Functional rescue of a constitutively desensitized β_2 AR through receptor dimerization

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We have recently demonstrated that wild-type β_2 -adrenergic receptors (β_2 AR) form homodimers and that disruption of receptor dimerization inhibits signalling via G_s [Hebert, Moffett, Morello, Loisel, Bichet, Barret and Bouvier (1996) *J. Biol. Chem.* **271**, 16384–16392]. Here taking advantage of the altered functional properties of a non-palmitoylated, constitutively desensitized mutant β_2 AR (C341G β_2 AR), we sought to study whether physical interactions between mutant and wild-type β_2 AR expressed in Sf9 cells could occur and have functional consequences. Using metabolic labelling with [³H]palmitate and co-immunoprecipitation we demonstrated the existence of heterodimerization between wild-type and C341G β_2 AR. Furthermore, we show that, in co-expression experiments, wild-type receptors have a dominant positive effect resulting in the functional complementation of C341G β_2 AR. Indeed, when

expressed alone, the mutant C341G receptor displays altered functional characteristics in that (1) the response of the receptor to agonist is reduced as compared to the wild-type receptor and (2) the desensitization of the receptor in response to prolonged exposure to agonist is minimal. In contrast, when C341G and the wild-type β_2 AR were expressed together, both the response to agonist and subsequent desensitization (at a constant level of total receptor) were equivalent to the wild-type β_2 AR expressed alone. This dominant positive effect was also seen when C341G was co-expressed with a second receptor mutant in which the two protein kinase A phosphorylation sites (S261, 262, 345, 346A β_2 AR) were mutated. Taken together these data suggest that intermolecular interactions between receptors may have both functional and structural implications for G-protein-mediated signalling.

INTRODUCTION

The β_2 -adrenergic receptor (β_2 AR) is perhaps the best characterized member of the family of G-protein-linked seven transmembrane segment receptors [1,2]. This receptor activates G_s , which subsequently activates adenylate cyclase. The stoichiometry of the interaction between the receptor, G protein and the effector molecule is assumed to be 1:1:1 [3]. Indeed, this is one of the assumptions of the ternary model classically used to describe this interaction [4]. However, evidence from several lines of inquiry suggests that this picture may be oversimplified. For example, studies on the cooperativity in binding patterns of agonists at G-protein-coupled receptors (GPCRs) have suggested that both the receptors and their cognate G proteins may be part of oligomeric arrays [5–14]. Structural studies, including photoaffinity labelling of muscarinic receptors [15], radiation inactivation of α - and β -AR [16], cross-linking of glucagon receptors [17] and hydrodynamic properties of cardiac muscarinic receptors [18] also lend credence to the idea that GPCRs can form dimers.

A number of recent functional studies have also lent support to the notion that GPCRs may form dimers. Co-expression of two binding defective angiotensin II receptor mutants was demonstrated to rescue ligand binding [19]. Maggio et al. [20] also demonstrated using co-transfection of chimaeric α_2 -adrenergic/m3-muscarinic receptors that intermolecular interactions occurs between receptors. Indeed, two chimaeras composed of the first five transmembrane domains of one receptor linked to the last two of the other could only form functional receptors with the normal binding properties of each parent receptor when co-transfected, while no binding was seen when each chimaeric receptor was expressed alone.

The role that such oligomeric GPCRs play in normal signal transduction remains poorly characterized. We have demonstrated previously that the β_2 AR can form homodimers that are involved in the activity of the receptor [21]. Using a peptide derived from TM VI of the receptor we also show that this region of the receptor may form part of the dimer interface. Functionally, this peptide specifically inhibits receptor-mediated adenylate cyclase stimulation [21]. Whether the peptide inhibited signalling by preventing receptor dimerization or else led to the formation of an inactive receptor by binding to monomer thus forming 'pseudodimers' remains an open question. In many instances where protein dimerization is an integral part of biological function, both dominant negative and positive effects of mutation can be observed when mutant proteins are co-expressed with their wild-type parents. If such functional complementation can be demonstrated for the β_2 AR it would suggest that dimerization is important for receptor-mediated signalling. Here, we take advantage of a previously characterized mutant (C341G; [22,23]) which, unlike wild-type β_2 AR, fails to incorporate [³H]palmitate and signals less efficiently than the wild type receptor. We use co-expression and immunoprecipitation of epitope-tagged wild-type and mutant β_2 ARs expressed in Sf9 cells using the baculovirus system to assess the possibility of physical and functional interactions between receptors.

