Oligomerization of the α1a- and α1b-Adrenergic Receptor Subtypes

POTENTIAL IMPLICATIONS IN RECEPTOR INTERNALIZATION*

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We combined biophysical, biochemical, and pharmacological approaches to investigate the ability of the α1a- and α1b-adrenergic receptor (AR) subtypes to form homo- and hetero-oligomers. Receptors tagged with different epitopes (hemagglutinin and Myc) or fluorescent proteins (cyan and green fluorescent proteins) were transiently expressed in HEK-293 cells either individually or in different combinations. Fluorescence resonance energy transfer measurements provided evidence that both the α1a- and α1b-AR can form homo-oligomers with similar transfer efficiency of ~0.10. Hetero-oligomers could also be observed between the α1a- and the α1b-AR subtypes but not between the α1a-AR and the β2-AR, the NK1 tachykinin, or the CCR5 chemokine receptors. Oligomerization of the α1b-AR did not require the integrity of its C-tail, of two glycophirin motifs, or of the N-linked glycosylation sites at its N terminus. In contrast, helix I and, to a lesser extent, helix VII were found to play a role in the α1b-AR homo-oligomerization. Receptor oligomerization was not influenced by the agonist epinephrine or by the inverse agonist prazosin. A constitutively active (A293E) as well as a signaling-deficient (R143E) mutant displayed oligomerization features similar to those of the wild type α1b-AR. Confocal imaging revealed that oligomerization of the α1-AR subtypes correlated with their ability to co-internalize upon exposure to the agonist. The α1a-selective agonist oxymetazoline induced the co-internalization of the α1a-AR and α1b-AR, whereas the α1b-AR could co-internalize with the NK1 tachykinin or CCR5 chemokine receptors. Oligomerization might therefore represent an additional mechanism regulating the physiological responses mediated by the α1a- and α1b-AR subtypes.

G protein-coupled receptors (GPCR),1 known also as heptahedral receptors, form the largest family of transmembrane signal-transducing proteins transducing signals arising from ions, hormones, neurotransmitters, odorants, chemoattractants, and photons. GPCRs were for a long time presumed to function as monomers according to the prevailing model: one ligand molecule—one receptor—one G protein. Recently, increasing complexity of GPCR function and regulation has progressively emerged. For example, one GPCR can adopt multiple conformational states able to interact differentially with signaling and regulatory proteins (1, 2). In addition, receptor cross-talks both at the G protein and at downstream signaling levels have often been described (3, 4). Recently, it was shown that cross-talk among GPCRs can also occur at the receptor level by means of receptor oligomerization (reviewed in Refs. 5–7). One of the early indications suggesting the capacity of GPCRs to oligomerize came from pharmacological studies showing positive or negative cooperativity of ligand binding curves. More recent support was provided by some “complementation” studies in which co-expression of two GPCR mutants could rescue their functional defects (8). These results were interpreted as evidence of inter-molecular interactions between two receptor mutants according to a “domain swapping” model (9). Further indication of GPCR oligomerization came from a large number of studies using co-immunoprecipitation of epitope-tagged receptors co-expressed in the same cells or fluorescence spectroscopy to monitor such oligomers in live cells (reviewed in Refs. 5–7).

The molecular basis of GPCR oligomerization is not yet fully understood. Co-immunoprecipitation relies on receptor solubilization, which can result in artifactual aggregation of the proteins. To overcome these limits fluorescence spectroscopy techniques, like fluorescence or bioluminescence resonance energy transfer, have been increasingly used. It is widely accepted that energy transfer measurements are well suited to monitor protein-protein interactions or proximity in live cells, as FRET only occurs when the distance between the two fluorophores falls below ~100 Å (10). So far, in virtually all studies an increased energy transfer signal between GPCRs has been interpreted as the evidence for receptor oligomerization. However, whether receptor oligomerization involves intramolecular interactions among receptors versus their increased proximity within the cell membrane without direct contact cannot be unequivocally demonstrated either by the energy transfer measurements or by the results of co-immunoprecipitation experiments. Therefore, in this study the term receptor “oligomerization” will be used bearing in mind that the precise molecular events underlying this phenomenon are not fully understood.
The Jackson Laboratories. Anti-rabbit and anti-mouse horseradish peroxidase from Santa Cruz Biotechnology. Fluorescein- and rhodamine-coupled protease inhibitor mixture, and restriction enzymes were from Roche from Invitrogen. [125I]HEAT and [3H]inositol were from PerkinElmer from Qiagen. DMEM, fetal calf serum, fungizone, and gentamycin were from GIBCO-BRL.

