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Biochemical Characterization of β_2-Adrenergic Receptor Dimers and Oligomers

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G Protein-coupled receptor dimerization/oligomerization has been well established during the last several years. Studies have demonstrated the existence of dimers/digomers both *in vitro* and in living cells. However, a thorough characterization of the biochemical nature of receptor dimers and oligomers as well as their occurrence at the cell surface has not been properly addressed. In this study, we show that both β_2-adrenergic receptor (β_2AR) dimers and oligomers exist at the plasma membrane and that the detection of such species, following receptor solubilization and resolution by denaturing polyacrylamide gel electrophoresis (SDS-PAGE), does not result from the formation of spurious disulfide bonds during cell lysis. Moreover, our results indicate that the biochemical nature of β_2AR dimers is different from that of the oligomers. Although both complexes are partially resistant to SDS denaturation, disulfide bonding is absolutely required for the stability of β_2AR oligomers but not dimers in SDS-PAGE. Indeed, dimeric species can be detected even in the presence of high concentrations of reducing and alkylating agents. Although the different biochemical nature of the dimers and oligomers may be indicative of distinct biological roles in cells, additional studies will be required to further elucidate the biosynthesis and function of these receptor forms.

Key words: Dimerization / Disulfide bonds / Cross-linking / G protein-coupled receptors.

Introduction

G protein-coupled receptors (GPCR) represent the largest family of membrane receptors (Bargmann, 1998) involved in signal transduction across biological membranes. Their physiological importance is evidenced by the wide spectrum of signaling molecules that rely on members of this family for their actions. These include neurotransmitters, peptide hormones, lipid mediators, odorant and taste molecules, and photons. Until recently, much of the classical pharmacology depicted these receptors as monomeric entities that interact with their cognate heterotrimeric G protein upon activation. However, there is now mounting functional and biochemical evidence suggesting that these receptors can exist and function as dimers and even larger oligomeric complexes (Angers *et al*., 2002). Obviously, the possibility of homodimerization raises important questions about the mechanisms involved in receptor activation and regulation, while the recent appreciation that GPCR can also engage in hetero-dimerization provides the basis for an added level of pharmacological diversity.

Despite the fundamental questions raised by the existence of GPCR oligomerization, very little is known about the biochemical properties of these complexes. The most direct and convincing evidence supporting the existence of GPCR homo and hetero-complexes comes from co-immunoprecipitation of differentially epitope-tagged receptors as well as biophysical approaches such as BRET and FRET (Bouvier, 2001; Angers *et al*., 2002). Although powerful, these techniques do not allow probing the biochemical nature of the oligomers formed. In contrast, Western blot analysis of GPCR expressed in various tissues and heterologous cellular systems has revealed immunoreactive bands that might correspond to complexes composed of two or more receptors that are resistant to SDS denaturation (Hebert *et al*., 1996; Ng *et al*., 1996). This characteristic is shared by several other integral membrane proteins that are known to form functional dimers (Furthmayr and Marchesi, 1976; Salhany *et al*., 1990; Danner *et al*., 1993). For one of these proteins, glycophorin A, mutagenesis and structural studies attributed SDS resistance of the complex to tight hydrophobic packing of the transmembrane domains engaged in dimer formation (Lemmon *et al*., 1992a,b; MacKenzie *et al*., 1997).

However, the fact that these putative dimers and larger oligomers were observed under denaturing conditions raised considerable suspicion regarding their biological significance. Non-specific aggregation resulting from incomplete solubilization as well as formation of spurious disulfide bonds were evoked as potential artefacts that could explain the appearance of multiple immunoreactive bands of the V2-vasopressine receptor (Innamorati *et al*., 1996). Nevertheless, Western blot analysis following SDS-PAGE remains a widely used approach to assess GPCR dimerization (for a review see Salahpour *et al*.,

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2000). Unfortunately, very little efforts have been made to demonstrate that these species truly correspond to receptor multimers that could exist as such in cells.

Therefore, the present study was undertaken to critically assess the biochemical characteristics and origin of the various immunoreactive species of the human β_2AR detected following SDS-PAGE. The roles of genuine disulfide bonds in the stabilization of dimers and multimeric complexes of the receptor were also assessed. We found that immunoreactive dimers and oligomers do not result from preparation artefacts and most likely exist in intact cells where higher order oligomers are stabilized by disulfide bonds.

