Monomers and Oligomers of the M_2 Muscarinic Cholinergic Receptor Purified from *Sf*9 Cells†

Paul S.-H. Park and James W. Wells*

Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada M5S 2S2

Received March 26, 2003; Revised Manuscript Received September 5, 2003

ABSTRACT: G protein-coupled receptors are known to form oligomers. To probe the nature of such aggregates, as well as the role and prevalence of monomers, epitope-tagged forms of the M_2 muscarinic receptor have been isolated as oligomers and monomers from *Sf*9 cells. Membranes from cells coexpressing the c-Myc- and FLAG-tagged receptor were solubilized in digitonin−cholate, and the receptor was purified by successive passage through DEAE-Sepharose, the affinity resin 3-(2′-aminobenzhydryloxy)tropane (ABT)−Sepharose, and hydroxyapatite. Coimmunoprecipitation of the two epitopes indicated the presence of oligomers at each stage of the purification up to but not including the fraction eluted specifically from ABT−Sepharose. The affinity-purified receptor therefore appeared to be monomeric. The failure to detect coimmunoprecipitation was not due to an ineffective antibody, nor did the conditions of purification appear to promote disaggregation. Receptor at all stages of purification bound *N*-[^{3}H]methylscopolamine and [^{3}H]quinuclidinylbenzilate with high affinity, but the capacity of receptors that were not retained on ABT−Sepharose was only 4% of that expected from densitometry of western blots probed with an anti-M_2 antibody. Similarly low activity was found with oligomers isolated by successive passage of coexpressed receptor on anti-c-Myc and anti-FLAG immunoaffinity columns. M_2 muscarinic receptors therefore appear to coexist as active monomers and largely or wholly inactive oligomers in solubilized extracts of *Sf*9 cells. A different pattern emerged when coinfected cells were treated with quinuclidinylbenzilate prior to solubilization, in that ABT-purified receptors from those cells exhibited coimmunoprecipitation. Treatment with the antagonist therefore led to oligomers in which at least some of the constituent sites were active and were retained by ABT−Sepharose.

It was noted some 20 years ago that G protein-coupled receptors may form oligomers (e.g., refs *1*−*4*). The early reports were largely disregarded, however, owing in part to uncertainty over the interpretation of the data. Also, there has been widespread acceptance of the notion that signaling occurs via a transient 1:1 complex between a monomeric receptor (R)[1] and the G protein (G) (*5*−*7*), although such schemes are problematic when examined quantitatively (*8*−*10*).

Further evidence for the existence of oligomers has emerged more recently from studies on modified receptors expressed in recombinant systems. Of particular note was the observation that complementary chimeras of the M_3 muscarinic receptor and the α_2 adrenergic receptor bound antagonists only when coexpressed (*11*). There subsequently have been numerous reports of multimeric forms on polyacrylamide gels, and both homo- and heterooligomers of various G protein-coupled receptors have been confirmed by means of coimmunoprecipitation (reviewed in refs *12*−*14*). The presence of oligomers at the surface of intact cells has been demonstrated by the detection of energy transfer between receptors tagged with fluorescent and bioluminescent probes (e.g., refs *15* and *16*). As the evidence for multimeric forms continues to accumulate, it appears that most if not all G protein-coupled receptors exist at least in part as oligomeric complexes.

Although the purpose of oligomers remains unclear, they appear to have a role in the trafficking of receptors within the cell. Studies on the PAF receptor and the V2 vasopressin receptor have shown that mutants inhibit cell-surface expression of the corresponding wild-type receptor when both are coexpressed in mammalian cells (*17*, *18*). Similarly, oligomerization of the R1 and R2 subunits of the $GABA_B$ receptor is necessary for trafficking of the R1 subunit to the cell surface (*19*−*23*). In that case, formation of the R1−R2 complex has been shown to mask a retention sequence found in the carboxy terminus of the R1 subunit that otherwise prevents its export from the endoplasmic reticulum (*23*). Oligomeric complexes of G protein-coupled receptors therefore appear to be formed within the cell and to be required

† This investigation was supported by the Canadian Institutes of Health Research (MOP43990) and the Heart and Stroke Foundation of Ontario (T4506 and T4914). P.S.-H.P. was the recipient of an Ontario Graduate Scholarship.

* Address correspondence to this author. Telephone: (416) 978-3068. Facsimile: (416) 978-8511. E-mail: jwells@phm.utoronto.ca.

[1] Abbreviations: ABT, 3-(2′-aminobenzhydryloxy)tropane; c-Myc-M_2, c-Myc-tagged M_2 muscarinic receptor; FLAG-M_2, FLAG-tagged M_2 muscarinic receptor; G, G protein; HEPES, sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; IB, immunoblot; IP, immunoprecipitation; NMS, *N*-methylscopolamine; PAF, platelet-activating factor; PMSF, phenylmethanesulfonyl fluoride; QNB, (−)-quinuclidinylbenzilate; R, receptor.

10.1021/bi034491m CCC: $25.00
Published on Web 10/18/2003

for the proper transport of the receptor to the plasma membrane.

Oligomers also may be a determinant of pharmacological specificity and of signaling per se insofar as they represent a unique species, distinct from the monomeric form, and offer a platform for cooperative interactions. Chimeric variants of the R1 and R2 subunits have been used to demonstrate that the oligomeric composition of the $GABA_B$ receptor affects both the binding of GABA and efficacy with respect to the regulation of inositol triphosphate (*22*). Cooperativity has been identified within a heteromer of the δ- and κ-opiate receptors, where the apparent affinity of the δ-selective agonist [D-Pen^2,D-Pen^5]enkephalin was at least 50-fold higher in the presence of the κ-selective agonist U69593 and vice versa (*24*). Similarly, cooperative interactions have been described between subtype-specific ligands to the β_1 and β_2 adrenergic receptors when the two proteins are coexpressed in HEK 293 cells (*25*).

Cooperative effects within homooligomers have been reported for the binding of ligands to both the M_2 muscarinic receptor from heart (*26*, *27*) and the D_2 dopamine receptor from transfected Chinese hamster ovary cells (*28*). Also, cooperativity can provide a mechanistically consistent account of the characteristic binding patterns revealed by agonists and guanyl nucleotides at the M_2 receptor and associated G proteins, in contrast to schemes based on the notion of a transient RG complex. Cooperative effects therefore offer an alternative mechanistic basis for the process of signaling between receptor and G protein (*26*, *27*, *29*, *30*).

Until recently, monomers have held a central and largely unchallenged position in the common view of G protein-mediated signaling (e.g., refs *5*−*7*). Their role and even their existence are placed in doubt, however, by the apparent ubiquity of oligomers and the evidence that oligomerization can have functional consequences. *Sf*9 cells have been widely used in studies of G protein-coupled receptors; they are particularly useful for biochemical work, owing in part to their high levels of expression. Muscarinic receptors from baculoviral-infected cells have been shown to exist as oligomers (*31*), to undergo posttranslational modifications such as glycosylation, palmitoylation, and phosphorylation (*32*−*34*), and to interact with G proteins to ellicit a cellular response (*35*, *36*). In the present investigation, we have purified monomers and oligomers of the M_2 muscarinic receptor from infected *Sf*9 cells and characterized each preparation for the binding of radiolabeled antagonists. A preliminary report of this work has appeared elsewhere (*37*).

MATERIALS AND METHODS

Ligands, Antibodies, and Other Materials. *N*-[^{3}H]Methylscopolamine chloride (lot 3474009, 83.5 Ci/mmol) and [^{3}H]-quinuclidinylbenzilate (lots 3329907 and 3363333, 42 Ci/mmol; lot 3467373, 39 Ci/mmol) were obtained from PerkinElmer Life Sciences. Unlabeled *N*-methylscopolamine bromide and (−)-quinuclidinylbenzilate were purchased from Sigma-Aldrich.

Agarose-conjugated anti-c-Myc antibody used for immunoprecipitation was purchased from Santa Cruz Biotechnology (9E10, mouse), and that used for immunochromatography was from Sigma-Aldrich (polyclonal, rabbit). A horseradish peroxidase-conjugated anti-c-Myc antibody (9E10, mouse) from Roche Diagnostics was used for western blots. Anti-FLAG antibodies (M2, monoclonal, mouse) conjugated to horseradish peroxidase and agarose were purchased from Sigma-Aldrich for use in western blots and immunochromatography, respectively. Ascites fluid containing monoclonal antibody directed against the porcine cardiac M_2 muscarinic receptor was purchased from Affinity Bioreagents, and a polyclonal antibody directed against the carboxyl terminus of the M_2 muscarinic receptor (positions 457−466) was from U.S. Biological. Peptides corresponding to the c-Myc (EQKLISEEDL) and FLAG epitopes (DYKDDDDK) were from Sigma-Aldrich.

SDS, glycine, and dithiothreitol were from BioShop Canada. All other materials were obtained from sources identified previously (*31*).

Epitope-Tagged Muscarinic Receptor from Sf9 Cells. Human M_2 muscarinic receptor tagged with the c-Myc or FLAG epitope was expressed in *Sf*9 cells and extracted in digitonin−cholate (0.86% digitonin, 0.17% cholate) as described previously (*31*). The solubilized receptor was purified via successive passage on DEAE-Sepharose, 3-(2′-aminobenzhydryloxy)tropane−Sepharose (ABT−Sepharose), and in some cases hydroxyapatite (CHT-II, Bio-Rad) (*26*). Receptor that was claimed after passage on ABT−Sepharose is referred to as ABT-purified receptor, and carbachol was removed on a column of Sephadex G-50 (fine) (0.8 × 5.0 cm) prequilibrated and eluted with buffer A (20 mM KH_2PO_4, 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.1% digitonin, 0.02% cholate, and NaOH to pH 7.4). Receptor that was claimed after hydroxyapatite is referred to as HPT-purified receptor and was transferred into buffer A by processing on an Econo-Pac P6 cartridge (Bio-Rad).