For other GPCRs, a role of oligomerization in receptor signal transduction has often represented an important reference for in vitro studies. However, much work is still required to elucidate how oligomerization is involved in these distinct processes. In addition, whereas the majority of studies was performed in recombinant systems, only in a few cases was evidence provided that GPCR oligomerization occurs in physiological systems. For example, in a recent study hetero-oligomers formed by the adenosine A1 and glutamate mGlur1 receptors were isolated from cerebellar neuronal cultures (12). In addition, hetero-oligomers formed by the angiotensin II AT1 and bradykinin B2 receptors were isolated in platelets of pre-eclamptic pregnant women (13).

Within the adrenergic receptor (AR) family, oligomerization has been extensively studied for the β2-AR (14–16), and these studies have often represented an important reference for investigating the oligomerization of other GPCRs. The β2-AR can form both homo- and hetero-oligomers with the δ- and κ-opioid receptors (17) as well as with the β1-AR (18). Homo-oligomerization of the β2-AR is constitutive, but it can also be enhanced by exposure available to the agonist (16). In contrast to the amount of information available for the β2-AR and several other GPCRs, nothing is known so far on the putative oligomeric state of the α1-AR subtypes.

In this study we extensively investigated the ability of the α1a- and α1b-AR subtypes to oligomerize using biophysical, biochemical, as well as pharmacological approaches. FRET measurements provided solid evidence that both recombinant α1a- and α1b-AR subtypes can selectively form homo- and hetero-oligomers. Our results suggest that the homo-oligomerization of the α1b-AR involves the participation of helix I and, to a lesser extent, of helix VII. The results of confocal imaging strongly suggest that receptor oligomerization plays a role in receptor endocytosis, which might have implications for the physiological responses mediated by the α1a- or α1b-AR subtypes.

**EXPERIMENTAL PROCEDURES**

**Materials**—The plasmid encoding the chemokine CCR5 receptor and RANTES were kind gifts of Dr. Jean-Luc Galzi, UPR 9050 CNRS, 40240 Dijon, France. The NK1-pEGFP-N1 plasmid was a kind gift of Dr. Bruno Meyer, EPFL, Lausanne, Switzerland. The pECDFP-N1, pECPF-N1, and pEYFP-N1 vectors were from Clontech. Immobilon membranes were from Millipore. Prolong mounting medium was from Molecular Probes. Monoclonal anti-c-Myc antibodies, protein A-Sepharose, and protein G-Sepharose were from Millipore. Prolong mounting medium was from Molecular Probes.

**Construction of Receptor-GFP and CFP Fusion Proteins and Site-directed Mutagenesis**—The full-length cDNA encoding the hamster α1b-AR (19) was PCR-amplified and inserted into the pEGFP-N1 and pECFP-N1 vectors using EcoRI/AgeI to give the α1b-EGFP-N1 and α1b-ECFP-N1 vectors, respectively. The cDNA encoding the α1a-AR fused to GFP (α1a-GFP) was also subcloned in the pRcRc5 vector to give the α1a-GFP-pRcK5 vector. The Thr369-GFP, Thr369-CFP, and Thr369-YFP constructs were obtained by PCR-amplifying a DNA fragment of the α1b-AR encompassing amino acids 1–369 and subcloning it at the EcoRI/AgeI sites into the α1b-EGFP-pRcK5 and pEYFP-N1 vectors, respectively. The R143E-GFP and A293E-GFP constructs were obtained by replacing the EcoRI-BstHII fragment of the previously described R143E and A293E mutants (20, 21) into the α1b-EGFP-pRcK5 vector. The G35L and G301L mutants were constructed by PCR mutagenesis and subcloned into the α1b-EGFP-pRcK5 vector to give the G35L-GFP and G301L-GFP constructs, respectively. The construction of the N-glycosylation-deficient mutant N4 fused to GFP (N4-GFP) was described previously (22).

