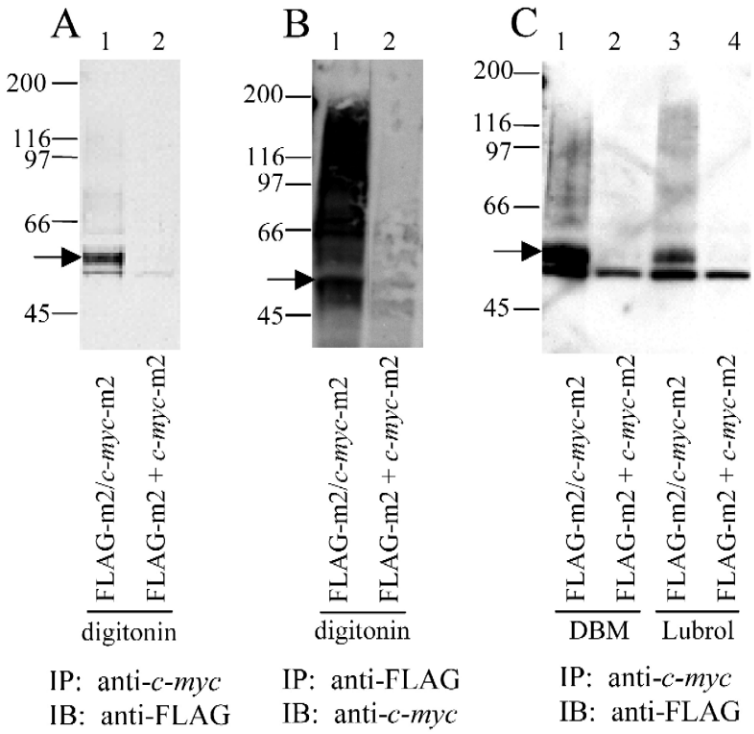

Fig. 2. Coimmunoprecipitation of tagged human muscarinic m2 receptors extracted in different detergents. *Sf*9 cells were singly infected or co-infected with the FLAG-tagged and *c-myc*-tagged m2 baculoviruses. Homogenates from co-infected cells were solubilised in digitonin (A and B, lanes 1), *n*-dodecyl-β-*D*-maltoside (C, lane 1) or Lubrol-PX (C, lane 3). Homogenates containing equal amounts of FLAG-tagged and *c-myc*-tagged receptor from singly infected cells were mixed and solubilised in the same detergents (A, lane 2; B, lane 2; C, lanes 2 and 4). The final concentrations were as follows, for co-infected and singly infected cells, respectively: in digitonin–cholate, 0.77g protein/l and 0.99 g protein/l (A), 39 nM receptor for both (B); in *n*-dodecyl-β-*D*-maltoside, 1.5 g protein/l and 1.3 g protein/l (C); and in Lubrol-PX, 0.72 g protein/l for both (C). An aliquot of each extract, containing 500 μg protein (A), 19 pmol receptor (B), 750 μg protein (C, lanes 1 and 2) or 370 μg protein (C, lanes 3 and 4), was mixed overnight with the agarose-conjugated anti-*c-myc* antibody (A, C) or the anti-FLAG antibody bound to protein G-PLUS-agarose (B). The complex was then precipitated, subjected to electrophoresis, blotted onto nitrocellulose and tested for immunoreactivity to the anti-FLAG antibody (A, C) or the anti-*c-myc* antibody (B). The band indicated by the arrow is the monomeric m2 receptor. The lower band is the heavy IgG chain of the anti-*c-myc* antibody, which is recognised by the anti-mouse secondary antibody.

coimmunoprecipitation was detected when homogenates from singly infected cells were premixed prior to the addition of detergent (Fig. 2). The failure to generate oligomers through mixing suggests that those found after co-infection were present in the cell and not created artifactually during the process of solubilisation. There also was no detectable coimmunoprecipitation when co-infected cells were extracted with cholate–NaCl.

The lifetime of the oligomers identified by coimmunoprecipitation and immunodetection was examined using receptors extracted from co-infected cells in digitonin–cholate. To test for dissociation into monomers, the extract was incubated at 30°C for different periods of time prior to immunoprecipitation by the agarose-conjugated anti-*c-myc* antibody. The amount of precipitated receptor detected by the anti-FLAG antibody on Western blots was independent of time for up to 60 min, the longest period of such experiments (Fig. 3). Also, the intensity of the band was unchanged when the receptor was incubated for 60 min at saturating concentrations of the agonist carbachol or the inverse agonist atropine (Fig. 3).