Solubilized receptor also was purified by immunochromatography on Poly-Prep columns (10 mL, Bio-Rad) packed with agarose-conjugated anti-c-Myc or anti-FLAG antibody to a bed volume of 1 mL. The columns were eluted by gravity flow, and all steps were performed at 4 °C. The anti-c-Myc resin was regenerated with ammonium hydroxide (15 mL, 0.1 M, pH 11−12), and the anti-FLAG resin was regenerated with glycine hydrochloride (3 mL, 0.1 M, pH 3.5). Both columns were equilibrated with buffer B (20 mM KH_2PO_4, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.1% digitonin, 0.02% cholate, and NaOH to pH 7.4) (anti-c-Myc, 15 mL; anti-FLAG, 5 mL). An aliquot of the solubilized extract (4−9 mL) was added to the equilibrated column, which then was capped and shaken for 2 h. The agarose was allowed to settle, and the supernatant then was drained and collected ("flow-through" fraction). The column was washed with 20 mL of buffer B ("wash" fraction), and bound receptor was recovered by applying an aliquot of buffer B (5 mL) supplemented with either the c-Myc peptide or the FLAG peptide (100 μg/mL), capping the column, and shaking for 2 h. The purified receptor then was eluted from the column, and that solution was used in binding or immunological assays. When necessary, the peptides were removed by concentrating the eluate 6−23-fold and desalting the concentrated material on Sephadex G-50 (fine) preequilibrated with buffer B.

Treatment of Sf9 Cells with Quinuclidinylbenzilate. Cells were supplemented with 10 μM quinuclidinylbenzilate at the time of baculoviral infection and processed as described previously (*31*). Alternatively, coinfected cells were harvested

3 days after infection by centrifugation at 1000*g*. The pellet was resuspended in buffer C (20 mM KH_2PO_4, 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 200 μg/mL bacitracin, 2 μg/mL leupeptin, 20 μg/mL pepstatin A, 156 μg/mL benzamidine, and NaOH to pH 7.4) supplemented with 10 μM quinuclidinylbenzilate. The mixture then was incubated for 2 h at 30 °C and centrifuged for 45 min at 4 °C and 100000*g*. The membranes were washed twice by resuspension in ice-cold buffer C without quinuclidinylbenzilate and subsequent centrifugation as described above.

Immunological Assays. Immunoprecipitation, electrophoresis, and western blotting were carried out essentially as described previously (*31*). Except where stated otherwise, samples being prepared for electrophoresis were heated at 65 °C for 5 min prior to being loaded on the polyacrylamide gel. Proteins were resolved on precast gels (7.5% or 10%) purchased from Bio-Rad (Ready Gel Tris-HCl). In tests for coimmunoprecipitation, solubilized receptor (300−500 μL) was immunoprecipitated with the agarose-conjugated anti-c-Myc antibody. Blots to be reprobed with a different antibody were stripped using the Re−Blot western blot recycling kit from Chemicon International.

The densities of bands were quantified by means of standard curves prepared with ABT-purified receptor. The standards comprised five samples that were assayed for specific binding at a saturating concentration of [^{3}H]-quinuclidinylbenzilate (∼100 nM) and were run in parallel with the sample of interest. Densitometric analyses were performed at a resolution of 600 dots per inch using 1D Image Analysis software (Kodak Digital Science). The densities of the standards increased linearly with the amount of receptor over a 7-fold range (49−392 fmol), as estimated by [^{3}H]quinuclidinylbenzilate, and the data were fit by linear regression. All bands corresponding to receptor were quantified, including multimeric forms and a 40 kDa fragment, and the amount of receptor was taken as the sum.

The specific binding activity of M_2 receptor recovered from ABT−Sepharose can vary from batch to batch of product. To examine the extent of the variability, samples of ABT-purified receptor (5 ng) from four different batches were run in parallel on a polyacrylamide gel. The blot was probed with the anti-M_2 antibody, and the intensities of the bands were estimated by densitometry; the amount of receptor applied to the gel was determined by [^{3}H]quinuclidinylbenzilate (∼100 nM). In three such comparisons, the mean ratio of intensity to binding capacity varied by up to 2-fold among the four preparations. The densities of bands revealed by the flow-through fraction from ABT−Sepharose therefore were quantified in most cases with reference to ABT-purified receptor eluted subsequently from the same column. When the flow-through and the standards were from different columns, a single sample of ABT-purified receptor from the same column as the flow-through also was applied to the gel. The number of sites calculated for the flow-through then was adjusted for the difference in the capacity of the purified receptor between that measured with [^{3}H]quinuclidinylbenzilate and that estimated from the standard curve. With untreated extracts and with receptor purified by immunochromatography, the densities were quantified throughout with standards from a single batch of ABT-purified receptor.

Binding Assays and Analysis of Data. Binding was measured essentially as described previously (*27*). Aliquots of solubilized receptor (3 μL) were added to assay buffer (20 mM HEPES, 20 mM NaCl, 1 mM EDTA, 5 mM $MgSO_4$, 0.1 mM PMSF, 0.1% digitonin, 0.02% cholate, and NaOH to pH 7.4) (50 μL) containing the radioligand and any unlabeled ligand as required. The reaction mixture was incubated at 30 °C for 45 min in assays with *N*-[^{3}H]-methylscopolamine and for 2 h in assays with [^{3}H]quinuclidinylbenzilate. Bound radioligand was separated on a column of Sephadex G-50 (fine) (0.8 × 6.5 cm). All of the eluant up to and including the void volume (1.65 mL) was collected and assayed for radioactivity. In assays with *N*-[^{3}H]-methylscopolamine, ethanol that accompanied the radioligand was evaporated under argon such that the final concentration in the reaction mixture was less than 1 vol % (*38*).

Binding at graded concentrations of the radioligand was measured over the range illustrated in Figure 4. Routine estimates of capacity were performed at a saturating concentration of [^{3}H]quinuclidinylbenzilate (∼100 nM). Binding at graded concentrations of ABT was measured with [^{3}H]-quinuclidinylbenzilate at a constant concentration of 1.1−1.7 nM. Nonspecific binding was taken throughout as total binding in the presence of 1 mM unlabeled *N*-methylscopolamine.

All data were analyzed with total binding taken as the dependent variable (B_{obsd}). Data acquired at graded concentrations of *N*-[^{3}H]methylscopolamine or [^{3}H]quinuclidinylbenzilate were analyzed according to eq 1, in which B_{sp} represents specific binding of the radioligand at the total concentration $[P]_t$, and B_{max} represents maximal specific binding. The parameter EC_{50} is the concentration of unbound radioligand that yields half-maximal occupancy; n_H is the Hill coefficient, and NS is the fraction of unbound radioligand that appears as nonspecific binding. Equation 1 was solved numerically (*39*), with the value of n_H fixed at 1 or optimized as required.

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

Data acquired at graded concentrations of ABT were analyzed according to eq 2, in which [PR] represents the labeled complex when [^{3}H]quinuclidinylbenzilate (P) and the unlabeled ligand (A) are assumed to compete for a homogeneous population of mutually independent sites (R). The equilibrium dissociation constants of P and A are K_P and K_A, respectively. The value of [PR] was calculated from the equation $[PR] = [P][R]/K_P$; the free concentrations of receptor ([R]) and each ligand ([P], [A]) were calculated numerically from the corresponding total concentrations ($[R]_t$, $[P]_t$, $[A]_t$) (*39*). To obtain an estimate of log K_A for ABT,

$$B_{obsd} = [PR] + NS([P]_t - [PR]) \quad (2)$$

data from three experiments were analyzed in concert, with the value of log K_P fixed at −9.02. Further details regarding the analyses and related statistical procedures have been described elsewhere (*26*, *27*, *30*, *39*).

RESULTS

M_2 muscarinic and other G protein-coupled receptors often appear to migrate as dimers and larger homooligomers on

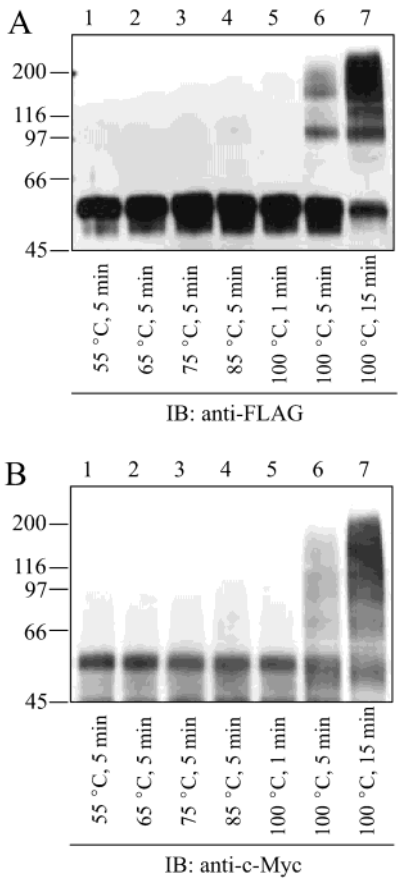

FIGURE 1: Temperature-induced aggregation of the M_2 muscarinic receptor. HPT-purified receptor from *Sf*9 cells expressing either FLAG-M_2 (A, 27 ng) or c-Myc-M_2 (B, 23 ng) was subjected to electrophoresis and detected with the appropriate antibody. Prior to electrophoresis, the samples were heated for 1, 5, or 15 min at the temperature shown in the figure.

western blots (e.g., ref *26*), but the conditions of electrophoresis can favor aggregation. The effect of temperature on the electrophoretic mobility of HPT-purified M_2 receptor from *Sf*9 cells is illustrated in Figure 1. Only the monomeric form was detected when samples were heated for 5 min at temperatures below 85 °C, while faint bands indicative of oligomers were visible after 5 min at 85 °C. When the samples were heated at 100 °C, oligomers represented an appreciable fraction of the total density after 5 min and the major fraction after 15 min. Except where noted otherwise, such aggregation was avoided in the present investigation by heating the samples for 5 min at 65 °C prior to loading them on the gel.