Results

As shown in Figure 1, Western blot analysis of human β_2AR resolved in non-reducing SDS-PAGE reveals the presence of multiple immunoreactivity species. These can be divided in three major groups of bands with apparent molecular masses corresponding to multiple assemblies of the monomeric species (~45 kDa). The heterogeneity within each group of bands most likely results from different glycosylation states of the receptor (Stiles *et al*., 1984). The apparent multimeric species observed do not result from the formation of spurious intermolecular disulfide bonds associated with oxidizing conditions since they were detected even in the presence of high concentrations of alkylating agents such as iodoacetamide and *N*-ethyl maleimide (NEM) during cell lysis (compare lane 1,2 and 3). The efficacy of the alkylating treatments is illustrated by the fact that the electrophoretic mobility of each individual species was decreased, most likely reflecting the ability of iodoacetamide and NEM to prevent the formation of intra-molecular bond(s) occurring during membrane preparation. This hypothesis is supported by the observation that a similar slower mobility was observed when SDS-PAGE was carried out under reducing conditions (see lanes 4–6). As can also be seen in these lanes, reducing conditions led to a complete dissociation of the large oligomeric species indicating that the higher order complexes require disulfide bonding. This reduction in the higher order oligomeric species was accompanied by an increase in the amount of monomers. In contrast, immunoreactivity was still detected for the dimeric species under these reducing conditions. In fact, densitometric analysis revealed that when considering the total population of immunoreactive species, the relative proportions of receptor detected as dimers were almost identical under reducing and nonreducing conditions (compare lanes 1 and 4). However, when compared to the monomer, the relative intensity of the dimeric species was significantly diminished suggesting that all higher oligomeric species were reduced to monomers.

In order to determine if the resistance of the dimers to β-mercaptoethanol treatment reflects disulfide bond-independent dimerization or results from incomplete reduction, the effect of increasing concentrations (0–50 mM) of dithiothreitol (DTT) was assessed. As seen in Figure 2, the amount of higher order oligomers steadily decreased as a function of the DTT concentration, reaching very low levels at 50 mM. However, only a modest reduction of the dimer immunoreactivity was observed, the maximal effect of DTT being already reached at 25 mM,

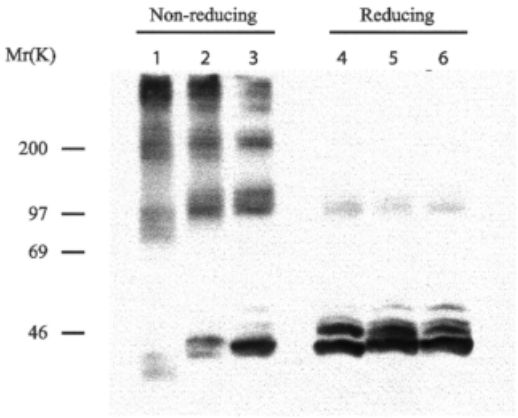

Fig. 1 β_2AR Dimers/Oligomers Do Not Result from Spurious Disulfide Bonding.
Sf9 cells expressing the myc-tagged β_2AR were lysed and membranes prepared as described in the Materials and Methods section in the absence of alkylating agent (lanes 1 and 4) or in the presence of 2 mM iodoacetamide (lanes 2 and 5) or 5 mM *N*-ethyl maleimide (lanes 3 and 6). The membrane preparations were then resolved on 8% SDS-PAGE in either non-reducing (lanes 1–3) or reducing (2.5% β-mercaptoethanol; lanes 4–6) conditions. Following transfer to nitrocellulose, the β_2AR was revealed by Western blot analysis using the 9E10 anti-myc monoclonal antibody.

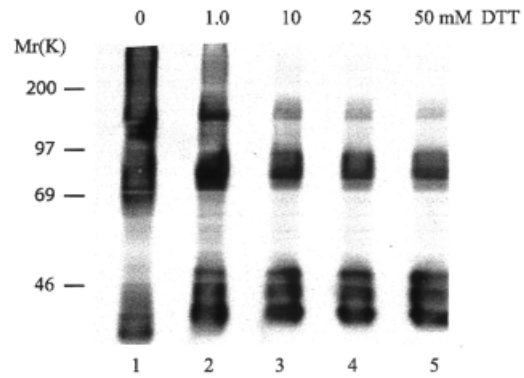

Fig. 2 β_2AR Oligomers Involve Disulfide Bond Formation.
Sf9 cells expressing the myc-tagged β_2AR were lysed in the presence of 2 mM iodoacetamide and membranes prepared as described in Materials and Methods. The membrane preparations were then resolved on 8% SDS-PAGE in sample buffer containing increasing concentration (0–50) mM of DTT in the absence of other reducing agents. Following transfer to nitrocellulose, the β_2AR was revealed by Western blot analysis using the 9E10 anti-myc monoclonal antibody.