To probe for the formation of oligomers in solution, *c-myc*-tagged and FLAG-tagged receptors extracted from singly infected cells were mixed and kept overnight at 4°C. The assays were performed at two concentrations of total receptor (i.e., 49 and 490 nM), and those at the lower concentration included parallel samples containing 1 mM carbachol or 1 mM atropine. No coimmunoprecipitation was detected with any of the samples (Fig. 4). Similar results have been reported for differently tagged forms of a human m3 receptor lacking the central portion of the third intracellular loop (Zeng and Wess, 1999). Also, no coimmunoprecipitation was detected when purified *c-myc*- and FLAG-tagged receptors were mixed in equal amounts at a total concentration of 39 or 78 nM.

To measure the efficiency of immunoprecipitation, extracts of singly infected and co-infected cells were tested for binding at a saturating concentration of [^{3}H]quinuclidinylbenzilate before and after addition of the agarose-conjugated anti-*c-myc* antibody. The results are summarised in Table 3, where the amount of receptor removed from the supernatant fraction is expressed relative to that

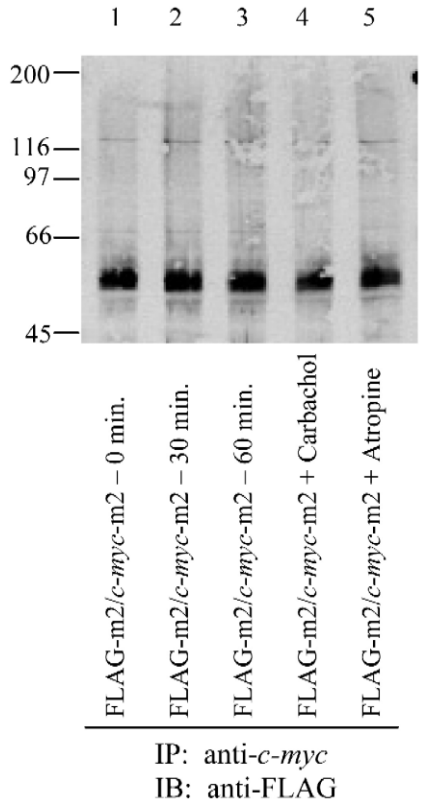

Fig. 3. Oligomeric stability of the muscarinic m2 receptor. *Sf*9 cells were co-infected with the FLAG-tagged and *c-myc*-tagged m2 baculoviruses and solubilised in digitonin–cholate. Aliquots of the extract were incubated at 30°C for 0, 30 and 60 min in the absence of ligand (lanes 1, 2 and 3, respectively) or for 60 min in the presence of 1 mM carbachol (lane 4) or 1 mM atropine (lane 5). The samples then were mixed overnight with the agarose-conjugated anti-*c-myc* antibody, and the precipitate was separated on polyacrylamide and tested for immunoreactivity to the anti-FLAG antibody. A band corresponding to the monomeric form of the receptor was detected under all conditions at essentially the same intensity.

present initially. The efficiency of immunoprecipitation from extracts of singly infected cells was highest in digitonin–cholate and *n*-dodecyl-β-D-maltoside, while a somewhat lower value was obtained in Lubrol-PX. Only 7% of the sites were precipitated from cholate–NaCl. With receptors extracted in digitonin–cholate, the efficiency obtained with co-infected cells was 87% of that obtained with singly infected cells (i.e., 30.6 ± 3.9 and 35.1 ± 2.8, respectively). Doubling the amount of anti-*c-myc* antibody had no appreciable effect on the efficiency of precipitation, as tested in one experiment on a preparation of singly infected cells solubilised in digitonin–cholate. The efficiency of the anti-FLAG antibody was not determined.