**Construction of Epitope-tagged Receptors**—The C-terminal fragment of the α1a-AR and the α1b-AR were amplified using primers encoding the HA (YPYDVPDYA) or Myc (EQKLISEEDL) epitopes at the N-terminus of the C-tail. Using this strategy, the following fragments were obtained for the α1b-AR: the HA epitope was inserted at the C-terminus of the α1b-AR and amplified using the forward primer encoding the Myc epitope at the 3'-end. The full-length cDNA encoding the human α1a-AR (23) was PCR-amplified and inserted into the pEGFP-N1 and pECFP-N1 vectors using EcoRI/AgeI (blunted) to give the α1a-EGFP and α1a-ECFP constructs, respectively. The full-length cDNA encoding the human β2-AR was PCR-amplified and subcloned into the pEGFP-N1 to give the β2-GFP construct. The full-length cDNA encoding the chemokine CCR5 receptor was PCR-amplified and subcloned into the pECFP-N1 using HindIII/AgeI to give the CCR5-CFP construct.

**Cell Culture and Transfection**—HEK-293 cells were grown in DMEM supplemented with 10% fetal calf serum and gentamicin (100 μg/ml) (37°C and 5% CO2) and transfected using the calcium-phosphate method or the transfection reagent Effectene following the manufacturer's protocol. For inositol phosphate determination, cells (0.15 × 10⁶) were seeded in 12-well plates and transfected with 0.2–0.5 μg/well using Effectene. For ligand binding, FRET, and immunoprecipitation experiments, cells were grown in 100-mm dishes and transfected with a maximum of 20 μg of DNA/dish using the calcium-phosphate method. Cells were grown on glass coverslips or polyclonal anti-HA antibody and scraped off the culture plates and dishes and transfected with 1–2 μg of DNA/well using the calcium-phosphate method. For cells transfected with different combinations of plasmids, the total amount of transfected DNA was kept constant in the samples using pRK5.

**Cell Preparation and Ligated Binding**—48 h after transfection, the cells were washed in PBS, scraped off the culture plates, and collected in ice-cold membrane buffer (5 mM Tris, 0.5 mM EDTA, pH 7.4). After centrifugation at 40,000 × g, the pellet was resuspended in ice-cold membrane buffer and Polytron-homogenized. Protein concentration was determined using the Bradford protein assay. For ligand binding of [125I]HEAT and [3H]inositol was used at a concentration of 250 pM for measuring receptor expression at a single concentration and of 80 pM for competition binding analysis. For saturation binding experiments, the radioligand concentration ranged between 10⁻⁸ and 10⁻⁴ M.
and 400 pm. Pratinol at 10⁻⁶ m was used to determine nonspecific binding. Saturation analysis and competition curves were analyzed using Prism 3.02 (GraphPad Software Inc., San Diego).

**Inositol Phosphate Accumulation**—24–36 h after transfection, cells were labeled for 12 h with myo-[³H]inositol at 4 µCi/ml in myo-inositol-free DMEM supplemented with 1% fetal bovine serum. Cells were preincubated for 10 min in PBS containing 20 mM LiCl and then stimulated for 5 min in medium with different concentrations of epinephrine. Total inositol phosphates were extracted and separated as described previously (19).

**SDS-PAGE and Western Blotting**—Samples were denatured in SDS-PAGE loading buffer (65 mM Tris, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, pH 6.8) for 1 h at room temperature, separated on 10% acrylamide gels, and electroblotted onto Immobilon membranes. Blots were incubated in TBS buffer (50 mM Tris, 150 mM NaCl, pH 8) containing 20% (v/v) methanol, and the primary antibody (mouse monoclonal anti-HA or anti-Myc) diluted 1:200. After the primary antibody was washed, the secondary anti-mouse antibody linked to horseradish peroxidase was added, and the blots were developed using the enhanced chemiluminescence (ECL) detection system.