EXPERIMENTAL

Materials

Radiolabelled compounds were obtained from DuPont–NEN. Tissue-culture reagents and goat anti-mouse alkaline

Abbreviations used: β_2 AR, β_2 -adrenergic receptor; GPCR, G-protein-coupled receptor; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HA, influenza haemagglutinin; MOI, multiplicity of infection; CYP, [¹²⁵I]cyanopindolol.

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phosphatase-coupled second antibody were obtained from GIBCO-BRL. Centriprep and Centricon cartridges were purchased from Amicon. The enhanced chemiluminescence detection system was purchased from Amersham. All other chemicals were obtained from either Sigma or BioRad.

Construction of recombinant baculoviruses

The recombinant baculoviruses containing either HA- or *c-myc*-tagged wild-type β_2 AR (HA β_2 AR, *c-myc* β_2 AR) or *c-myc*-tagged C341G β_2 AR (*c-myc* C341G) were constructed as described [24]. Briefly, HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) and *c-myc* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) tags containing initiator methionine residues were introduced into the receptor cDNAs immediately before their initiator methionines by subcloning the corresponding double-stranded oligonucleotides. These tagged receptor coding sequences were inserted into the Sf9/baculovirus recombination plasmid pJV-*c-myc* β_2 AR as described previously [24]. Additional constructs, encoding mutant forms of the receptor, used in this study (pBC12BI- β_2 AR-C341G [23], pTZ18R- β_2 AR-S261, 262, 345, 346A [25], were subsequently inserted into the tagged vector construct. Briefly, purified plasmids were digested by *Nco*I-*Ppu*M1 (NEB). The fragments coding for either C341G β_2 AR or S261, 262, 345, 346A β_2 AR were inserted in the pJV-*c-myc* fragment by cohesive end ligation. Positive clones were confirmed by dideoxy sequencing [26]. Recombination of the receptor-bearing plasmids into the baculovirus genome was achieved by co-transfection into Sf9 cells and subsequent screening, purification and amplification of viral plaques [27]. Confirmation of β_2 AR mutant protein expression was assayed by whole cell radio-ligand binding as previously described [24].

Sf9 cell culture

Sf9 cells were maintained at 27 °C in serum-supplemented [10 % (v/v) fetal bovine serum (FBS)] Grace's insect medium. Cells were grown either as monolayers in T-flasks or in suspension in spinner bottles supplemented with pluronic acid to prevent cell tearing due to agitation. Cells were infected at exponential phase at a density of 1×10^6 – 2×10^6 cells per ml for 48 h with multiplicities of infection (MOI) from 2 to 6.

Membrane preparation

Membranes were prepared as described previously [21]. Briefly, cells were washed twice with ice-cold PBS. They were then disrupted by homogenization with a Polytron in 10 ml of ice-cold buffer containing 5 mM Tris/HCl, pH 7.4, 2 mM EDTA, 5 μ g/ml leupeptin, 10 μ g/ml benzamidin and 5 μ g/ml soybean trypsin inhibitor. Lysates were centrifuged at 500 *g* for 5 min at 4 °C, the pellet homogenized and centrifuged as before, then supernatants from both centrifugations pooled. The supernatant was then centrifuged at 45000 *g* for 20 min and the pellet washed twice in the same buffer. This membrane preparation was then immediately used for adenylate cyclase and binding assays or processed further as described below.