indicating the presence of a dimer population that is resistant to DTT. In fact, increasing the concentration of the reducing agent up to 500 mM (data not shown) did not promote any additional reduction of the dimers. Densitometric analysis reveals that in comparison to the monomers the intensity if the higher oligomeric species declined by 79% upon DTT treatment while that of the dimer was only reduced by 36%. This difference in sensitivity between the dimers and the higher order oligomers to the reducing agents suggests that they have distinct structural organizations that render the intermolecular disulfide bonds more resistant within the dimer or that some dimers do not require disulfide bridges to remain stable in SDS.

It should be emphasized here that direct detection of dimers and oligomers on SDS-PAGE is a reflection of the stability of the complexes under denaturing conditions and not a quantitative assessment of their relative amount in native membranes. This obviously raises the question of whether stable dimers on SDS-PAGE could be an artefact of SDS solubilization. Indeed, it is often suggested that hydrophobic transmembrane segments associate more readily among themselves than with SDS thus leading to aggregation that could be mistaken for dimerization or oligomerization. If this were the case for the β_2AR, one would predict that isolated monomeric species would not be stable in SDS and would readily convert to dimers and higher oligomeric forms. In order to test this hypothesis, receptors were solubilized using the non-ionic detergent, dodecyl maltoside (which maintains the ligand binding properties of the receptor), and subjected to ultrafiltration using a filter with a molecular cut-off of ~100 kDa (Microcon 100). Figure 3 shows the species that were found in the total soluble fraction (lane 1), the retained fraction (lane 2) and the ultrafiltrate (lane 3) following denaturation in SDS for 30 minutes. Clearly, the monomeric species could be isolated and was stable in SDS since no dimeric or oligomeric species were detected in the ultrafiltrate (lane 3) following SDS-PAGE under reducing conditions. This indicates that dimers and oligomers cannot spontaneously form when monomeric receptors are isolated, essentially ruling out the possibility that dimers and oligomers result from non-specific aggregation due to SDS denaturation and PAGE. The increased proportion of dimeric forms observed in the retained fraction (compare lane 2 where the dimers represent 43% of the total receptor population to lane 1 where it represents 30%) could result either from an enrichment of the SDS-stable dimers or from some aggregation due to the filtration itself. In any case, the detection of a significant amount of monomers in this fraction and the complete absence of dimers in the ultrafiltrate (lane 3), even following a 30 minute incubation in SDS, is consistent with the idea that SDS most likely promotes dissociation of preformed dimers rather than favoring the spurious formation of dimers from monomeric β_2AR.

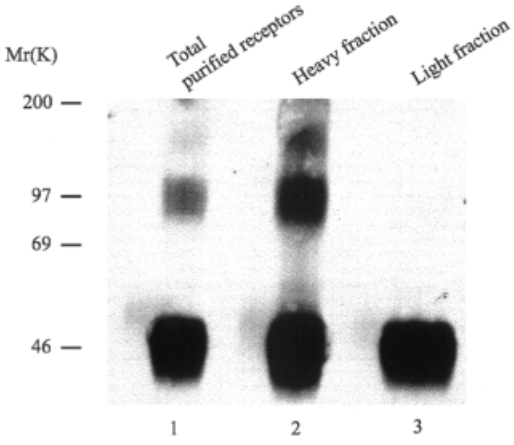

Fig. 3 Isolation of β_2AR Monomers by Ultrafiltration.
Myc-tagged β_2AR were solubilized using 0.3% dodecyl-maltoside and affinity-purified by alprenolol-Sepharose chromatography. Five pmol of the purified receptors was then subjected to ultrafiltration using a Microcon 100. The retained fraction and the ultrafiltrate were then concentrated on a centricon 30 and resolved on 8% SDS-PAGE in sample buffers containing 50 mM DTT. Lane 1: one pmol of the starting purified material; lane 2: concentrated retained fraction; lane 3: concentrated ultrafiltrate. Following transfer to nitrocellulose, the β_2AR was revealed by Western blot analysis using the 9E10 anti-myc monoclonal antibody.