**Immunoprecipitation of Receptors**—36 h after transfection, cells were washed twice with PBS and scraped off the culture plates in ice-cold buffer containing 5 mM Tris and 5 mM EDTA, pH 7.4. After centrifugation at 40,000 × g, the pellet was resuspended in 1 ml of ice-cold lysis buffer (20 mM Tris, 0.5 mM EDTA, 1% digitonin, 100 mM NaCl, pH 7.4) containing a complete protease inhibitor mixture. Solubilization was carried out for 3 h at 4°C on a spinning wheel. Unsolubilized material was pelleted by centrifugation for 15 min at 20,000 × g on a bench-top centrifuge. The supernatant was incubated with 5 μl of anti-HA polyclonal antibody overnight on the spinning wheel at 4°C. After addition of 20% (v/v) protein A-Sepharose, the incubation was continued for 2 h at 4°C, followed by a brief centrifugation on a bench-top centrifuge. The pellet was washed three times in the lysis buffer, once in PBS, and then dissolved in SDS-PAGE loading buffer for 1 h at 37°C.

**Fluorescence Spectroscopy**—48 h after transfection, the cells were washed in PBS and detached from the plates using PBS containing 0.5 mM EDTA. The cells from one 10-cm culture dish were resuspended in PBS containing 1% BSA and for another hour in PBS containing 0.1% BSA. The cells were labelled with different epitopes (HA and Myc) or fluorescent proteins (GFP and CFP), the receptors were expressed in HEK-293 cells and used in spectrofluorimetric measurements. Cells were routinely washed three times in PBS, fixed with formaldehyde, and permeabilized for 5 min in 0.2% Triton. For immunostaining of cells expressing the HA-tagged receptors, the cells were incubated for 1 h in PBS containing 1% BSA and for another hour in PBS containing 0.1% BSA, and the polyclonal anti-HA antibody was diluted at 1:100. After a second incubation with the anti-rabbit rhodamine-coupled antibody diluted at 1:100 in PBS containing 0.1% BSA, the coverslips were washed three times in PBS and mounted in Prolong mounting medium. For immunostaining of cells co-expressing the α₁-HA and myc-CR5, the polyclonal anti-HA and the monoclonal anti-Myc antibodies were used, followed by the anti-rabbit FITC-coupled and the anti-mouse rhodamine-coupled secondary antibodies. Cells immunostained with fluorescent antibodies were analyzed by confocal imaging.

**Confocal Imaging**—Cells expressing different fluorescent receptors were imaged using a Zeiss LSM510 confocal microscope equipped with a C-apochromat ×63/1.2-watt water immersion objective (Zeiss, Germany). The following laser lines were used for excitation: 458 nm for CFP, 488 nm for GFP and FITC, and 543 nm for rhodamine. The following Zeiss filter sets were used to detect the fluorescence of a particular fluorophore: LP475 for CFP, BP505-550 for GFP and FITC, and LP543 for rhodamine. The pinhole was kept at ~1 airy unit for all recordings.