In order to estimate the stoichiometry between immunoprecipitated receptor and loss of binding, the intensities of the bands on Western blots were measured by densitometric analysis and compared with the decrease in the capacity of the digitonin-solubilised extract for [^{3}H]quinuclidinylbenzilate. The amount of *c-myc*-tagged receptor immunoadsorbed from extracts of singly infected cells was determined by means of a standard curve prepared with four samples of purified receptor (5–60 ng) that were blotted and assayed in parallel with the extract, as described above. The total intensity in each lane was taken as the sum of the individual intensities of all bands corresponding to the full-length monomeric receptor and multiples thereof. Various amounts of extracted receptor were used within the range defined by the standard curve, and the total intensity of the blot increased linearly with the decrease in capacity for [^{3}H]quinuclidinylbenzilate. Quantified in this manner, the ratio of precipitated protein to the loss of ligand-specific sites from the supernatant fraction was 2.3 (S.E.M. = 0.3, $N = 16$). [^{3}H]Quinuclidinylbenzilate therefore appears to underestimate the amount of

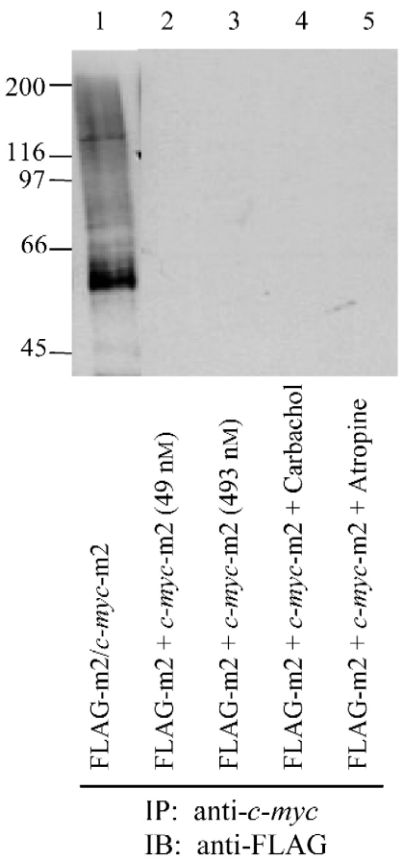

Fig. 4. Failure of differently tagged human m2 receptors to form nascent oligomers. *Sf*9 cells were singly infected or co-infected with the FLAG-tagged and *c-myc*-tagged m2 baculoviruses and solubilised in digitonin–cholate. Samples containing equal amounts of FLAG-tagged and *c-myc*-tagged receptor from the single infections were mixed at a total receptor concentration of either 49 (lane 2) or 493 nM (lane 3) and kept overnight at 4°C. Parallel samples at the lower concentration of receptor contained 1 mM carbachol (lane 4) or 1 mM atropine (lane 5). The concentration of receptor in a control from co-infected cells was 39 nM (lane 1). Receptors from all samples were mixed overnight with the agarose-conjugated anti-*c-myc* antibody and, following electrophoresis, were tested for immunoreactivity to the anti-FLAG antibody.

Table 3
Efficiency of immunoprecipitation from solubilised *Sf*9 cells
An aliquot of solubilised extract (500 μl) containing the amount of receptor listed in the table was mixed with anti-*c-myc* antibody conjugated to agarose beads (40 μg, 20 μl). Prior to the addition of the antibody, a sample of the same extract was removed for subsequent use as a control. The mixture was shaken overnight at 4°C and then centrifuged for 5 min at 4°C and 1000 × *g*. The quantity of receptor in the supernatant was estimated from the amount of specific binding at a saturating concentration of [^{3}H]quinuclidinylbenzilate (0.3 μM). The efficiency of immunoprecipitation was taken as the difference in binding between the antibody-treated sample and the control, measured in parallel, and expressed relative to the amount of specific binding in the control. Binding in the control was unaffected by shaking overnight and subsequent centrifugation. Values are listed as the mean ± S.E.M., and the number of experiments is shown in parentheses. The results for co-expressed *c-myc*- and FLAG-tagged receptors are from three batches of co-infected cells.

Extract	Receptor (ng)	Efficiency (%)
c-myc-m2 receptor		
Digitonin–cholate (9)	170–928	35 ± 3
n-Dodecyl-β-D-maltoside (3)	88–165	33 ± 3
Cholate–NaCl (3)	13–25	7 ± 3
Lubrol-PX (3)	75	24 ± 0.3
Co-expressed c-myc-m2 and FLAG-m2 receptor		
Digitonin–cholate (12)	165–846	31 ± 4

receptor removed by immunoprecipitation, suggesting that the extracts contain a subpopulation of latent sites or otherwise non-functional protein.