Oligomers also have been inferred from coimmunoprecipitation, which has been shown to occur with FLAG- and c-Myc-tagged forms of the M_2 muscarinic receptor in detergent-solubilized extracts from coinfected *Sf*9 cells (*31*). Similar results were obtained in the present study when digitonin-solubilized membranes from coinfected cells were precipitated with the anti-c-Myc antibody and probed with the anti-FLAG antibody (Figure 3A, lane 1). In contrast, no coimmunoprecipitation was detected with coexpressed receptor passed successively through DEAE-Sepharose, ABT−Sepharose, and hydroxyapatite (Figure 2A, lane 1). It follows that purified M_2 receptors from *Sf*9 cells are predominantly if not wholly monomeric.

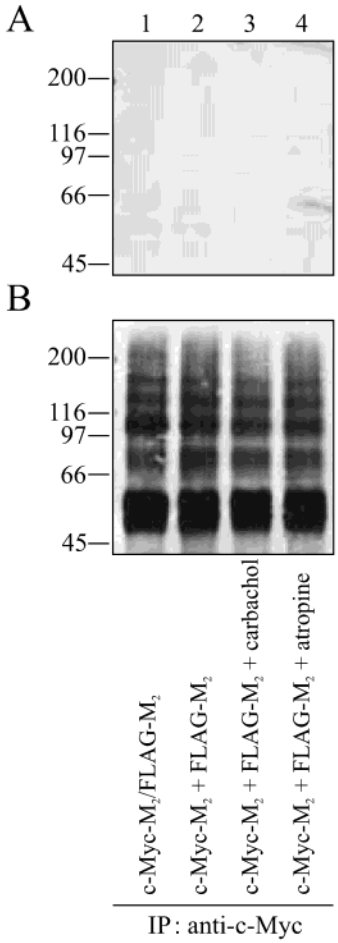

FIGURE 2: Immunoprecipitation of purified M_2 muscarinic receptor. Cells were infected singly (c-Myc-M_2 or FLAG-M_2) or were coinfected (c-Myc-M_2/FLAG-M_2) with baculovirus coding for c-Myc- and FLAG-tagged M_2 receptor. Membranes were solubilized in digitonin−cholate and purified on DEAE-Sepharose, ABT−Sepharose, and hydroxyapatite. The HPT-purified receptor was immunoprecipitated with the anti-c-Myc antibody, subjected to electrophoresis, and transferred to a nitrocellulose membrane. Samples were boiled for 5 min prior to electrophoresis. (A) The blot was probed with the anti-FLAG antibody. (B) The blot shown in panel A was stripped and reprobed with the anti-c-Myc antibody. The anti-c-Myc antibody used to precipitate the receptor was not detected by either the anti-FLAG antibody or the anti-c-Myc antibody on western blots. Each sample contained either coexpressed receptor (lane 1) or equal amounts of singly expressed receptor mixed in the absence of ligand (lane 2) or in the presence of either 1 mM carbachol (lane 3) or 1 mM atropine (lane 4). In each case, the total concentration of receptor was 40 nM in a volume of 500 μL, as determined by [^{3}H]QNB (~100 nM). Mixed samples containing singly expressed c-Myc-M_2 and FLAG-M_2 were incubated for 1 h at 30 °C prior to the addition of the immobilized antibody.

The failure to detect a c-Myc- and FLAG-containing heteromer after purification was not due to insufficient immunoprecipitation, since bands corresponding to the receptor were evident when the blot shown in Figure 2A was stripped and reprobed with the anti-c-Myc antibody (Figure 2B). To confirm the effectiveness of the agarose-coupled anti-c-Myc antibody, preparations of HPT-purified c-Myc-tagged receptor were assayed for the removal of specifically bound [^{3}H]quinuclidinylbenzilate from the supernatant fraction. Measured in this manner, the efficiency of immunoprecipitation was 32 ± 5% (N = 4), in good agreement with the value reported previously for removal

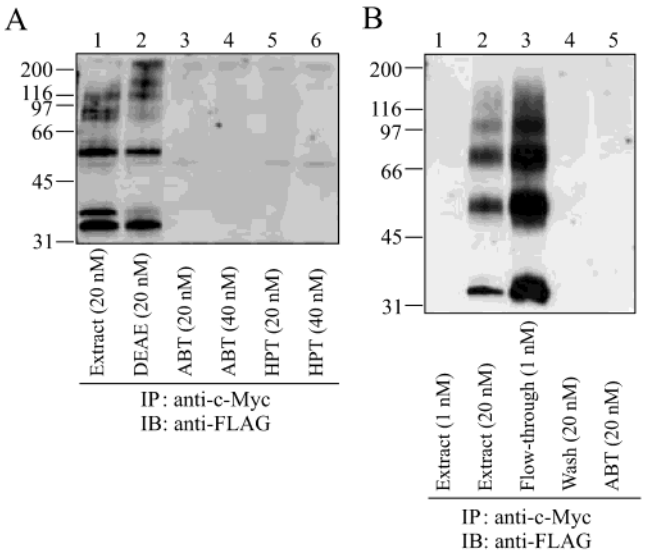

FIGURE 3: Detection of coimmunoprecipitation in fractions collected at each stage of purification. *Sf*9 cells coexpressing c-Myc-M_2 and FLAG-M_2 were solubilized in digitonin−cholate, and the extract was processed successively on DEAE-Sepharose, ABT−Sepharose, and hydroxyapatite. (A) Aliquots of the solubilized extract (lane 1), the final eluate from DEAE-Sepharose (lane 2), the carbachol-containing eluate from ABT−Sepharose (lanes 3 and 4), and the final eluate from hydroxyapatite (lanes 5 and 6) were mixed with the immobilized anti-c-Myc antibody, and the precipitate was blotted with the anti-FLAG antibody. (B) Aliquots of the solubilized extract (lanes 1 and 2) and different fractions eluted from ABT−Sepharose (lanes 3−5) were subjected to immunoprecipitation and blotting as in panel A. The fractions from ABT−Sepharose are as follows: receptor not adsorbed on the column (flow-through, lane 3), receptor leached from the column during the wash with buffer (wash, lane 4), and receptor eluted specifically in the presence of carbachol (lane 5). The wash yielded bands with some preparations but not others. The volume used for immunoprecipitation was 500 μL throughout, and the values shown in parentheses indicate the concentration of receptor as determined by [^{3}H]QNB (∼100 nM).

of the same receptor from solubilized extracts (cf. 35 ± 3%, ref *31*). The monomers obtained after purification appear not to associate spontaneously, nor is oligomerization promoted by muscarinic ligands. No coimmunoprecipitation was detected when equal amounts of c-Myc- and FLAG-tagged receptor purified from singly infected cells were mixed in the absence of ligand and in the presence of either carbachol (1 mM) or atropine (1 mM) (Figure 2A, lanes 2−4).

The product from each chromatographic step in the purification was tested in order to determine the point at which the heteromeric complex is lost. Coimmunoprecipitation was detected with coexpressed receptor recovered from DEAE-Sepharose but not with receptor that was retained by and subsequently eluted from ABT−Sepharose (Figure 3A, lanes 3 and 4). Similar results were obtained when either carbachol (0.3 M) or atropine (0.1 M) was used to dislodge the receptor from the affinity column. Thus, M_2 receptors first appear exclusively as monomers upon their recovery from ABT−Sepharose. Their retention on the column and specific desorption imply that they remain functional, and receptor in the eluted fraction was found to bind [^{3}H]-quinuclidinylbenzilate and *N*-[^{3}H]methylscopolamine with high affinity (Table 1, Figure 4B).

Adsorbed M_2 receptor generally is recovered from ABT−Sepharose by elution at a high concentration of the agonist carbachol (i.e., 300 mM). It has been shown previously that the coimmunoprecipitation of c-Myc- and FLAG-tagged receptor from digitonin-solubilized extracts of coinfected *Sf*9 cells is unaffected by either carbachol or atropine at a concentration of 1 mM (*31*). Similarly, the level of coimmunoprecipitation was unaffected by preincubation of the solubilized receptor at saturating concentrations of either carbachol (300 mM) or ABT (10 μM) (Figure 5). There also was no effect when the receptor was preincubated successively with ABT and then carbachol, which were added in sequence in order to mimic the conditions of the affinity column. Since not all sites of an oligomer are likely to engage an immobilized ligand at the same time, ABT was included at saturating and subsaturating concentrations (i.e., 10 and 0.1 μM) (Figure 5). The binding of ABT to the M_2 receptor in digitonin-solubilized extracts of *Sf*9 cells revealed a single class of sites (i.e., eq 2), and the inferred affinity was 35 nM (i.e., $\log K_A = -7.45 \pm 0.04$). It follows that the conditions of chromatography on ABT−Sepharose do not promote disaggregation of the receptor; rather, the monomers that account for most, if not all, of the receptor after purification appear to be present in the unprocessed solubilized extract.

To examine further the fate of the oligomers, each fraction collected from ABT−Sepharose was probed for coimmunoprecipitation of the two epitopes (Figure 3B). In contrast to the negative result obtained with receptor that was retained on the column, there was pronounced coimmunoprecipitation in the fraction containing unadsorbed receptor (i.e., the flow-through) (Figure 3B, lane 3). Moreover, detection was possible at much lower levels of receptor, as determined by [^{3}H]quinuclidinylbenzilate, than were required to obtain a signal with the solubilized extract (Figure 3B, cf. lanes 1−3). The flow-through therefore was enriched in oligomers per unit of binding, and [^{3}H]quinuclidinylbenzilate appears to underestimate the amount of receptor in that fraction.