Additional evidence suggesting that β_2AR dimers and oligomers do not represent an artefact of cell lysis or SDS-PAGE include the observation that covalent cross-linking carried out in whole cells increased the amount of the dimers and higher oligomeric species. As shown in Figure 4A and B, treatment with BS3, a membrane-impermeable cross-linker, led to a concentration-dependent increase in the amount of both dimer and higher order oligomeric species detected following SDS-PAGE, reaching a maximum at cross-linker concentrations between 0.5 and 1 mM. At higher concentrations (1 to 5 mM) the amount of dimers, oligomers as well as monomers tended to decrease. This is probably due to the fact that very high concentrations of BS3 favor the formation of larger complexes that cannot be properly resolved by SDS-PAGE. Identical patterns were obtained whether the cross-linking reaction was performed using intact cells (Figure 4A) or crude membrane preparations (4B), indicating that these molecular species exist at the cell surface where they are accessible to the chemical cross-linking reagent. Interestingly, pre-treatment of the membrane with the reducing agent DTT prior to the cross-linking experiments affected the efficacy of BS3 to stabilize both the dimer and higher oligomeric forms (Figure 4C). The effect of DTT on the ability of BS3 to stabilize dimers and oligomers is illustrated in Figure 5. As can be appreciated, densitometric analysis revealed that the reducing conditions had a greater effect on inhibiting the action of BS3 on the higher oligomeric forms than on the dimers. This result again points to a difference between the biochemical properties of the dimer and higher order oligomers, the latter being more sensitive to reducing agents. The fact that each species retains the same sen-

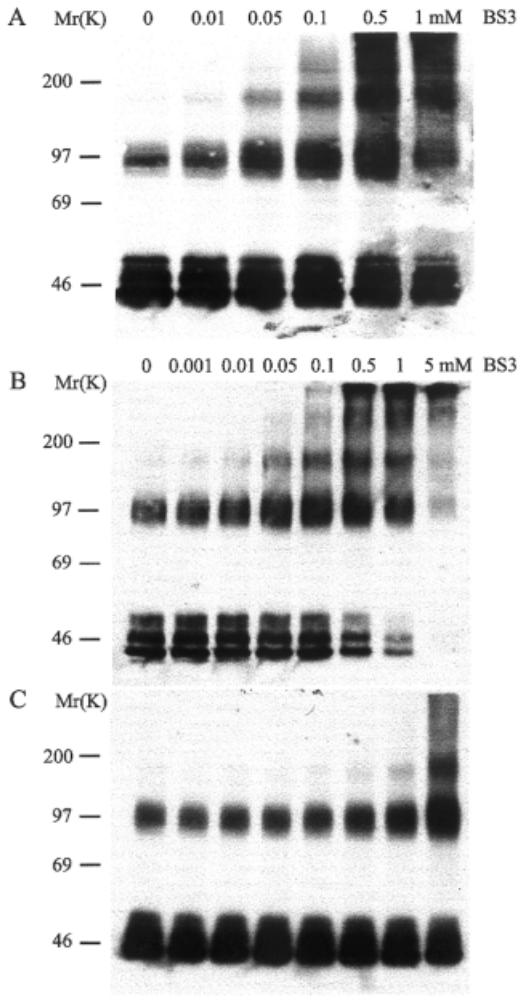

Fig. 4 Membrane and Whole Cell Cross-Linking of β_2AR Expressed in Sf9 Cells.
Increasing concentration of the membrane-impermeable cross-linking agent BS3 was added to intact Sf9 cells (A) or membrane preparation (B and C) expressing the myc-tagged β_2AR. In (C), the membrane preparation was treated with 50 mM DTT prior to the addition of BS3. Following transfer to nitrocellulose, the β_2AR was revealed by Western blot analysis using the 9E10 anti-myc monoclonal antibody.

sitivity to reducing agents, whether receptors are treated within membranes (Figure 4C) or are solubilized by SDS (Figure 2), further supports the notion that SDS-resistant dimers and oligomers are not artefacts of protein denaturation.