4. Discussion

Muscarinic receptors expressed in *Sf*9 cells form oligomers that appear to retain their multimeric status under a variety of conditions. When *c-myc*- and FLAG-tagged receptors were co-expressed, the two epitopes could be coimmunoprecipitated from extracts in digitonin–cholate, *n*-dodecyl-β-D-maltoside and Lubrol-PX. The receptors therefore exist as oligomers in the original extracts, and presumably in the cells, but their oligomeric status is unclear. On the basis of the immunoblot alone, there could be a unique oligomeric state of indeterminate size or a mixture of monomers and various multimeric forms. Virtually no coimmunoprecipitation was observed with extracts prepared in cholate–NaCl, perhaps owing to the low efficiency of immunoprecipitation in that detergent. Opioid receptors also exist as oligomers under a variety of conditions, since κ homodimers and κ–δ heterodimers have been shown to coimmunoprecipitate from extracts in deoxycholate–NaCl, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), *n*-dodecyl-β-D-maltoside, and Triton-X (Jordan and Devi, 1999).

Further tests in digitonin–cholate suggest that the oligomeric status of the receptor is static, at least in that detergent. No coimmunoprecipitation was observed when cells infected separately with the FLAG-m2 and *c-myc*-m2 baculoviruses were disrupted and mixed prior to solubilisation; similarly, there was no effect when the two receptors were solubilised and then mixed, either with or without muscarinic receptor ligands. It follows that no sustained interaction occurred between monomers or any of the oligomers present in the original extracts. The absence of any time-dependent decrease in the degree of coimmunoprecipitation from extracts of co-infected cells indicates that preformed oligomers do not fragment into monomers. New aggregates therefore may be precluded by a scarcity of monomers within a receptor population composed largely of oligomers. Alternatively, monomers may coexist with oligomers but fail to interact owing to interference from the detergent or to local concentrations well below those in native membranes, the long reaction time notwithstanding. Finally, oligomers may be formed and stabilised prior to their insertion in the cellular membrane. In studies on the platelet-activating factor receptor (Le Gouill et al., 1999) and the vasopressin V2 receptor (Zhu and Wess, 1998), for example, mutants have been shown to inhibit the surface expression of the corresponding wild-type receptor in CHO and COS-7 (African green monkey kidney) cells. Moreover, the localisation of muscarinic m3 receptors and dopamine D_{2S} receptors in *Sf*9 cells is primarily intracellular (Vasudevan et al., 1995; Grünewald et al., 1996), although β-adrenoceptors and m3 receptors both have been shown to elicit G protein-mediated responses in *Sf*9 cells (e.g., Hébert et al., 1996; Parker et al., 1991; Vasudevan et al., 1992). It follows that oligomers identified in the present investigation are likely to derive mostly from intracellular compartments, such as the endoplasmic reticulum, and further oligomerisation may not occur elsewhere. In any event, the data indicate that oligomers neither form nor dissociate to an appreciable extent under the conditions of the assays.

The efficiency of immunoprecipitation from extracts of co-infected cells was 87% of that from extracts containing only the *c-myc*-tagged receptor. This value is comparatively high, as described below, and it suggests that at least some of the receptors are trimeric or larger. Since the two forms of the receptor are identical, apart from the tags, it is likely that all possible combinations within the oligomer emerge in a random fashion. The relative amount of each combination therefore is given by the binomial distribution, provided that the complement of tagged forms within the pool of available receptors remains unchanged as oligomerisation proceeds. The expected efficiency of immunoprecipitation can be calculated according to Eq. (2) for an oligomer in which x out of n subunits contain the epitope recognised by the immobilised antibody and expressed at mole fraction P. The other epitope therefore is expressed at mole fraction $1 - P$.

$$E(P) = 100 \sum_{x=1}^{n} \frac{n!}{x!(n-x)!} P^x (1-P)^{n-x}. \qquad (2)$$

The ratio of *c-myc* to FLAG epitopes in co-infected cells was assumed to be 1:1 (i.e., $P = 0.5$), as inferred from densitometric analysis of the electrophoretic bands obtained with receptor purified from co-infected and singly infected cells. If the anti-*c-myc* antibody were to remove 100% of the sites from extracts containing the *c-myc*-receptor alone, and if the receptors existed exclusively as dimers, Eq. (2) predicts that 75% of the sites would be removed from extracts of co-infected cells (Table 4). Similarly, the predicted efficiency is 88% for trimers and 94% for tetramers. The measured value of 87% therefore points to a trimer, at least in digitonin–cholate, if the oligomeric status of all receptors is the same and the measured efficiency is taken at face value.