The binding of [^{3}H]quinuclidinylbenzilate and *N*-[^{3}H]-methylscopolamine to M_2 receptor in the flow-through fraction is illustrated in Figure 4C, and the parametric values obtained from eq 1 are listed in Table 1. The curves reveal a uniform population of sites (i.e., $n_H \approx 1$) with affinities that are indistinguishable from those obtained for receptor in the solubilized extract and after purification on ABT−Sepharose (Table 1). To estimate the apparent specific activity of the receptor, the capacity for [^{3}H]quinuclidinylbenzilate was compared with that determined from the density of western blots prepared with an M_2-specific antibody. In the densitometric assays, ABT-purified receptor was taken as the standard. In the flow-through fraction, [^{3}H]-quinuclidinylbenzilate bound to only 4% of the receptors identified on the western blots (Table 2). The oligomers identified by coimmunoprecipitation of the c-Myc and FLAG epitopes (Figure 3B) therefore appear to be inactive, at least in binding assays.

Reduced binding also was typical of unprocessed extracts, in which the measured capacity for [^{3}H]quinuclidinylbenzilate was 54−74% of that predicted from the density of western blots with ABT-purified receptor taken as the standard (Table 2). Such extracts also contained oligomers, as indicated by coimmunoprecipitation (Figure 3). The shortfall in capacity therefore may be due to a mixture of monomers, which bind [^{3}H]quinuclidinylbenzilate and *N*-[^{3}H]-methylscopolamine with characteristic muscarinic affinities,

Table 1: Empirical Characterization of Specific Binding to Tagged M_2 Muscarinic Receptor from *Sf*9 Cells[a]

preparation	*N*-[^{3}H]methylscopolamine			[^{3}H]quinuclidinylbenzilate			$B_{max,[^3H]NMS}/B_{max,[^3H]QNB}$
	log EC_{50}	n_H	B_{max} (pM)	log EC_{50}	n_H	B_{max} (pM)	
c-Myc-M_2/FLAG-M_2							
soluble extract (4, 7)	-8.46 ± 0.12	0.80 ± 0.07	160−329	-9.02 ± 0.14	0.92 ± 0.06	162−721	0.83 ± 0.08
ABT−Sepharose, flow-through (1, 4)	−8.43	0.94	218	-8.93 ± 0.04	1.22 ± 0.09	105−327	0.88
ABT−Sepharose, purified (4, 4)	-8.37 ± 0.18	0.87 ± 0.05	302−716	-9.07 ± 0.09	1.01 ± 0.10	382−721	0.83 ± 0.07
anti-FLAG-/anti-c-Myc−agarose, purified (0, 3)	*b*	*b*	*b*	-9.82 ± 0.07	0.85 ± 0.15	4−37	
c-Myc-M_2							
anti-c-Myc−agarose, purified (0, 1)	*b*	*b*	*b*	−9.57	1.26	654	
FLAG-M_2							
anti-FLAG−agarose, purified (0, 1)	*b*	*b*	*b*	−9.64	1.14	503	

[a] Solubilized extracts from *Sf*9 cells coexpressing (c-Myc-M_2/FLAG-M_2) or singly expressing (c-Myc-M_2 or FLAG-M_2) the c-Myc- or FLAG-tagged M_2 receptor were processed as indicated in the table. Binding was measured at graded concentrations of [^{3}H]NMS and [^{3}H]QNB, as illustrated in Figure 4, and the data were analyzed in terms of eq 1 to obtain estimates of log EC_{50}, n_H, and B_{max}. The number of experiments involving [^{3}H]NMS or [^{3}H]QNB is shown in parentheses ([^{3}H]NMS, [^{3}H]QNB). [^{3}H]NMS was used throughout in parallel with [^{3}H]QNB in order to determine the relative capacity for the two radioligands (i.e., $B_{max,[^3H]NMS}/B_{max,[^3H]QNB}$). The values of log EC_{50}, n_H, and $B_{max,[^3H]NMS}/B_{max,[^3H]QNB}$ obtained from individual experiments were averaged to obtain the means (±SEM) listed in the table. The values of B_{max} refer to the concentration in the binding assays and indicate the range among individual experiments. [b] Not measured.

and oligomers that are wholly or largely inactive. In that event, the decrease in specific binding activity from 54−74% in extracts to 4% in the flow-through fraction derived from the removal of active monomers on ABT−Sepharose. Although the purified receptor was predominantly monomeric, those preparations may have contained a minor population of active oligomers that were adsorbed by ABT−Sepharose. With some batches of purified material, a faint band corresponding to a molecular mass somewhat greater than 200 kDa was detected when immunoprecipitates obtained with the anti-c-Myc antibody were blotted with the anti-FLAG antibody.

To isolate oligomeric forms of the receptor, digitonin-solubilized extracts from cells coexpressing the c-Myc- and FLAG-tagged proteins were processed successively on columns of anti-FLAG and anti-c-Myc antibody conjugated to agarose. The two epitopes were found to coimmunoprecipitate in the fraction obtained from the second column (Figure 6), indicating the presence of oligomers. To control for nonspecific effects, extracts containing equal amounts of c-Myc- or FLAG-tagged receptor from singly infected cells were mixed and processed on the two immunocolumns in the same manner. The final eluate from the second column showed no detectable reactivity to the anti-FLAG antibody (Figure 6), the anti-c-Myc antibody, or the anti-M_2 antibody; similarly, there was no detectable coimmunoprecipitation of the c-Myc and FLAG epitopes. Successive passage of coexpressed receptor on the two columns therefore appears to yield a preparation of oligomers in which monomers are absent or at least below the limit of detection on western blots.

The amount of receptor recovered from the two immunocolumns also was monitored in assays with [^{3}H]quinuclidinylbenzilate. With tagged receptor from coinfected cells, the capacity for [^{3}H]quinuclidinylbenzilate in the c-Myc-sensitive fraction eluted from the second column represented 0.4% of the sites applied to that column and about 0.1% of those applied to the first (Table 3). With the 50:50 mixture of receptors from singly infected cells, the capacity for [^{3}H]-quinuclidinylbenzilate in the final eluate from the second column represented 0.2% of the sites applied to that column and about 0.06% of those applied to the first (Table 3). The preparation therefore was cleared or almost cleared of functional sites irrespective of whether the product of the second column was essentially devoid of receptor or enriched in oligomers. With the preparation of oligomers obtained from extracts of coinfected cells, the capacity for [^{3}H]-quinuclidinylbenzilate was only 2% of that predicted from the density of western blots prepared with the anti-M_2 antibody and calibrated with standards of ABT-purified receptor (Table 2). Since most of the sites existed within oligomers, it follows that the oligomers were largely inactive.

The low specific binding activity of immunopurified oligomers from extracts of coinfected cells was not due to an effect of the columns themselves. The affinity of [^{3}H]-quinuclidinylbenzilate for coexpressed receptor recovered from both immunocolumns taken in succession and for singly expressed receptor recovered from one column or the other, as appropriate, was somewhat higher than that for receptors in the solubilized extract and after purification on ABT−Sepharose (Table 1). With FLAG-tagged receptor from singly infected cells, the specific activity for [^{3}H]quinuclidinylbenzilate was the same, at about 0.3 mol of the radioligand/mol of receptor, regardless of whether the sample was passed down the anti-FLAG column twice or only once (Table 2). Also, 97% of the FLAG-tagged receptor retained by the anti-FLAG column, as estimated from the binding of [^{3}H]-quinuclidinylbenzilate, was recovered upon elution with the FLAG peptide (Table 3).

In the case of the anti-FLAG column, the high yield and the reproducibility upon double passage confirm the benign nature of the column itself. A similar confirmation was not practicable with the anti-c-Myc column, since the lower yield of 35% precluded an accurate estimate of the specific activity after more than one passage (Table 3). It appears that the c-Myc peptide is comparatively inefficient at eluting the c-Myc-tagged receptor, perhaps owing to the high affinity of the latter for the immobilized anti-c-Myc antibody (*40*). Neither column was assessed for its adsorptive capacity. If the capacity was exceeded, the efficiency of elution listed in Table 3 is an underestimate of the value that would have been obtained with a smaller amount of receptor.

A further characteristic of the anti-c-Myc immunocolumn was the comparatively poor retention of sites as detected by