In the present study, Sf9 cells were used because the higher level of expression obtained in this system facilitated the detection of the various receptor species. However, it could be argued that oligomer formation could result from non-specific aggregation due to the very high level of expression or to the heterologous nature of the insect cells. Thus, in order to validate the observations made in Sf9 cells, membranes derived from human HEK293 cells stably expressing human β_2AR were treated with increasing concentrations of BS3. As shown in Figure 6, BS3 promoted the stabilization of higher molecular mass species, presumably corresponding to the dimeric form of the receptor indicating that this species also exists in human cells expressing a human receptor. As observed for the Sf9 cells (Figure 4), the stabilizing ef-

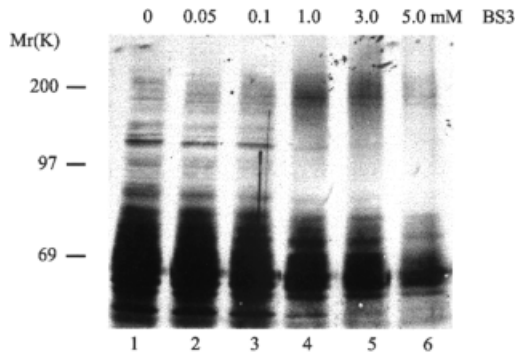

Fig. 6 Cross-Linking of β_2AR Expressed in HEK-293 Cells.
Increasing concentration (0–5 mM) of the membrane-impermeable cross-linking agent BS3 was added to membrane preparations derived from HEK-293 cells stably expressing the β_2AR. Following transfer to nitrocellulose, the β_2AR was detected by Western blot analysis using the H-20 anti-β_2AR polyclonal antibody.

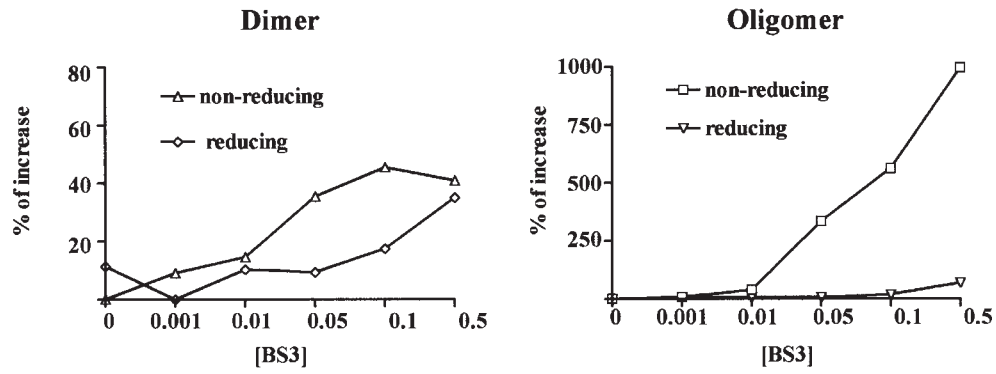

Fig. 5 Quantitative Analysis of β_2AR Cross-Linking on Membranes.
Densitometric analysis comparing the effect of DTT pretreatment (Figure 4B *versus* 4C) on the ability of BS3 to stabilize the dimeric and higher oligomeric species. The data are expressed as percent of increase of each species as a function of BS3 concentration.

fect of BS3 was biphasic most likely as a result of the formation of very large complexes at the highest concentrations used. The fact that the oligomeric forms could be stabilized by performing the cross-linking before the addition of SDS confirms that the detection of these species in mammalian cells is not an artefact of solubilization, as was also observed with the insect cells. The cross-linking reaction could not proceed after SDS solubilization and denaturation since BS3 was inactivated by the addition of an excess of reactive amine groups. It should be noted that the apparent molecular mass of the monomeric species detected in the HEK293 cells (66 kDa) is different from that of the receptor expressed in Sf9 cells due to the different glycosylation processes occurring in the two cell types (Luckow and Summers, 1988). The discrete species detected within the broad band corresponding to the monomeric receptor most likely reflect the glycosylation heterogeneity characteristic of GPCR (Stiles *et al*., 1984).

Discussion

Although several authors have interpreted the presence of higher molecular weight receptor species following SDS-PAGE as evidence of GPCR dimerization and multimerization, the notion that they may represent artefacts that have little to do with normal receptor structure is still prevalent. The present study clearly indicates that the different molecular weight forms detected on SDS-PAGE do not represent biochemical artefacts of cell lysis, spurious disulfide bonding or SDS-promoted aggregation but rather are the reflection of oligomeric assemblies of the β_2AR. Consistent with this notion, the occurrence of β_2AR and other GPCR oligomerization in living cells has recently been confirmed using energy transfer approaches such as BRET or FRET (Angers *et al*., 2000; Cornea *et al*., 2000; Rocheville *et al*., 2000; Overton and Blumer, 2000; Kroeger *et al*., 2001). However, the biochemical properties of these complexes have not been thoroughly investigated.