The estimated ratio of *c-myc*- to FLAG-tagged receptor is accompanied by some uncertainty, which derives in part from the semi-quantitative nature of densitometric assays and from the possibility of small, undetected differences in the recovery of differently tagged proteins after solubilisation and purification. Also, the standard error on the efficiency of immunoprecipitation is comparatively high (i.e., 87 ± 13), and an estimate of the 95% confidence interval encompasses the value predicted for a population of dimers (i.e., 87 ± 25). Finally, the interpretation of efficiency is less straightforward if there are multiple oligomeric states or co-existing populations of monomers and oligomers. The observed value of 87% therefore is consistent with various scenarios, but most point to at least a subpopulation of receptors that are trivalent or larger.

An accompanying population of monomers would reduce the overall efficiency of immunoprecipitation to a minimum of 50%. If the sites were divided equally between monomers and dimers, for example, the predicted efficiency of immunoprecipitation is 63% of that in the control rather than 75% with dimers alone; with monomers and tetramers, the predicted efficiency is 72% rather than 94% with tetramers alone (Table 4). Monomers therefore tend to increase the oligomeric size required of at least some receptors if the predictions of Eq. (2) are to agree with the measured efficiency of 87%. More complex mixtures similarly point to a sub-population of comparatively large oligomers (Table 4).

The use of Eq. (2) and the values listed in Table 4 are based on several assumptions regarding the process of immunoprecipitation and its dependence on the oligomeric status of the receptor. It is assumed that the affinity of the agarose-coupled antibody for the corresponding epitope is the same regardless of whether the tagged receptor exists within an oligomer or as a monomer. It also is assumed that the efficiency of immunoprecipitation is the same regardless of the number of tagged receptors or their position within the oligomer. Since clusters seem more likely than monomers to impede access of the antibody to the epitope or otherwise to interfere with adsorption, exceptions to these assumptions might be expected to decrease the observed efficiency of immunoprecipitation.

Table 4
Efficiency of immunoprecipitation for different combinations of monomers and oligomers
The expected efficiency of immunoprecipitation was calculated according to Eq. (2) for a 50:50 mixture of two, differently tagged receptors in various states of aggregation. The antibody was assumed to recognise only one of the tags. The immunoprecipitation was assumed to be complete for all receptors bearing that tag, regardless of their oligomeric status, and for receptors bearing the unrecognised tag within the same oligomer.

Status of individual sites within the extract (%)				Efficiency (%)
Monomer	Dimer	Trimer	Tetramer	
100	0	0	0	50.00
0	100	0	0	75.00
0	0	100	0	87.50
0	0	0	100	93.75
50	50	0	0	62.50
70	30	0	0	57.50
90	10	0	0	52.50
50	0	50	0	68.75
50	0	0	50	71.88
33 1/3	33 1/3	33 1/3	0	70.83
25	25	25	25	76.56

The uncertainty arising from such effects diminishes as the efficiency of immunoprecipitation from extracts of co-infected cells approaches that from singly infected controls, and as the efficiency of the control approaches 100%.

The consequences of non-random immunoprecipitation are illustrated in Table 5 for a mixture in which the sites are distributed equally between monomers and dimers. If the antibody does not discriminate between the two forms, the predicted efficiency is 63% (Table 4). If the ratio of adsorption for monomers and dimers is 2:1, however, the predicted efficiency is 58% relative to that obtained with singly infected controls (Table 5, cf. Cases 1 and 2). The prevalence of dimers therefore would be underestimated if the preferential adsorption were not recognised (cf. Table 4). The efficiency would be reduced to less than 50% of the control value, which in itself points to some degree of oligomerisation, if the adsorption of dimers were limited to those bearing two *c-myc* epitopes (Table 5, Cases 3 or 4). Oligomers would be overestimated if the antibody were biased against monomers (Table 5, cf. Cases 1 and 5).