Metabolic labelling of cells with [3 H]palmitate or [32 P]phosphate

Metabolic labelling with tritiated palmitate was performed as described previously [28,29]. Briefly, at 30 h post-infection, cells were transferred to serum-free Grace's medium. At 47 h cells were counted and adjusted to 1 % FBS at a density of approximately 50 million cells/20 ml. At 48 h post-infection,

[9,10- 3 H]palmitic acid (60 Ci/mmol) dissolved in 50 μ l of DMSO was added to the culture at a final concentration of 0.2 mCi/ml for 60 min. Metabolic labelling with [32 P] P_i was performed as described [23] with the following modifications; 2 h prior to labelling cells were incubated in phosphate-free Grace's medium. Cells were then incubated with 5 mCi of [32 P] P_i in Grace's medium for 2 h. In either experiment, labelling was terminated by centrifugation at 500 *g* for 5 min at 4 °C followed by two washes with ice-cold PBS. The cells were then disrupted by sonication (two 5 s bursts), membranes prepared and receptors purified by alprenolol-Sepharose affinity chromatography or by immunoprecipitation as described below.

Immunoprecipitation of β_2 AR

Epitope-tagged β_2 AR was immunoprecipitated with a mouse anti-*c-myc* monoclonal antibody (9E10 [30]) or a mouse anti-haemagglutinin monoclonal antibody (12CAS) as described previously [24]. Briefly, receptors were solubilized in 2 % digitonin for 2 h at 4 °C with gentle agitation. Removal of digitonin and concentration of the solubilized receptor was performed by dialysis using Centriprep cartridges against an ice-cold solution (Buffer A) containing 100 mM NaCl, 10 mM Tris/HCl, pH 7.4, and 2 mM EDTA (including the protease inhibitors described above) until the digitonin concentration was reduced below ≈ 0.05 %. Purified 9E10 or 12CA5 antibodies (1:1000 dilution) were added to the concentrate and gently agitated for 2 h at 4 °C. Anti-mouse IgG-agarose (at an 11:1 molar ratio of secondary to primary antibodies) was then added. The reaction was allowed to proceed overnight at 4 °C with gentle agitation. The immunoprecipitate was centrifuged at 12000 rev./min in a micro-centrifuge for 10 min at 4 °C. The pellet was washed three times in buffer A and finally resuspended in 200 μ l of non-reducing SDS/PAGE loading buffer for 30 min, sonicated and centrifuged at 12000 rev./min. The supernatant was then subjected to SDS/PAGE as described below. The efficiency of our immunoprecipitation protocol was determined by soluble [125 I]cyanopindolol (CYP) binding before and after specific immunoprecipitation as previously described [24,33] and was 75 ± 8 %.

Affinity purification of β_2 AR

Receptor solubilized as above was purified using alprenolol-Sepharose chromatography [31] and the affinity purified preparations were concentrated using Centriprep and Centricon cartridges. The amount of receptor was determined in soluble CYP radioligand-binding assays as described [24,33].

SDS/PAGE and Western blotting

Membranes or purified β_2 AR were prepared for non-reducing SDS/PAGE on 10 % slab gels as described [32]. Samples were dissolved in 10 % glycerol/8 % SDS/0.005 % Bromophenol Blue, 25 mM Tris, pH 6.5 and heated to 75 °C for 10 min. For labelling studies, equal amounts of receptor as determined by soluble binding assays using CYP as radioligand were loaded in each lane. Following metabolic labelling with [32 P] P_i , gels were fixed, dried and exposed to Kodak XAR-5 film at -70 °C for several days. In experiments where receptors were metabolically labelled with [3 H]palmitate gels were fixed, treated with Enlightning (DuPont), dried and exposed to Kodak XAR-5 film at room temperature for several weeks. For Western blotting, proteins were transferred to nitrocellulose and blotted with either a mouse anti-*c-myc* monoclonal antibody (9E10) or a mouse anti-HA monoclonal (12CA5) antibody at dilutions of 1:1000.

Immunoblots were revealed with a goat anti-mouse alkaline phosphatase-coupled second antibody (Gibco-BRL) or using the ECL system (Amersham).