**RESULTS**

**Functional Characterization of the Tagged Receptors**—To investigate the pharmacological properties of the α₁-AR subtypes tagged with different epitopes (HA and Myc) or fluorescent proteins (GFP and CFP), the receptors were expressed in HEK-293 cells and tested for their ability to bind the radioligand [¹²⁵I]HEAT and epinephrine. Saturation binding experiments indicated that the KD values of [¹²⁵I]HEAT were similar at the various tagged or non-tagged α₁-AR constructs (results not shown). As shown in Table I, the expression levels of the
various receptors ranged between 300 and 900 fmol/well. The IC_{50} values of epinephrine were similar at the tagged or non-tagged receptors. The baseline differences for the non-tagged receptor mutants (21, 20, 24). Coupling of the different α1-AR constructs to the G_{i}/phospholipase C pathway was assessed as their ability to mediate epinephrine-stimulated IP accumulation (Table I). The IP accumulation mediated by the α1a-AR or α1a-AR-differentially tagged was overall similar to that of the wild type receptor. The A293E-GFP was constitutively active, whereas the R143E-GFP was signaling-similar to that of the wild type receptor. The α1b-AR was co-expressed with the α1b-CFP or α1b-GFP expressed individually (results not shown). Based on the results of these calibration curves, we assessed that in the FRET experiments the amount of the α1b-CFP and α1b-GFP co-expressed in each sample was about 1–2 pmol/mg of protein for each construct.

Table I

Pharmacological properties of different α1-AR constructs

| Receptor | Expression | Basal IP | Epi-stimulated IP | IC_{50} | Epi |
|----------|------------|----------|-------------------|--------|-----|
| wt α1b   | 961 ± 100  | 104 ± 13 | 896 ± 28          | 5.31 ± 0.36 |
| α1b-GFP  | 898 ± 354  | 105 ± 7  | 784 ± 82          | 5.73 ± 1.3  |
| α1b-CFP  | 1055 ± 102 | 102 ± 12 | 809 ± 40          | 22.37 ± 4.7 |
| T369-GFP | 892 ± 341  | 122 ± 2  | 1068 ± 107        | 4.14 ± 0.7  |
| A293E-GFP| 382 ± 188  | 1478 ± 144* | 1923 ± 55*       | 0.12 ± 0.02*|
| R143E-GFP| 511 ± 107  | 133 ± 18 | 125 ± 16*         | 0.04 ± 0.01*|
| G53L-GFP | 875 ± 55   | 112 ± 5  | 492 ± 96          | 1.76 ± 0.1  |
| G301L-GFP| 665 ± 103  | 118 ± 6  | 321 ± 41          | 2.23 ± 0.3  |
| α1b-HA   | 653 ± 127  | 181 ± 21 | 1068 ± 87         | 5.92 ± 1.7  |
| α1b-myc  | 556 ± 21   | 150 ± 29 | 1181 ± 170        | 5.38 ± 2.7  |
| wt α1a   | 658 ± 116  | 147 ± 29 | 1953 ± 90         | 6.87 ± 0.4  |
| α1a-GFP  | 253 ± 111  | 161 ± 20 | 2260 ± 287        | 10.72 ± 3.3 |
| α1a-HA   | 508 ± 24   | 126 ± 8  | 1223 ± 318        | 8.51 ± 0.3  |
| α1a-myc  | 656 ± 180  | 133 ± 3  | 1947 ± 186        | 8.27 ± 0.9  |

* Paired Student’s t test, p < 0.05 compared with wt α1b.

To assess whether the fluorescent receptor constructs were properly localized at the plasma membrane, we used confocal imaging of fixed HEK-293 cells transiently expressing the different receptors fused to GFP or CFP. As shown in Fig. 1, all the fluorescent receptor constructs listed in Table I were expressed at the cell surface as indicated by the sharp fluorescence at the plasma membrane. The fluorescent receptor constructs also displayed some intracellular fluorescence which was however more pronounced for the A293E-GFP (Fig. 1g). This is consistent with previous findings suggesting that the constitutively active A293E mutant is also constitutively internalized (21).

Homo-oligomerization of the α1a-AR—To explore whether the α1a-AR can oligomerize, the α1a-CFP and α1a-GFP were co-expressed in HEK-293 cells, and FRET was measured on cell suspensions (as described under “Experimental Procedures”). This fluorophore couple fulfills the conditions necessary for FRET to occur because the fluorescence spectrum of CFP (acting as a donor) largely overlaps with the excitation spectrum of GFP (acting as acceptor).