A further assumption is that all forms of the receptor are saturated by [^{3}H]quinuclidinylbenzilate at a concentration of about 300 nM; that is, the values listed in Table 3 presuppose a 1:1 stoichiometry between the number of binding sites lost from the solubilised extract and the amount of *c-myc*-tagged receptor adsorbed by the immobilised antibody. This contrasts with the value of 2.3 obtained when the immunoreactive bands were quantified by densitometry. The difference may arise in part from a background signal that is higher with extracts than with the purified receptor used to construct the standard curve. Also, some receptors may be inactive in binding assays performed on the extract. In a mixture of non-interconvert-

Table 5
Overall efficiency of immunoprecipitation for a mixture of monomers and dimers when the efficiency depends upon the oligomeric status of the receptor
The overall efficiency of immunoprecipitation was calculated for a 50:50 mixture of *c-myc*- and FLAG-tagged receptors distributed equally between monomers and dimers. The immobilised antibody was assumed to recognise only the *c-myc* epitope. The efficiencies of immunoprecipitation for individual species within the mixture are as follows: case 1, monomer—100%, dimer—100%; case 2, monomer—100%, dimer—50%; case 3, monomer—100%, homodimer (i.e., *c-myc*/*c-myc*)—100%, heterodimer (i.e., *c-myc*/FLAG or FLAG/*c-myc*)—0%; case 4, monomer—100%, homodimer—50%, heterodimer—0%; case 5, monomer—50%, dimer—100%.

	Single infection (*c-myc*)			Co-infection (*c-myc* + FLAG)						Efficiency (%) (A/B)
	Monomer	Dimer	Total (A)	Monomer		Dimer			Total (B)	
				c-myc	FLAG	*c-myc*/*c-myc*	*c-myc*/FLAG FLAG/*c-myc*	FLAG/FLAG		
Relative number of sites	50	50	100	25.0	25	12.50	12.50 + 12.50	12.50	100.00	
Adsorption, case 1	50	50	100	25.0	0	12.50	12.50 + 12.50	0	62.50	62.50
case 2	50	25	75	25.0	0	6.25	6.25 + 6.25	0	43.75	58.33
case 3	50	50	100	25.0	0	12.50	0	0	37.50	37.50
case 4	50	25	75	25.0	0	6.25	0	0	31.25	41.67
case 5	25	50	75	12.5	0	12.50	12.50 + 12.50	0	50.00	66.67

ing monomers and dimers, for example, the overall efficiency would be overestimated if monomers were not to bind the radioligand. If the sites were distributed equally between the two forms, the efficiency would be 62.5% overall but 75% with respect to the number of labelled sites. Since the non-binding species is transparent, the measured efficiency is the same as that predicted for the oligomer alone (cf. Table 4).

A potentially more complex situation arises if the non-binding but tagged protein can form oligomers either with itself or with the functional receptor. If the different tagged species aggregate randomly, the probability of obtaining a hetero-oligomer of size n and comprising x_j units of each species X_j ($j = 1, 2, \ldots, k$) is given by the multinomial distribution function. The relationship is shown in Eq. (3), where k is the number of different tagged species, and P_j is the mole fraction contributed by those of type j (DeGroot, 1989). The expected efficiency of immunoprecipitation will be the sum of the probabilities for all possible combinations of functional receptor in which the oligomer contains one or more equivalents of the epitope recognised by the antibody.

$$Pr(X_1 = x_1, \ldots, X_k = x_k) = \frac{n!}{\prod_{j=1}^{k} x_j!} \prod_{j=1}^{k} P_j^{x_j}. \quad (3)$$

A mixture of two forms of the receptor, one active (R_A) and one inactive (R_I), would yield four species under the conditions of the present experiments (i.e., *c-myc*–R_A, FLAG–R_A, *c-myc*–R_I, FLAG–R_I). The effect of the single non-binding component is illustrated for a dimer and a trimer in Table 6, where the efficiency of immunoprecipitation is seen to be unchanged provided that the ratio of active to inactive receptor is the same for both the *c-myc*-tagged and the FLAG-tagged species (i.e., *c-myc*–R_A/*c-myc*–R_I = FLAG–R_A/FLAG–R_I, or *c-myc*–R_A/FLAG–R_A = *c-myc*–R_I/FLAG–R_I). If the ratios differ, however, the efficiency of immunoprecipitation will differ from that expected for the functional receptor alone. The efficiency also will differ if the aggregation is not random.

A special case of the situation described above arises if the inactive form of the receptor occurs as a consequence of aggregation. The system may be intrinsically asymmetric owing to differences in affinity that emerge when the monomeric units assemble to form the oligomer. Alternatively, asymmetry may be induced in an otherwise symmetric system as a result of negative cooperativity between successive equivalents of the radioligand. In either case,

Table 6
Effect of a non-binding receptor on the efficiency of immunoprecipitation
The effect of a non-binding, tagged receptor on the efficiency of immunoprecipitation was calculated according to Eq. (3). Four tagged species were present in the relative amounts listed in the table ($k = 4$), and the receptor was assumed to exist exclusively as a dimer ($n = 2$) or a trimer ($n = 3$). The immobilised antibody was assumed to recognise only the *c-myc* epitope, and the efficiency was taken as the expected decrease in the capacity of the solubilised extract for the radioligand. Other conditions were as described in the legend to Table 4.