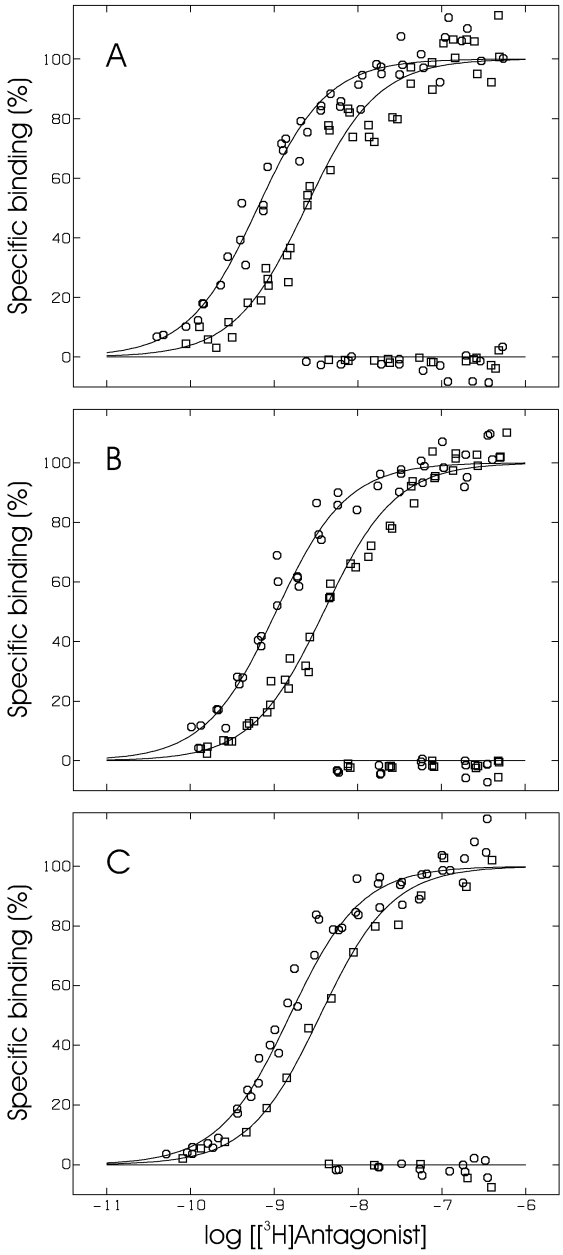

FIGURE 4: Binding to coexpressed M_2 muscarinic receptor at different stages of purification. *Sf*9 cells coexpressing c-Myc- and FLAG-tagged receptor were solubilized in digitonin−cholate and purified by successive passage on DEAE-Sepharose and ABT−Sepharose. Receptor in the solubilized extract (A), ABT-purified receptor (B), and receptor in the flow-through from ABT−Sepharose (C) were characterized for binding at graded concentrations of [^{3}H]QNB (○) or [^{3}H]NMS (□), either alone (upper curves) or in the presence of 1 mM unlabeled NMS (baseline). Each curve represents the best fit of eq 1 ($n_H = 1$) to the pooled data from three separate experiments except in the case of [^{3}H]NMS in panel C, where the data are from a single experiment. A single value of log EC_{50} was common to all of the data in each analysis. Separate values of B_{max} and NS were assigned to the data from each experiment, and the value of NS was common to data acquired in the absence and presence of unlabeled NMS within the same experiment. Values plotted on the *y*-axis are shown as a percentage of B_{max} for that radioligand.

[^{3}H]quinuclidinylbenzilate, which was 3-fold less than the level of retention on the anti-FLAG immunocolumn (*D*, Table 3); moreover, the specific binding activity of c-Myc-tagged receptor recovered from the anti-c-Myc column was 3-fold less than that of FLAG-tagged receptor recovered from

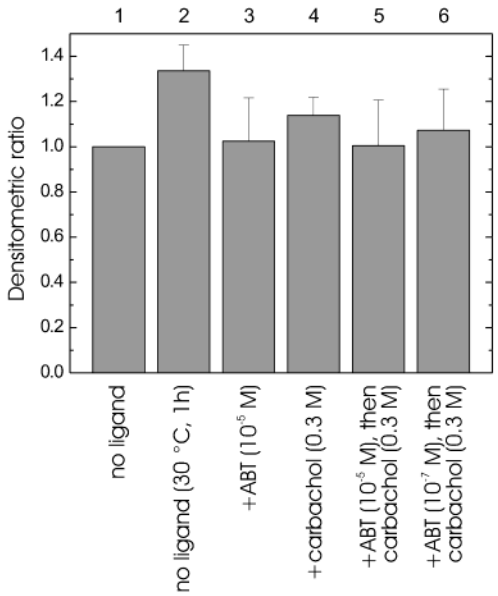

FIGURE 5: Effect of ABT and carbachol on the level of coimmunoprecipitation. *Sf*9 cells coexpressing c-Myc- and FLAG-tagged receptor were solubilized in digitonin−cholate. Aliquots of the extract (200 μL) containing 20−30 nM receptor, as determined by [^{3}H]QNB, were supplemented with ligand as shown in the figure and incubated at 30 °C for 1 h. Samples containing both ligands were supplemented first with ABT (30 °C, 1 h) and subsequently with carbachol (30 °C, 1 h). Each sample then was processed on a column of Sephadex G-50 (fine) (0.8 × 5.0 cm) preequilibrated with buffer A in order to remove unbound ligand. Receptor was recovered in the void volume (500 μL) and immunoprecipitated with the anti-c-Myc antibody. The immunoprecipitate was resolved by SDS−PAGE and transferred onto nitrocellulose, and oligomers were detected by the anti-FLAG antibody. Blots then were stripped and reprobed with the anti-c-Myc antibody as described in the legend to Figure 2B. Densitometric analyses were performed on both blots, and the level of coimmunoprecipitation of the two tags was expressed as the ratio of the intensity of bands detected by the anti-FLAG antibody to that of bands detected by the anti-c-Myc antibody. Each ratio was expressed relative to that obtained from sample 1, in which the extract was left on ice in the absence of ligand. The experiment was performed three times, and the values shown in the figure are the means (±SEM).

the anti-FLAG column (Table 2). The conditions of elution were the same for both immunocolumns, and it seems unlikely that one would denature the receptor or otherwise degrade function more than the other. Since monomers were active and oligomers were essentially inactive, an alternative explanation may be that the anti-c-Myc column preferentially binds oligomers.

*Sf*9 cells were found to yield functional oligomers if a lipophilic antagonist was included either at the time of coinfection or when the cells were harvested 3 days later. Coimmunoprecipitation of the c-Myc and FLAG epitopes was readily detected in samples of ABT-purified receptor from cells treated either way with quinuclidinylbenzilate (Figure 7A). Since the oligomers were retained by the affinity resin, the antagonist appears to have fostered activity in at least some of the constituent sites. There was comparatively little coimmunoprecipitation with ABT-purified receptor from cells coinfected in the presence of the hydrophilic antagonist *N*-methylscopolamine.

To assess the effect of quinuclidinylbenzilate, the degree of coprecipitation before and after purification was quantified by densitometry. The results are summarized as a ratio in

Table 2: Specific Binding Activity of Tagged M_2 Muscarinic Receptor before and after Purification[a]

sample	receptor	relative specific binding activity
ABT−Sepharose, flow-through	c-Myc-M_2/FLAG-M_2	0.04 ± 0.01 (8, 2)
ABT−Sepharose, purified	c-Myc-M_2/FLAG-M_2	1.00
anti-FLAG/anti-c-Myc, purified	c-Myc-M_2/FLAG-M_2	0.02 ± 0.01 (9, 3)
anti-c-Myc, purified	c-Myc-M_2	0.11 ± 0.01 (5, 3)
anti-FLAG, purified	FLAG-M_2	0.33 ± 0.04 (7, 3)
anti-FLAG/anti-FLAG, purified	FLAG-M_2	0.35 ± 0.07 (5, 2)
extract in digitonin−cholate	c-Myc-M_2	0.54 ± 0.16 (2, 1)
extract in digitonin−cholate	FLAG-M_2	0.69 ± 0.20 (4, 2)
extract in digitonin−cholate	c-Myc-M_2/FLAG-M_2	0.74 ± 0.21 (6, 4)

[a] Tagged receptor expressed in coinfected cells (c-Myc-M_2/FLAG-M_2) or in singly infected cells (c-Myc-M_2 or FLAG-M_2) was extracted in digitonin−cholate (extract). The solubilized receptor was processed on DEAE- and ABT−Sepharose or on agarose bearing the anti-c-Myc or anti-FLAG antibody. Further details are described in Materials and Methods. The eluate from ABT−Sepharose was collected before (flow-through) and after the inclusion of carbachol (purified). The immunoaffinity columns were used separately or in succession to obtain the eluate from one or both as shown in the table. The flow-through fraction from ABT−Sepharose and the final eluates from immunochromatography were assayed for the binding of [^{3}H]QNB (∼100 nM) and for the density of bands identified by the anti-M_2 antibody on western blots. Densities were converted to their molar equivalents by means of standard curves prepared with ABT-purified receptor as described in Materials and Methods. Specific binding activity was calculated as the capacity for [^{3}H]QNB divided by the number of receptors determined from the density of western blots. ABT-purified receptor was taken as the standard for the quantification of western blots, and the activity shown for those preparations therefore is 1. Receptor in the flow-through from ABT−Sepharose was quantified with standards of purified receptor from the same run; all other preparations were quantified with ABT-purified receptor from a single batch. Since the true specific activity of the ABT-purified standards can be less than 1, the values listed in the table represent upper limits on the activities of the different preparations. Estimates of the specific activity from individual experiments were averaged to obtain the means (±SEM) listed in the table. The numbers in parentheses indicate the number of experiments followed by the number of different preparations.

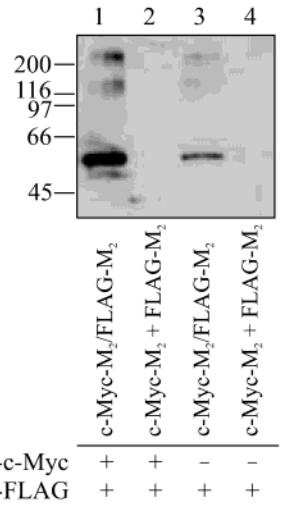

FIGURE 6: Purification of the oligomeric receptor. Solubilized extracts of *Sf*9 cells coexpressing the c-Myc and FLAG-tagged receptors (c-Myc-M_2/FLAG-M_2, lanes 1 and 3) and mixed extracts from cells separately expressing the two forms (c-Myc-M_2 + FLAG-M_2, lanes 2 and 4) were applied successively to columns of agarose-conjugated anti-FLAG and anti-c-Myc antibody. The mixed samples contained equal amounts of c-Myc- and FLAG-tagged receptor, as estimated at a saturating concentration of [^{3}H]QNB (∼100 nM). The product from the second (anti-c-Myc) immunocolumn (300 μL) was treated with the immobilized anti-c-Myc antibody, and the precipitate was blotted with the anti-FLAG antibody (lanes 1 and 2); parallel samples were blotted with the anti-FLAG antibody without prior immunoprecipitation (lanes 3 and 4). The concentration of receptor in the samples subjected to immunoprecipitation was 0.19 nM (lane 1) and 0.20 nM (lane 2). The amount of receptor applied directly to the gel was 78 pg (lane 3) and 82 pg (lane 4).