Receptor quantification and adenylate cyclase assay

Membranes were prepared and washed as described above. Total receptor number was calculated from binding experiments using CYP as the radioligand [33]. Briefly, 10 μ l of an Sf9 membrane preparation (3–5 μ g of protein) in a total volume of 0.5 ml was labelled with 250 pmol of CYP which is at a near saturating concentration. To determine the efficiency of immunoprecipitation or to calculate amounts of affinity-purified β_2 AR soluble receptor binding assays were performed as described previously [24,33]. In all cases non-specific binding was defined using 10 μ M alprenolol. Adenylate cyclase activity was assayed by the method of Salomon et al. [34]. Enzyme activities were determined in the presence of 1 nM–100 μ M isoproterenol. Data were calculated as pmol cAMP produced per min/mg protein and were analysed by least squares regression using SigmaPlot 4.17 (Jandel Scientific).

RESULTS

Characterization of C341G mutant β_2 AR in Sf9 cells

In previous studies, we have shown that wild-type β_2 AR expressed in the Sf9/baculovirus system has properties that are similar to receptors expressed in mammalian cells [21,24,25,35]. In order to study co-expression of the wild-type β_2 AR with the C341G mutant in Sf9 cells, we first wanted to confirm that this mutant exhibits similar properties when expressed in Sf9 cells as when expressed in mammalian cells. As shown in Figure 1, the previously described reduction in signalling efficacy of C341G [22,23] was also observed in Sf9 cells expressing this receptor. Indeed, at all levels of receptor expression, maximal isoproterenol-stimulated adenylate cyclase activity was higher for the wild-type than for the mutant receptor (Figure 1B). Furthermore, the mutant was found to have constitutively higher basal level of phosphorylation (in the absence of agonist) than the wild-type receptor (Figure 1A, inset) and to be resistant to agonist-promoted desensitization (Table 1) as previously reported in mammalian cells [23,36].

In Figure 1(A) (inset) phosphorylated receptors appeared as a single molecular species of approx. 46–54 kDa when purified by alprenolol-Sepharose affinity chromatography. However, when receptors were immunoprecipitated, phosphorylated molecular forms corresponding to monomeric and dimeric species were detected (Figure 2). These results are consistent with a previous report indicating that receptor antagonists (such as alprenolol used in the affinity purification scheme) reduced the level of the dimeric species [21]. This also indicates that receptor dimers can either act as substrates for regulatory protein kinases or that phosphorylated monomeric receptors can form dimers as the level of phosphorylation increases upon stimulation.

Functional interaction between different β_2 ARs

To determine if wild-type and C341G β_2 ARs interacted with each other in a functional manner, *c-myc* C341G β_2 AR and the HA β_2 AR were either expressed individually or co-expressed in Sf9 cells. Figure 3(A) shows dose-response curves from cells expressing each receptor alone or in combination at a total receptor density of ~ 4 pmol of receptor per mg of membrane protein. The isoproterenol-stimulated adenylyl cyclase activity

Figure 1 Expression of wild-type β_2 AR and C341G in Sf9 cells

Sf9 cells were infected with recombinant baculoviruses encoding either wild-type (WT) or C341G β_2 AR at different MOI (varying from 2–4 for wild-type and 3–6 for C341G). Membranes were prepared from these cells 48 h post-infection and adenylate cyclase assays were performed. (a) Isoproterenol-stimulated adenylate (adenylyl) cyclase activity was determined in membranes from wild-type (5.6 ± 1.3 pmol/mg membrane protein, closed circles, $n = 11$) or C341G (3.2 ± 1.4 pmol/mg membrane protein, closed squares, $n = 3$). Values are expressed as % maximum of wild-type receptor stimulation. Inset: basal phosphorylation level of wild-type and C341G β_2 AR. Cells expressing either receptor were metabolically labelled with [32 P]P $_i$. Solubilized receptors were purified by affinity chromatography and subjected to 10% SDS/PAGE. 100 fmol of receptor were loaded into each lane. (b) Net (maximal-basal) adenylate cyclase stimulation (pmol cAMP $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ membrane protein) by isoproterenol is plotted versus receptor number. Receptor number was determined by saturation binding with CYP as radioligand.

measured in cells infected with C341G alone was 19 % of that observed in cells expressing a similar number of the wild-type receptor (Figure 3A). Surprisingly, cells co-infected with equal amounts of each virus showed a similar level of stimulation as cells expressing the wild-type β_2 AR alone. Assuming no functional interactions between the two receptors and that each receptor represented half of the total receptor population, an intermediate phenotype of adenylyl cyclase stimulation would have been expected.