Relative level of expression (%)				Efficiency (%)	
Functional receptor		Non-binding receptor		Dimer	Trimer
c-myc-m2	FLAG-m2	*c-myc*-m2	FLAG-m2		
50	50	0	0	75.00[a]	87.50[a]
25	25	25	25	75.00	87.50
35	35	15	15	75.00	87.50
15	15	35	35	75.00	87.50
35	35	25	5	80.00	92.00
25	5	35	35	93.33	97.33
60	40	0	0	84.00[a]	93.60[a]
40	26 2/3	20	13 1/3	84.00	93.60
40	60	0	0	64.00[a]	78.40[a]
26 2/3	40	13 1/3	20	64.00	78.40

[a]The expected efficiency of immunoprecipitation in the absence of non-binding receptor (i.e., $k = 2$), when Eq. (3) becomes equivalent to the binomial distribution (Eq. (2)).

the presence of latent sites in the extract but not in the purified preparation would lead to a discrepancy between the amount of immunoprecipitated protein and the loss of binding from the supernatant fraction. The efficiency of immunoprecipitation as inferred from the latter is unaffected, however, provided that the different proteins aggregate randomly and the ratio of *c-myc*-tagged to FLAG-tagged species is constant as described above. If all of the receptors were tetrameric, for example, the efficiency would be the same at 93.75% irrespective of whether each oligomeric unit failed to bind one, two or three equivalents of the radioligand.

Studies here and elsewhere have provided some evidence that G protein-linked receptors indeed can adopt a low-affinity or apparently latent state, the prevalence of which is associated with low levels of cholesterol. Membranes from *Sf*9 cells contain cholesterol at levels that are 10–20-fold lower than those in plasma membranes from mammalian cells (Gimple et al., 1995; Marheineke et al., 1998). With *Sf*9 cells expressing the human oxytocin receptor, the number of receptors in a state of high affinity for the agonist [^{3}H]oxytocin was increased more than threefold when a complex of cholesterol and methyl-β-cyclodextrin was added to the culture medium or to the membranes obtained from a sucrose gradient (Gimple et al., 1995). Similarly, the capacity for [^{3}H]quinuclidinyl-benzilate has been found to increase twofold or more when cholesterol hemisuccinate is added to muscarinic m2 receptors extracted from *Sf*9 cells in digitonin–cholate (Park, P., Pawagi, A.B., Wells, J.W., unpublished observations). In contrast, the high-affinity binding of [^{3}H]oxytocin and the antagonist [^{3}H]propionyl-[1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyltyrosine),4-threonine,8-ornithine,9-tyrosylamide]vasotocin ([^{3}H]propionyl-OTA) to the oxytocin receptor was reduced when myometrial plasma membranes were depleted of cholesterol through treatment with methyl-γ-cyclodextrin. The reduction was unaffected by GTPγS, indicating that the effect of cholesterol was independent of the G protein (Klein et al., 1995). Latent sites therefore may account for the stoichiometric excess of *c-myc*-tagged protein identified on the Western blots over the loss of capacity from the extract.

It has been shown previously that the binding properties of cardiac muscarinic receptors can be rationalised in terms of cooperative interactions provided that the receptor is at least tetravalent, presumably a tetramer. Moreover, agreement is achieved with the simplifying assumption that the receptor retains its oligomeric integrity under the conditions of the assays; that is, either the tetramer does not dissociate into smaller oligomers or monomers, or dissociated subunits regroup without exchanging partners. The implication that binding is independent of the local concentration of receptors is supported by similarities between receptors in sarcolemmal membranes and purified receptors in solution. (Wreggett and Wells, 1995; Chidiac et al., 1997). The stability of the supposed oligomer is indicated more directly by the present data. There is no exchange of subunits among any of the species present, and aggregates are retained after solubilisation in at least three of the four detergents tested. The notion of a tetramer is supported by the comparatively high efficiency of immunoprecipitation and the attendant implication that the oligomer can be larger than a dimer.

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