For some receptors, disulfide bonding has been proposed to play an important role in dimer and oligomer formation (Romano *et al*., 1996; Bai *et al*., 1998; Zeng and Wess, 1999). However, the molecular forms corresponding to β_2AR dimers in this study were found to be resistant to both exhaustive reduction and alkylation indicating that interactions distinct from disulfide bonds contribute to stabilization of the dimers. By analogy with glycophorin A, a single transmembrane domain protein which also forms SDS-resistant dimers, we previously suggested that hydrophobic interaction involving the transmembrane domains of the β_2AR may play a role in dimer formation (Hebert *et al*., 1996). Similar roles for TM domains were also invoked for dimerization of the D2 (Ng *et al*., 1996) and D1 receptors (Lee *et al*., 2000). The resistance of these complexes to SDS denaturation could be attributed to tight hydrophobic packing of the transmembrane domains engaged in dimer interface as suggested for glycophorin A (MacKenzie *et al*., 1997). Even for the calcium-sensing and muscarinic receptors, where dimerization has been shown to involve disulfide bonds (Pace *et al*., 1999; Zeng and Wess, 1999), non-disulfide interactions have also been reported to be sufficient for dimer formation. Indeed, co-immunoprecipitation could still be obtained using receptor mutants that lacked the cysteines involved in intermolecular disulfide bonding. However, in these cases the dimeric species could not be detected on SDS-PAGE as a stable complex, indicating that calcium and muscarinic receptor dimers lacking disulfide bonds are not sufficiently stable to resist SDS denaturation. For the β_2AR, partial resistance of dimers to disulfide bond disruption suggests that hydrophobic stacking between TM domains may be sufficient for dimer stability in SDS. Taken together, these results suggest that at least two types of interactions, disulfide bonds and hydrophobic packing, are involved in maintaining the dimeric structure of GPCR.

In contrast to β_2AR dimers, larger oligomers were completely dissociated upon β-mercaptoethanol treatment suggesting that such oligomeric assembly involves the formation of disulfide bond. This may indicate that dimers and higher oligomeric assemblies have distinct structural arrangements. One possibility is that dimers can self assemble into larger structure *via* disulfide bonding. Alternatively, they may represent an entirely different type of association. Indeed, although the formation of intermolecular disulfide bonds has been suggested for several receptors (Romano *et al*., 1996; Bai *et al*., 1998; Zeng and Wess, 1999), one could argue that conformational changes imposed by intramolecular bonds could promote other types of interaction that would underlie oligomerization.

The fact that BS3 treatment stabilized both dimers and higher order oligomers in whole cells indicates that these species can co-exist at the cell surface. Although several studies documented that receptor dimers could be detected at the cell surface (Cvejic and Devi, 1997; Bai *et al*., 1998), this report presents the first direct evidence supporting the existence of multimers at the cell surface. In fact, upon BS3 treatment in whole cells, these became the prevalent species, an observation that may suggest that oligomers represent an important fraction of the receptor population. The existence of large oligomers has also been suggested by other investigators. In particular, both pharmacological and biochemical analysis of the m2 muscarinic receptor have suggested that it may exist as an octamer (Wreggett and Wells, 1995). More recently, oligomerization rather than simple dimerization of the μ-opioid receptor has been linked to the internalization process (He *et al*., 2002). Whether dimers and higher order oligomers could have distinct biological roles remains to be further investigated.

However, the study of these various forms is complicated by the lack of adequate methodologies to determine their relative abundance. Indeed, neither co-im-

munoprecipitation nor energy transfer approaches used to study receptor oligomerization allow to distinguish between dimers and higher order structures. Our study excluded as much as possible the likelihood that the higher oligomeric species could represent biochemical artefacts thus suggesting they may indeed exist in whole cells. However, the SDS-PAGE approach used can hardly be considered quantitative. In particular, the resistance to SDS denaturation is most likely only partial and not identical for all oligomeric forms. Consequently, the dimeric and monomeric entities detected could result from partial SDS promoted dissociation of the larger oligomeric arrangements. Future work will therefore be needed to quantitatively assess the proportion of the different receptor species in cells and to determine their specific roles in receptor function.