Based on the observed phenotype for each receptor expressed alone (Figure 1B) we calculated a relation between receptor number and net (maximal-basal) adenylate cyclase stimulation. From Figure 3(A), at 4 pmol of receptor the net stimulation (maximal-basal) by C341G is 76 pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ and for the WT receptor it is 398 pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$. By linear regression of the data in Figure 1(B), we calculated that the net activity ob-

Table 1 Desensitization of wild-type and mutant β_2 AR

Prior exposure of Sf9 cells expressing various β_2 AR to agonist (1 μ M isoproterenol for 30 min) leads to a reduction of response to ligand subsequently applied to membranes prepared from these cells for measurement of adenylate cyclase stimulation.

	% Desensitization* (maximal)	% Desensitization† (at 10^{-8} M ISO)
Wild-type	30 ± 4.5	21 ± 2.7
C341G	10 ± 5.2	9 ± 1.5
Wild-type/C341G	27.5 ± 6.1	23.6 ± 3.2

* The extent of desensitization is expressed as the % reduction in the maximal isoproterenol-stimulated adenylate cyclase activity induced by pretreatment relative to control. Maximal reduction of response relative to control is plotted ($\pm$ S.E.M., $n = 3$) for wild-type, C341G, and co-expressed C341G/wild-type.

† The extent of desensitization is expressed as the % reduction in the adenylate cyclase activity stimulated by 10^{-8} M isoproterenol (ISO) after pretreatment with 10^{-6} M isoproterenol relative to control. The isoproterenol stimulation was determined using full dose-response curves of isoproterenol-stimulated adenylate cyclase activity. The concentration of total receptor was ≈ 5 pmol/mg membrane protein for all experiments. Adenylate cyclase activity is measured as described in the Experimental section.

Figure 2 Phosphorylation of monomeric and dimeric β_2 AR

c-myc β_2 AR were immunoprecipitated from cells metabolically labelled with [32 P]P_i were treated with vehicle (lane 1) or 1 μ M isoproterenol (lane 2) for 30 min prior to cell harvesting. After SDS/PAGE, incorporation of label was determined by exposing dried gels to Kodak XAR film at -70°C . Positions of the monomeric and dimeric species are denoted by arrows and molecular-mass markers are shown on the right. Data are representative of three independent experiments.

tained at an expression level of 2 pmol receptor (i.e. when the two receptors are co-expressed at equivalent levels) should be 0.62 and 0.65-fold of that found at 4 pmol of receptor for WT and C341G expressed alone, respectively. It follows that, if the receptors are not interacting, co-expression should lead to a net activity of $297 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ [$(0.62 \times 398) + (0.65 \times 76)$]. The measured value in Figure 3(A) for the co-expressed receptors was $425 \text{ pmol cAMP} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein thus suggesting a dominant-positive effect of the wild-type receptor.

Further supporting the notion that functional interactions between the two receptor species occurred, we found that the maximal isoproterenol-stimulated adenylate cyclase activity measured in co-expression experiments in which the relative stoichiometries of the two viruses were varied (up to 2:1,

C341G:wild-type) was always higher than the predicted simple summation of wild-type and mutant activity alone (data not shown).

To confirm that infection of cells with equal titres of viruses led to the expression of the two proteins, Western blot analysis was carried out. Crude membranes from cells infected with HA β_2 AR and *c-myc* C341G β_2 AR were immunoblotted with either anti-HA or anti-*c-myc* antibodies. As shown in the inset of Figure 3(A), both receptors were detected and thus co-expression was confirmed. Unfortunately, direct quantitative comparison is prevented by the use of different primary antibodies.