Figure 7C, where the underlying densities were determined relative to the number of available binding sites and the total amount of receptor. Normalization to the number of binding sites is implicit in the density per se, since [^{3}H]quinuclidinylbenzilate was used to determine the amount subjected to immunoprecipitation. To obtain values normalized to total receptor, densities from blots performed with the anti-FLAG antibody (Figure 7A) were divided by those measured when the samples were stripped and reblotted with the anti-c-Myc antibody (Figure 7B). The ratio plotted in Figure 7C is therefore an indicator of the number of ABT-purified oligomers relative to the number of oligomers in the unprocessed extract. The increase from about 0.1 for untreated cells to 1.1−1.6 for treated cells points to an increase in the level of functional oligomers upon treatment of the cells with quinuclidinylbenzilate. Uncertainty over such properties as the size of the oligomers and the distribution of active and inactive sites precludes a more specific interpretation of the data.

The specific binding activity of the purified material represented in lanes 4 and 6 of Figure 7A was calculated as the capacity for [^{3}H]quinuclidinylbenzilate relative to the density of western blots probed with the anti-M_2 antibody. The value found for ABT-purified receptor from cells treated with quinuclidinylbenzilate during or after coinfection was about 25% of that for purified receptor from untreated cells or from cells treated with *N*-methylscopolamine during coinfection. Also, the inclusion of quinuclidinylbenzilate decreased the retention of functional sites by ABT−Sepharose from 91% to about 63% (D, Table 3).

A lower specific activity suggests that the purified oligomers contain a mixture of functional sites and sites that are occluded or otherwise inaccessible to the radioligand. Some degree of occlusion is expected owing to the slow dissociation of unlabeled quinuclidinylbenzilate, which would be retained by some of the sites even after purification; in addition, some of the sites may be nonfunctional or in a latent state (e.g., ref *27*). The purification is based on the specific binding of the receptor to immobilized ABT, however, and the purified oligomers detected in Figure 7 must contain at least some sites that are both functional and devoid of quinuclidinylbenzilate. A complement of inactive sites or occlusion by the ligand also may account for the decreased retention by ABT−Sepharose of receptor from treated cells, perhaps by causing an oligomer to bind less tightly than a monomer.

We have noted previously that a 40 kDa immunoreactive species was detected along with the full-length protein in preparations of tagged M_2 receptor from *Sf*9 cells (*31*). That species also has been detected in the present investigation, both in solublilized extracts and after purification. The 40

Table 3: Efficiency of Purification[a]

column	receptor	initial (pmol) (A)	receptor at different stages of purification (%) flow-through (B)	wash (C)	bound (D)	specific elution (E)	recovery of adsorbed receptor (%) (F = 100E/D)
ABT−Sepharose (4)	c-Myc-M_2/FLAG-M_2	130−590	0.6 ± 0.3	8 ± 3	91 ± 3	45 ± 8	49 ± 3
ABT−Sepharose (3)	c-Myc-M_2/FLAG-M_2 + QNB (infection)[b]	22−96	24 ± 7	14 ± 3	62 ± 10	24 ± 3	41 ± 12
ABT−Sepharose (3)	c-Myc-M_2/FLAG-M_2 + QNB (harvest)[b]	10−50	2 ± 1	35 ± 4	64 ± 5	18 ± 3	29 ± 6
anti-c-Myc−agarose (3)	c-Myc-M_2	111−117	49 ± 3	28 ± 2	24 ± 4	8 ± 1	35 ± 3
anti-FLAG−agarose (5)	FLAG-M_2	61−173	15 ± 6	16 ± 1	70 ± 6	65 ± 5	97 ± 12
anti-FLAG−agarose/ anti-c-Myc−agarose[c] (3)	c-Myc-M_2/FLAG-M_2	134−213	47 ± 3	9 ± 2	44 ± 2	30 ± 2	68 ± 1
		46−61	83 ± 1	16 ± 2	2 ± 2	0.4 ± 0.2	*d*
anti-FLAG−agarose/ anti-c-Myc−agarose[c] (2)	c-Myc-M_2 + FLAG-M_2	178−238	53 ± 1	9 ± 1	38 ± 2	25 ± 1	67 ± 38
		47−58	70 ± 8	23 ± 6	7 ± 3	0.24 ± 0.03	4 ± 2

[a] Solubilized extracts containing coexpressed receptor (c-Myc-M_2/FLAG-M_2), singly expressed receptor (c-Myc-M_2 or FLAG-M_2), and singly expressed receptor mixed in equal amounts (c-Myc-M_2 + FLAG-M_2) as determined by [^{3}H]QNB were processed on ABT−Sepharose or an immunoaffinity resin as shown in the table. Material applied to ABT−Sepharose was processed first on DEAE-Sepharose. The immunoaffinity columns were loaded with unpurified extract. The amount of receptor at different stages of purification was estimated from 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 carbachol or the appropriate peptide (E, specific elution). Receptor adsorbed to the column was taken as the difference between that applied and that recovered either in the flow-through or during washing. The yield of purified receptor is shown both as a percentage of the amount applied initially (E) and as a percentage of that adsorbed (F). Each type of purification was carried out two to five times, as shown in parentheses, and the percentages from individual runs were averaged to obtained the means (±SEM) listed in the table (B−F). [b] The *Sf*9 cells were supplemented with QNB (10 μM) either at the time of coinfection (infection) or when the cells were harvested 3 days later (harvest). [c] Receptor was processed first on the anti-FLAG column, and the entire yield from specific elution (E) then was applied to the anti-c-Myc column. [d] The mean is indistinguishable from 0.

kDa fragment varied in proportion from preparation to preparation, as noted by others (*33*, *41*), and underwent coimmunoprecipitation (Figure 3). Since the fragment was detected by antibodies raised against the c-Myc and FLAG epitopes, which are located at the amino terminus of the receptor, it most likely represents the first five transmembrane domains. This was confirmed using an antibody directed against the carboxyl terminus of the M_2 receptor, which did not detect the 40 kDa band. In contrast, the same antibody detected two smaller fragments in ABT-purified preparations (16 and 26 kDa), one of which may represent transmembrane domains 6 and 7.

DISCUSSION

Studies on tagged M_2 muscarinic receptors at different stages of purification suggest that they coexist as monomers and oligomers in solubilized extracts of coinfected *Sf*9 cells. As monitored by coimmunoprecipitation, oligomers containing the c-Myc and FLAG epitopes were present upon solubilization of the cells in digitonin−cholate and throughout the initial stages of purification on DEAE-Sepharose. They also occurred in the flow-through from ABT−Sepharose but were absent from the fraction eluted subsequently in the presence of carbachol. Labeling by *N*-[^{3}H]-methylscopolamine and [^{3}H]quinuclidinylbenzilate was found at all stages of purification. It follows that M_2 receptors eluted specifically from ABT−Sepharose were wholly or largely monomeric.

Whereas binding was ubiquitous, and the affinities of both radioligands were the same throughout, the specific activity of the receptor varied 50-fold among the different fractions. It was highest after purification on ABT−Sepharose. It was lowest in the flow-through from ABT−Sepharose and in the fraction obtained from successive passage on anti-c-Myc- and anti-FLAG−agarose. The receptor therefore was most active when it appeared to be monomeric, and it was virtually inactive when predominantly oligomeric.

Since the degree of coimmunoprecipitation from unprocessed extracts was unaffected by ABT or carbachol, added either separately or in sequence, monomers do not appear to have been formed during chromatography on ABT−Sepharose. Also, no oligomers were detected when HPT-purified monomers obtained from cells singly expressing the c-Myc- or FLAG-tagged receptor were mixed in solution, either with or without a muscarinic ligand; similarly, nascent oligomers were not detected in mixtures of unprocessed solubilized extracts from singly infected cells (*31*). It follows that neither monomers nor oligomers were formed under the conditions of purification, nor was there any exchange of monomers among preexisting oligomers. Solubilization in digitonin−cholate therefore appears to yield a static mixture of monomers and oligomers. The latter are inactive and on that basis were separated from monomers on ABT−Sepharose.

Since the affinity of residual sites in either of the oligomer-rich preparations was comparable to that of purified monomers, the multimeric status of those sites is unclear. There may have been a comparatively small population of monomers that coeluted as a contaminant or, in the case of ABT−Sepharose, were leached from the column by the eluting buffer; alternatively, a minor fraction of the oligomers or a fraction of the sites within an oligomer may retain activity. Since there very likely was a mix of monomeric and oligomeric forms throughout, except perhaps for the last fraction eluted from ABT−Sepharose, the residual activity of those fractions enriched in oligomers may have more than one source.