Materials and Methods

Materials

All cell culture materials were purchased from Invitrogen (Burlington, ON, Canada). *N*-ethyl maleimide, iodoacetamide, benzamidine and soybean trypsin inhibitor were purchased from Sigma (Saint Louis, MO, USA). Dodecyl maltoside and leupeptin were obtained from Alexis while bis(sulfosuccinimidyl)suberate (BS3) was from Pierce (Rockford, IL, USA).

Sf9 and HEK 293 Cell Culture

Sf9 cells were maintained at 27 °C in serum-supplemented (10% fetal bovine serum, v/v) Grace's insect medium with gentamycin and fungizone. Cells were grown either as monolayers in T flasks or in suspension in spinner bottles supplemented with pluronic acid to prevent cell tearing during agitation. Cells were infected with a recombinant baculovirus encoding the myc-tagged human β_2AR at a density of 1 to 2×10^6 cells/ml as previously described (Mouillac *et al*., 1992). Cells were harvested 48 hours post-infection and used either directly or to prepare membrane as described below. Mammalian HEK 293 cells stably expressing the human β_2AR were grown in Dulbecco's modified Eagle's medium supplemented with L-glutamate, 10% fetal bovine serum, gentamycin, fungizone and 450 µg/ml G418.

Membrane Preparation and Solubilization

Sf9 or HEK 293 cells were washed twice with ice-cold phosphate-buffered saline. Cells 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 and a protease inhibitor mixture consisting of 5 mg/ml leupeptin, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor. The lysate was centrifuged at 500 *g* for 5 min at 4 °C, the pellet homogenized and recentrifuged as before and the supernatants pooled. The supernatant was then centrifuged at 45 000 *g* for 20 min and the pellet washed twice in the same buffer. In some cases receptors were then solubilized in 0.3% dodecyl maltoside by agitation at 4 °C for 2 hours. When applicable, iodoacetamide or *N*-ethyl maleimide were added into the homogenization buffer as well as in all the following steps.

Affinity Purification and Ultrafiltration of β_2ARs

Solubilized receptors were affinity-purified by alprenolol-Sepharose chromatography as described (Mouillac *et al*., 1992). The affinity-purified preparations were desalted on Sephadex G-50 columns and concentrated using Centriprep and Centricon cartridges (Amicon, Millipore, Nepean, ON, Canada). The amount of β_2AR in each sample was determined by binding assays using [^{125}I]-Cyanopindolol as radioligand, as previously described (Mouillac *et al*., 1992). Ultrafiltration was then carried out by applying the purified receptor to Microcon100 filtration units and centrifuged at 500 *g* for 15 min at 4 °C. The retained fraction and ultrafiltrate were then concentrated for SDS-PAGE and Western blot analyses.

Cross-Linking of β_2ARs

Cross-linking reactions using the membrane impermeable BS3 were carried out both on whole cells or membrane preparations. For intact Sf9 cells, β_2AR expressing cells were harvested and resuspended in phosphate buffered saline (pH 7.4) at 4 °C in the presence of 0 – 1 mM BS3. After 15 minutes, the reaction was stopped by the addition of 50 mM tris (pH 7.4) and cells incubated on ice for an additional 10 minutes. Cells were then lysed and membranes prepared for SDS-PAGE analysis. For cross-linking carried out in Sf9 and HEK 293 membrane preparations, the reactions were carried out in a 50 mM potassium phosphate buffer (pH 7.4) as described for whole cells.

SDS-PAGE and Western Blotting

Membrane preparations from Sf9 or HEK 293 cells or in some cases affinity-purified β_2AR were separated for SDS-PAGE on 8% slab gels. For Western blot analyses, proteins were transferred to nitrocellulose and blotted with either the 9E10 mouse anti-c-myc monoclonal antibody (1:1000) or H-20 anti-β_2AR rabbit polyclonal antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as indicated. The immunoreactivity was revealed using goat horseradish peroxidase-conjugated anti-mouse or anti-rabbit antisera (Sigma) respectively. Western blots were developed using a chemiluminescent substrate for hoseradish peroxidase (Western lightning from PerkinElmer Life Sciences, Boston, MA, USA).

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