To determine if the apparent dominant positive effect of the β_2 AR over the C341G mutant was a unique feature of the wild-type receptor, we co-expressed C341G with another receptor mutant displaying a distinct phenotype. Toward this end we used S261, 262, 345, 346A β_2 AR, which has two consensus protein kinase sites mutated and thus is somewhat resistant to agonist-promoted desensitization but stimulates adenylate cyclase with an efficacy that is similar to that of the wild-type β_2 AR (cf. Figures 3A and 3B; [25,37]). As for the wild-type receptor, the phenotype of the S261, 262, 345, 346A β_2 AR mutant dominated over that of C341G. Indeed, when the two mutants were co-expressed, the agonist-stimulated adenylate cyclase activity measured was similar to that of the S261, 262, 345, 346A β_2 AR expressed alone (Figure 3B). These data indicate that both the wild-type β_2 AR and S261, 262, 345, 346A β_2 AR acted in a dominant positive fashion, complementing the C341G phenotype.

In order to assess if the dominant positive effect extended to other properties of the receptor, the profiles of agonist-promoted desensitization were studied in cells infected with wild-type and C341G receptors. Prolonged exposure to agonist leads to a desensitization of the β_2 AR which is associated with an increased phosphorylation of the receptor [38]. As described above, the C341G β_2 AR is constitutively desensitized and agonist-stimulation results in little additional agonist-promoted desensitization (Table 1). As demonstrated for the efficacy of signalling, co-expression of receptors led to a dominant effect on the desensitization profile. The extent of desensitization observed in the co-expression experiments of C341G with wild-type β_2 AR was similar to those measured when wild-type β_2 AR was expressed alone (Table 1) confirming the dominant-positive effect of the wild-type receptor.

Physical interactions between β_2 ARs

In a previous study we demonstrated using an immunoblotting approach that wild-type β_2 AR can form homodimers [21]. To determine if C341G β_2 AR can also form homodimers, membranes prepared from cells expressing *c-myc* C341G β_2 AR or *c-myc* β_2 AR were subjected to 10% non-reducing SDS/PAGE, transferred to nitrocellulose and blotted with the anti-*c-myc* antibody. Figure 4 shows that molecular species representing monomeric and dimeric *c-myc*-tagged wild-type β_2 AR (lane 2) and *c-myc* C341G β_2 AR (lane 1) were detected. As we have demonstrated previously for the wild-type β_2 AR [21] higher molecular mass forms of the C341G receptor were detected as well suggesting the possibility of higher order structures such as receptor tetramers. However, these structures were not seen consistently in all preparations, in contrast to the monomeric and dimeric forms.

In order to determine if there was a physical correlate to the functional interactions between wild-type and C341G β_2 AR, we specifically assessed whether heterodimers of wild-type and C341G receptors could form. Toward this end, we adopted a variation of the co-immunoprecipitation approach that we recently used to demonstrate β_2 AR homodimerization [21]. We

Figure 3 Adenylate cyclase stimulation after co-expression of wild-type or S261, 262, 345, 346A β_2 AR with C341G β_2 AR

Equivalent amounts of total receptor (approx. 4 pmol/mg membrane protein) were used in all curves shown. Receptor number was determined by saturation binding with CYP as radioligand. Stimulation of adenylate cyclase activity is measured as described in Experimental section. (a) Wild-type β_2 AR (4.1 ± 0.2 pmol/mg membrane protein) is represented by open circles, C341G (4.2 ± 0.4 pmol/mg membrane protein) is represented by open squares and co-expressed wild-type β_2 AR and C341G (3.8 ± 0.4 pmol/mg membrane protein) are represented by closed circles. Inset: crude membrane preparations (2 pmol receptor/mg membrane protein, 500 fmol/lane) of wild-type HA- β_2 AR (lane 1) or c-myc C341G (lane 2) which were immunoblotted following SDS/PAGE with either the anti-HA monoclonal 12CA5 or the anti-c-myc monoclonal 9E10 antibodies, respectively. Arrows are used to denote monomeric and dimeric forms. (b) C341G is represented by open squares, S261, 262, 345, 346A β_2 AR (4.3 ± 0.6 pmol/mg membrane protein) is represented by open triangles and co-expressed S261, 262, 345, 346A β_2 AR and C341G (4.4 ± 0.5 pmol/mg membrane protein) receptors are represented by closed squares. Data are presented as means of five independent experiments $\pm$ S.E.M.