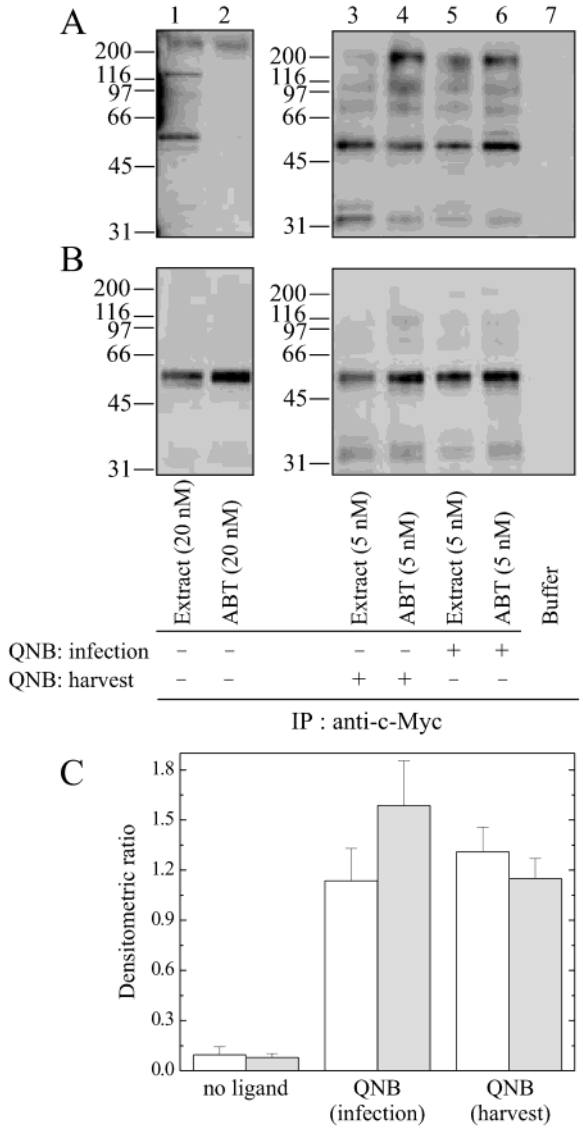

FIGURE 7: Recovery of functional oligomers by quinuclidinylbenzilate. *Sf*9 cells coexpressing c-Myc- and FLAG-tagged receptor were solubilized in digitonin−cholate (lanes 1, 3, and 5), and the receptor was purified by successive passage on DEAE-Sepharose and ABT−Sepharose (lanes 2, 4, and 6). The cells were treated with QNB (10 μM) either at the time of coinfection (lanes 5 and 6) or when the cells were harvested 3 days later (lanes 3 and 4). (A) Aliquots (500 μL) containing receptor at the concentration shown were incubated with immobilized anti-c-Myc antibody, and the immunoprecipitate was blotted with the anti-FLAG antibody. (B) The blot in panel A was stripped and probed with anti-c-Myc antibody. An aliquot of buffer A (500 μL) was incubated with immobilized anti-c-Myc antibody and processed in the same manner (lane 7). The concentration of receptor was adjusted with reference to the binding of [^{3}H]QNB (~100 nM). (C) The degree of coimmunoprecipitation was measured by densitometry of the bands shown in panel A. The bars indicate the ratio of the density obtained with the ABT-purified preparation (lanes 2, 4, and 6) to that obtained with the unprocessed extract (lanes 1, 3, and 5). The densities used to calculate each ratio were normalized to the capacity for [^{3}H]QNB (white bars) or to total receptor (gray bars) as described in the text. Values plotted in the figure are the means (±SEM) from six (no ligand, QNB infection) or three (QNB harvest) experiments. Several preparations of receptor are represented in the data (no ligand, 6; QNB infection, 4; QNB harvest, 2).

A minor population of contaminating monomers is consistent with the similarly low yields, about 0.1% or less in terms of the capacity for [^{3}H]quinuclidinylbenzilate, that were obtained when coexpressed receptor or a mixture of singly expressed receptors was processed sequentially on anti-c-Myc- and anti-FLAG−agarose (Table 3). Since the product obtained with coexpressed receptor was enriched in oligomers, any property of the oligomer ought to have been greater in that preparation. The alternative possibility that some residual binding is intrinsic to the oligomer is suggested by the recovery of functional oligomers from cells treated with quinuclidinylbenzilate. Also, a weak band corresponding to a molecular mass of about 200 kDa was detected with ABT-purified receptor on some occasions when immunoprecipitates obtained with the immobilized anti-c-Myc antibody were blotted with the anti-FLAG antibody.

It has been noted previously that the oligomeric size of the receptor can be inferred from the efficiency of immunoprecipitation, taken as the fraction of specific binding removed by an immobilized antibody from the supernatant in a solubilized preparation of coexpressed, differently tagged receptors (*31*). Such an approach is uninformative, however, if most or all of the aggregated sites are inactive. A further complication relates to the use of the binomial expansion, which requires an estimate of the ratio of c-Myc- to FLAG-tagged receptor within the preparation. The amounts of tagged protein were estimated previously by means of densitometry and standard curves prepared with HPT-purified receptor (*31*). In the present study, however, different batches of ABT-purified receptor have been found to vary by up to 2-fold in their specific activity. The quantitation of c-Myc- and FLAG-tagged receptors therefore requires confirmation that the specific activities of the corresponding standards are the same.

A 40 kDa fragment corresponding to the first five transmembrane domains of the receptor was detected at all stages of purification. The same fragment has been observed by others, who have suggested that it is a product of proteolysis at a position within the third intracellular loop (*33*, *41*). Both the 40 kDa peptide and the full-length receptor were identified by the anti-FLAG antibody in the precipitate obtained with the anti-c-Myc antibody from extracts of coinfected cells. The cleavage therefore does not appear to affect the ability of the resulting fragments to incorporate into oligomeric complexes. The 40 kDa fragment also was detected in purified preparations obtained from cells untreated with muscarinic ligand, indicating that it was retained on ABT−Sepharose and eluted specifically in the presence of carbachol. Since no coimmunoprecipitation of either the full-length receptor or the 40 kDa fragment was detected with such purified material, the fragment must associate directly with immobilized ABT.

Truncated forms of the M_2 and M_3 muscarinic receptors corresponding to the first five transmembrane domains have been shown not to bind ligand when expressed alone in COS-7 cells (*42*). Binding was regained, however, when the missing carboxy terminus was coexpressed with the amino-terminal fragment; moreover, the complementary fragments of the M_3 receptor were shown to regulate the production of inositol phosphates. This suggests that the 40 kDa species is able to bind ligand only if a corresponding carboxy-terminal fragment is also present. The proteolytic fragments therefore appear to form a functional binding pocket that, upon solubilization, is sufficiently stable to be retained on ABT−Sepharose (*33*, *41*). Bands corresponding to the 40

kDa species were included in densitometric analyses on the assumption that they represented functional receptor in the binding assays.

Treatment of the cells with quinuclidinylbenzilate led to a gain of function, in that oligomers were retained by ABT−Sepharose and purified in an active state. Since it was sufficient to add the ligand when the cells were harvested, the effect was on events immediately before or during solubilization. It has been shown previously that the M_2 receptor does not aggregate during extraction (*31*). Quinuclidinylbenzilate therefore appears to increase the number of oligomers that were functional in the membrane or remain so in solution; accordingly, it may promote oligomerization in the membrane, slow or prevent the fragmentation of preexisting oligomers, or exert a protective effect on their activity. A protective effect has been demonstrated previously with the M_3 muscarinic receptor expressed in *Sf*9 cells, where treatment of the membranes with *N*-methylscopolamine prior to solubilization increased the yield of solubilized receptor as measured by *N*-[^{3}H]methylscopolamine (*43*). Also, the muscarinic ligands *N*-methylscopolamine, quinuclidinylbenzilate, and carbachol have been shown to prevent or delay the thermal inactivation of purified M_2 receptor from porcine atria (*38*).

Details of the process whereby solubilization in digitonin−cholate leads to active monomers and inactive oligomers remain unclear. The latter may include misfolded aggregates that occur as a byproduct of overexpression. Also, oligomers that were functional in the membrane may have become inactivated during the process of solubilization or by a direct effect of the detergent itself. Digitonin has been shown to inactivate membrane-bound Na^+,K^+-ATPase without affecting its multimeric status (*44*). Also, solubilization has been shown to alter the affinities of various ligands for muscarinic receptors expressed in *Sf*9 cells (*45*). Similarly, detergents have been shown to modulate the binding properties of muscarinic receptors from mammalian sources, and the measured affinity for some ligands can vary 20-fold from one detergent to another (ref *27* and references cited therein). In some cases, detergent-related effects on binding have been indicative of changes in cooperative interactions among linked sites, presumably within an oligomer (*27*).

Oligomers of the M_2 receptor from *Sf*9 cells may be unusually sensitive to dissociation or inactivation owing to the absence of an additional element that imparts stability in other systems. Insect cells are deficient in cholesterol and in G proteins that couple to the M_2 receptor (*35*, *46*−*48*), either of which may be important to the functionality or the structural integrity of oligomers upon solubilization. Such effects may account for the binding properties and inferred oligomeric status of the M_2 receptor from porcine atria, which has been studied in various detergents (*27*) and as a purified RG complex in digitonin−cholate (*26*). Both the purified complex and an unprocessed extract in cholate−NaCl have revealed a shortfall in the apparent capacity for *N*-[^{3}H]-methylscopolamine, which was about 50% of that for [^{3}H]-quinuclidinylbenzilate; moreover, the difference in capacity was accompanied by a discrepancy in the apparent affinity of labeled and unlabeled *N*-methylscopolamine. Such a pattern points to cooperative effects, and a quantitative description of the data in those terms has required at least four interacting sites (*26*, *27*). A shortfall in capacity also was found with a purified M_2 receptor devoid of G protein (*26*), although the difference was smaller and less definitive (*38*). No comparable effects have been observed with monomeric receptors from *Sf*9 cells (Table 1). Cooperativity and the implication of linked sites suggest that the oligomeric integrity of M_2 receptors from porcine atria is more robust than that of receptors from *Sf*9 cells.

Quinuclidinylbenzilate was added to *Sf*9 cells at the time of coinfection in order to explore the possibility that the nonfunctional aggregates detected in solution were a byproduct of overexpression, presumably at the level of the endoplasmic reticulum. The proper assembly and functioning of some multimeric proteins, such as the nicotinic acetylcholine receptor and the Shaker potassium channel, are known to be dependent upon molecular chaperones (*49*−*51*). Similarly, ligands have been shown to act as pharmacological chaperones by assisting in the maturation and transport of some G protein-coupled receptors to the cellular surface (*52*−*54*). The overall effect of quinuclidinylbenzilate was the same, however, regardless of whether it was added with the baculovirus or when the cells were harvested. The recovery of binding sites from ABT−Sepharose was comparable, and purified receptor showed a similar degree of coimmunoprecipitation. It therefore seems likely that quinuclidinylbenzilate added during coinfection acted primarily by stabilizing the complex at the time of solubilization.