Figure 4 Dimeric species are seen in both wild-type and C341G β_2 AR

Crude membrane preparations (2 pmol receptor/mg membrane protein) of wild-type c-myc β_2 AR (lane 1) or c-myc C341G (lane 2) were immunoblotted following SDS/PAGE with the anti-c-myc monoclonal 9E10 antibody. The blots reveal immunoreactive bands corresponding to the expected monomeric form (43–50 kDa) and dimeric species (85–95 kDa) forms as well as possible higher molecular mass forms. Position of the monomeric and dimeric species are denoted by arrows and molecular-mass markers are shown on the right. Data are representative of three independent experiments.

co-expressed a c-myc-tagged C341G receptor with a non epitope-tagged wild-type β_2 AR and metabolically labelled the cells with [3 H]palmitate. Receptors were solubilized and immunoprecipitated with the anti-c-myc antibody and then subjected to SDS/PAGE and fluorography. Since it has been previously demonstrated that the C341G mutant does not incorporate [3 H]palmitate [22,23], labelled palmitoylated wild-type receptor should only be detected if it is co-immunoprecipitated with the tagged-non-palmitoylated C341G mutant. Figure 5 (lane 1) shows

Figure 5 Co-immunoprecipitation of c-myc-tagged C341G and wild-type β_2 AR

Cells expressing either wild-type c-myc β_2 AR (lane 1), c-myc C341G and untagged wild-type β_2 AR together (lane 2) or c-myc C341G receptors alone (lane 3) were metabolically labelled with tritiated palmitate as described in the Experimental section. Receptors were solubilized from crude membrane preparations and immunoprecipitated with an anti-c-myc monoclonal antibody followed by SDS/PAGE (10%) under non-reducing conditions (1.5 pmol receptor were loaded in each lane). The gel was then fixed, dried and exposed to XAR film at -70°C . Data are representative of three independent experiments. A parallel experiment using cells co-expressing HA- β_2 AR and c-myc C341G was performed where solubilized receptors were immunoprecipitated with the anti-HA antibody and blotted with either anti-HA (lane 4) or anti-c-myc (lane 5). Data are representative of two independent experiments. Position of the receptor bands are denoted by arrows and molecular-mass markers are shown on the right.

incorporation of labelled palmitate into the immunoprecipitated c-myc wild-type β_2 AR when expressed alone. It also demonstrates the incorporation of labelled palmitate into immunoprecipitates obtained from cells expressing the c-myc C341G and a non-tagged wild-type β_2 AR (lane 2). Noticeably, it is mainly the dimeric species which is immunoprecipitated consistent with the notion that the two molecular species were co-immunoprecipitated as part of a complex. When the c-myc-tagged C341G β_2 AR was expressed alone, no [3 H]palmitate labelling

could be immunoprecipitated, confirming that C341G cannot incorporate tritiated palmitate (Figure 5, lane 3). In a parallel experiment we also show that immunoprecipitation of HA- β_2 AR results in a co-immunoprecipitation of *c-myc*-tagged C341G. When cells co-expressing the two receptors are solubilized and HA- β_2 AR are immunoprecipitated with the anti-HA antibody both the monomeric and dimeric species are detected in Western blots using the anti-HA antibody (Figure 5, lane 4). When the same immunoprecipitate is blotted with the anti-*c-myc* antibody it is the dimeric species which again predominates (Figure 5, lane 5), confirming that the C341 β_2 AR is associated with the HA- β_2 AR as part of a heterodimeric complex.

DISCUSSION

To study the functional and/or physical interactions between β_2 ARs we have used co-expression and immunoprecipitation of receptors bearing epitope tags. We also took advantage of a mutated β_2 AR (C341G) which cannot be palmitoylated and is impaired in its ability to couple to G_s and to undergo desensitization. When C341G is co-expressed with the wild-type β_2 AR at equal levels the wild-type receptor exhibits a dominant positive effect complementing the defects in the mutant. Indeed, both signalling and desensitization properties were rescued. Similar functional complementation was also seen between the S261, 262, 345, 346A β_2 AR and C341G β_2 AR as the signalling phenotype of the former dominated when the two receptors were co-expressed. The existence of heterodimeric receptor species was confirmed using a co-immunoprecipitation approach coupled with a differential palmitate labelling paradigm (Figure 5). The observation that C341G and wild-type receptors can be co-immunoprecipitated suggests that the functional complementation most likely is a manifestation of physical interactions between the two receptor species.

Receptor dimerization has long been recognized to be important for most families of signal transducing receptors. These include many growth factor receptors with tyrosine kinase activity, members of the growth hormone receptor family, bacterial sensory receptors and cytokine receptors (see [39,40] for review). However, until recently the oligomeric nature of GPCRs was not generally appreciated despite a significant amount of circumstantial evidence. The data reported in the present study brings additional support to recent reports suggesting that GPCRs also exist physically and functionally as oligomers (see Introduction). The idea that signal transduction through GPCR systems occurs via the formation of higher order structures is also supported by the recent isolation of oligomeric G protein and GPCR complexes [14,41–43] as well as by recent observations that structural cytoskeletal elements may be required for proper signal transduction by organizing such complexes ([44]; reviewed in [42]).

Whether or not the oligomeric receptor and receptor–G protein complexes represent the active signalling forms of these proteins or a passive organizational network remains an open question. In an recent study we demonstrated that a peptide corresponding to transmembrane domain VI of the β_2 AR inhibits both receptor activation and dimerization arguing that dimerization plays a functional role rather than simply a structural role [21]. However, it was not clear from this study whether (1) the dimeric species of the receptor is the active form of the receptor and the peptide deactivates the receptor by inhibiting dimerization or (2) if the peptide causes the formation of a ‘pseudodimer’ which inactivates the monomeric form of the receptor. The results obtained here in our co-expression studies show that wild-type β_2 AR and S261, 262, 345, 346A β_2 AR can complement the functional

defects in C341G receptors, suggesting that the dimer is the active species of the receptor.

Also consistent with the notion that receptor dimers are important for receptor function is an elegant study demonstrating the rescue of ligand binding by co-expression of two different binding-defective angiotensin II receptor mutants [19]. Indeed, intermolecular interaction between the two receptors is the simplest explanation for the complementation of binding seen as genetic recombination events were carefully controlled for in this study.

Although our data are consistent with the idea that dimerization may be important for receptor activation, one could still posit a role for dimerization in subsequent regulatory events such as desensitization and down-regulation. If this were the case, one would expect the extent of dimerization to be altered in a mutant receptor which is constitutively desensitized such as C341G β_2 AR. However, the data obtained suggest that dimerization and desensitization are independent phenomena, since C341G was found to form dimers to a similar extent as the wild type receptor (Figure 4). A role for dimerization in longer term regulatory processes such as sequestration and/or down-regulation cannot be ruled out by the present study.

Interestingly, our results also suggests that dimerization between a constitutively desensitized and a wild-type receptor yields a fully functional receptor and thus that phosphorylation of a single receptor polypeptide chain may not be sufficient to regulate receptor responsiveness. Further, our observations show that dimerization is not regulated by processes such as phosphorylation and palmitoylation, which are intimately involved in the regulation of receptor desensitization, since a non-palmitoylated receptor which is constitutively phosphorylated can form both homodimers as well as heterodimers with wild-type receptor.

Taken together the results presented here provide a link between complementation of receptor function and physical interaction between receptor moieties suggesting that dimerization plays an important role in GPCR signalling.

T.E.H. was supported by fellowships from the Heart and Stroke Foundation of Canada and the Fonds de la Recherche en Santé du Québec and thanks J. Popper and S. Earle for inspiration. T.P.L. and L.A. were supported by scholarships from the Medical Research Council of Canada (MRCC). M.B. is an MRCC Scientist. This work was supported by grants from the Heart and Stroke Foundation of Québec and the MRCC to M.B. and T.E.H.

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