The monomers purified from untreated *Sf*9 cells may preexist in the membrane, or they may derive at least in part from the disaggregation of functional oligomers during solubilization. Studies on the extraction of cytochrome *c* oxidase from mitochondrial membranes have shown that the native dimeric structure of the enzyme may be lost in solution, depending upon the nature and the concentration of the detergent (*55*, *56*). M_2 monomers can be purified nonetheless, albeit from *Sf*9 cells, and they appear to be functional insofar as they bind antagonists with typical muscarinic affinity. That in turn raises the possibility that monomers participate in the signaling process, as implied by mechanistic schemes based on the notion of a transient complex between receptor and G protein (e.g., refs *5* and *6*). While there is little direct evidence that monomers can activate a G protein or elicit a cellular response, affinity-purified and presumably monomeric M_2 receptor from *Sf*9 cells has been shown to stimulate the binding of [^{35}S]GTPγS upon reconstitution with G_o or G_i in phospholipid vesicles (*35*). Reconstituted receptors may form oligomers once colocalized within the vesicles, although preliminary studies using c-Myc- and FLAG-tagged receptor purified from singly infected *Sf*9 cells have failed to detect coimmunoprecipitation from preparations of receptor reconstituted either with or without G protein.[2] Comparative studies with receptor purified from *Sf*9 and mammalian cells may help to clarify these issues and to provide further insight into the role of monomers and oligomers in the signaling process.

ACKNOWLEDGMENT

We thankfully acknowledge Dr. Hubert H. M. Van Tol of the Centre for Addiction and Mental Health and Dr. David

[2] P. S.-H. Park and J. W. Wells, unpublished observations.

B. Williams of the Department of Biochemistry, University of Toronto, for helpful discussions.

REFERENCES

1. Avissar, S., Amitai, G., and Sokolovsky, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 156−159.
2. Berrie, C. P., Birdsall, N. J. M., Haga, K., Haga, T., and Hulme, E. C. (1984) *Br. J. Pharmacol. 82*, 839−851.
3. Dadi, H. K., and Morris, R. J. (1984) *Eur. J. Biochem. 144*, 617−628.
4. Peterson, G. L., Rosenbaum, L. C., Broderick, D. J., and Schimerlik, M. I. (1986) *Biochemistry 25*, 3189−3202.
5. De Lean, A., Stadel, J. M., and Lefkowitz, R. J. (1980) *J. Biol. Chem. 255*, 7108−7117.
6. Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615−649.
7. Birnbaumer, L., Abramowitz, J., and Brown, A. M. (1990) *Biochim. Biophys. Acta 1031*, 163−224.
8. Lee, T. W. T., Sole, M. J., and Wells, J. W. (1986) *Biochemistry 25*, 7009−7020.
9. Neubig, R. R. (1994) *FASEB J. 8*, 939−946.
10. Green, M. A., Chidiac, P., and Wells, J. W. (1997) *Biochemistry 36*, 7380−7394.
11. Maggio, R., Vogel, Z., and Wess, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3103−3107.
12. Gomes, I., Jordan, B. A., Gupta, A., Rios, C., Trapaidze, N., and Devi, L. A. (2001) *J. Mol. Med. 79*, 226−242.
13. Milligan, G. (2001) *J. Cell Sci. 114*, 1265−1271.
14. Angers, S., Salahpour, A., and Bouvier, M. (2002) *Annu. Rev. Pharmacol. Toxicol. 42*, 409−435.
15. Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M., and Bouvier, M. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 3684−3689.
16. Rocheville, M., Lange, D. C., Kumar, U., Sasi, R., Patel, R. C., and Patel, Y. C. (2000) *J. Biol. Chem. 275*, 7862−7869.
17. Zhu, X., and Wess, J. (1998) *Biochemistry 37*, 15773−15784.
18. Le Gouill, C., Parent, J. L., Caron, C.-A., Gaudreau, R., Volkov, L., Rola-Pleszczynski, M., and Stanková, J. (1999) *J. Biol. Chem. 274*, 12548−12554.
19. White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M., and Marshall, F. H. (1998) *Nature 396*, 679−682.
20. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2000) *Neuron 27*, 97−106.
21. Calver, A. R., Robbins, M. J., Cosio, C., Rice, S. Q., Babbs, A. J., Hirst, W. D., Boyfield, I., Wood, M. D., Russell, R. B., Price, G. W., Couve, A., Moss, S. J., and Pangalos, M. N. (2001) *J. Neurosci. 21*, 1203−1210.
22. Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prezeau, L., and Pin, J. P. (2001) *EMBO J. 20*, 2152−2159.
23. Pagano, A., Rovelli, G., Mosbacher, J., Lohmann, T., Duthey, B., Stauffer, D., Ristig, D., Schuler, V., Meigel, I., Lampert, C., Stein, T., Prezeau, L., Blahos, J., Pin, J., Froestl, W., Kuhn, R., Heid, J., Kaupmann, K., and Bettler, B. (2001) *J. Neurosci. 21*, 1189−1202.
24. Jordan, B. A., and Devi, L. A. (1999) *Nature 399*, 697−700.
25. Lavoie, C., and Hebert, T. E. (2003) *Can. J. Physiol. Pharmacol. 81*, 186−195.
26. Wreggett, K. A., and Wells, J. W. (1995) *J. Biol. Chem. 270*, 22488−22499.
27. Park, P. S.-H., Sum, C. S., Pawagi, A. B., and Wells, J. W. (2002) *Biochemistry 41*, 5588−5604.
28. Armstrong, D., and Strange, P. G. (2001) *J. Biol. Chem. 276*, 22621−22629.
29. Chidiac, P., and Wells, J. W. (1992) *Biochemistry 31*, 10908−10921.
30. Chidiac, P., Green, M. A., Pawagi, A. B., and Wells, J. W. (1997) *Biochemistry 36*, 7361−7379.
31. Park, P., Sum, C. S., Hampson, D. R., Van Tol, H. H. M., and Wells, J. W. (2001) *Eur. J. Pharmacol. 421*, 11−22.
32. Richardson, R. M., and Hosey, M. M. (1992) *J. Biol. Chem. 267*, 22249−22255.
33. Nakata, H., Kameyama, K., Haga, K., and Haga, T. (1994) *Eur. J. Biochem. 220*, 29−36.
34. Hayashi, M. K., and Haga, T. (1997) *Arch. Biochem. Biophys. 340*, 376−382.
35. Parker, E. M., Kameyama, K., Higashijima, T., and Ross, E. M. (1991) *J. Biol. Chem. 266*, 519−527.
36. Vasudevan, S., Premkumar, L., Stowe, S., Gage, P. W., Reilander, H., and Chung, S. H. (1992) *FEBS Lett. 311*, 7−11.
37. Park, P. S.-H., and Wells, J. W. (2002) Program No. 141.3 in *2002 Abstract Viewer/Itinerary Planner*, Society for Neuroscience, Washington, DC (CD-ROM).
38. Sum, C. S., Park, P. S.-H., and Wells, J. W. (2002) *J. Biol. Chem. 277*, 36188−36203.
39. Wells, J. W. (1992) in *Receptor−Ligand Interactions: A Practical Approach* (Hulme, E. C., Ed.) pp 289−395, Oxford University Press, Oxford.
40. Hillman, M. C., Yang, L. S., Sun, S., Duke, J. L., O'Neil, K. T., Kochie, J. E., Karjoo, A., Nath, P., Breth, L. A., Murphy, K., Ross, O. H., Burn, T. C., Hollis, G. F., and Wynn, R. (2001) *Protein Expression Purif. 23*, 359−368.
41. Weill, C., Autelitano, F., Guenet, C., Heitz, F., Goeldner, M., and Ilien, B. (1997) *Eur. J. Pharmacol. 333*, 269−278.
42. Maggio, R., Vogel, Z., and Wess, J. (1993) *FEBS Lett. 319*, 195−200.
43. Vasudevan, S., Hulme, E. C., Bach, M., Haase, W., Pavia, J., and Reiländer, H. (1995) *Eur. J. Biochem. 227*, 466−475.
44. Powell, L. D., and Cantley, L. C. (1980) *Biochim. Biophys. Acta 599*, 436−447.
45. Rinken, A. (1995) *J. Pharmacol. Exp. Ther. 272*, 8−14.
46. Gimpl, G., Klein, U., Reilander, H., and Fahrenholz, F. (1995) *Biochemistry 34*, 13794−13801.
47. Heitz, F., McClue, S. J., Harris, B. A., and Guenet, C. (1995) *J. Recept. Signal Transduction Res. 15*, 55−70.
48. Marheineke, K., Grunewald, S., Christie, W., and Reilander, H. (1998) *FEBS Lett. 441*, 49−52.
49. Chang, W., Gelman, M. S., and Prives, J. M. (1997) *J. Biol. Chem. 272*, 28925−28932.
50. Wang, J. M., Zhang, L., Yao, Y., Viroonchatapan, N., Rothe, E., and Wang, Z. Z. (2002) *Nat. Neurosci. 5*, 963−970.
51. Higgins, M. K., Demir, M., and Tate, C. G. (2003) *Biochim. Biophys. Acta 1610*, 124−132.
52. Morello, J. P., Salahpour, A., Laperriere, A., Bernier, V., Arthus, M. F., Lonergan, M., Petaja-Repo, U., Angers, S., Morin, D., Bichet, D. G., and Bouvier, M. (2000) *J. Clin. Invest. 105*, 887−895.
53. Petaja-Repo, U. E., Hogue, M., Bhalla, S., Laperriere, A., Morello, J. P., and Bouvier, M. (2002) *EMBO J. 21*, 1628−1637.
54. Noorwez, S. M., Kuksa, V., Imanishi, Y., Zhu, L., Filipek, S., Palczewski, K., and Kaushal, S. (2003) *J. Biol. Chem. 278*, 14442−14450.
55. Musatov, A., Ortega-Lopez, J., and Robinson, N. C. (2000) *Biochemistry 39*, 12996−13004.
56. Musatov, A., and Robinson, N. C. (2002) *Biochemistry 41*, 4371−4376.

BI034491M