In preliminary experiments, we established that transfection of cells with equal amounts of DNA encoding the α1b-CFP or α1b-GFP resulted in a GFP:CFP expression ratio ranging from 0.8 to 1.3 (results not shown). Because the radioligand [125I]HEAT could not discriminate between the two fluorescent constructs, we determined the relationship of fluorescence intensity versus receptor expression measured by radioligand binding for the α1b-CFP and α1b-GFP expressed individually (results not shown). Based on the results of these calibration curves, we assessed that in the FRET experiments the amount of the α1b-CFP and α1b-GFP co-expressed in each sample was about 1–2 pmol/mg of protein for each construct.

The energy transfer efficiency between α1b-CFP and α1b-GFP

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[See full document for detailed scientific content and tables.]
calculated over 28 independent experiments was 0.09 ± 0.02, the average value of the GFP:CFP ratio being 1.25 ± 0.27. As expected, increasing the acceptor:donor ratio resulted in an increased energy transfer efficiency (results not shown). However, all the FRET measurements considered in this study were from experiments in which the GFP:CFP ratio was close to 1.

Hetero-oligomerization of the α1b-AR and Its Selectivity—To test whether the α1b-AR could form hetero-oligomers with other GPCRs, we selected a number of receptors belonging to the rhodopsin-like sub-family: the α1a-AR, the β2-AR, the NK1 tachykinin receptor, and the CCR5 chemokine receptor. The CFP- or GFP-tagged receptor constructs were all inserted into the plasma membrane of HEK-293 cells as shown in Fig. 1.

FRET experiments in HEK-293 cells co-expressing the α1a-CFP and α1b-GFP constructs indicated that, like the α1b-AR, the α1a-AR subtype can form homo-oligomers (Fig. 3). In addition to the FRET measurements, we also investigated the selectivity of the α1b-AR. To this end, we co-expressed the wild type α1b-AR or myc-CCR5 with the α1b-CFP and α1b-GFP constructs. The results are shown in Fig. 2C, where the FRET efficiency is plotted against the wavelength. The error bars represent the standard error of the mean (S.E.) from three independent experiments. **, paired Student’s t test, p < 0.001 compared with α1b-CFP/α1b-GFP.
from the homo-oligomerization of the indicated by the value of the energy transfer efficiency of the oligomers with the results are the mean ± S.E. from 5 to 10 independent experiments. **, unpaired Student’s t test, p < 0.01 compared with α1b-CFP/α1a-GFP; $, unpaired Student’s t test, p < 0.01 compared with α1a-CFP/α1b-GFP.

As mentioned above, the HA- or Myc-tagged its C-tail; and the CCR5 receptor with Myc at its N terminus. was tagged with HA at its C-tail; the NK1 receptor with Myc at co-expression of the wild type CCR5 receptor with ble FRET signal was detected. In agreement with these results, the immunoprecipitated samples. As expected, the anti-Myc anti-HA (Fig. 4 A–B) was unable to decrease the FRET signal resulting from cells co-expressing either the receptor oligomerization using the co-immunoprecipitation approach employed in a large number of studies to document oligomerization of GPCRs. The receptors were tagged as follows: the α1b-AR with either HA or Myc at its C-tail; the α1a-AR was tagged with HA at its C-tail; the NK1 receptor with Myc at its C-tail; and the CCR5 receptor with Myc at its N terminus. As mentioned above, the HA- or Myc-tagged α1b-AR subtypes displayed functional properties similar to those of the non-tagged receptors (Table I).

In the experiment shown in Fig. 4, differently tagged receptors were expressed either alone or in combination in HEK-293 cells, and a polyclonal anti-HA antibody was used to immunoprecipitate the receptors. Monoclonal anti-Myc (Fig. 4A) or anti-HA (Fig. 4B) antibodies was used for the immunoblot of the immunoprecipitated samples. As expected, the anti-Myc antibodies did not reveal any bands on Western blots of the immunoprecipitates from cells expressing the individual receptors α1b-AR, α1b-HA, α1b-myc, α1a-HA, or myc-CCR5 (Fig. 4A, lanes 1–5). In contrast, a predominant band of ~67 kDa corresponding to the α1b-myc could be detected in the immunoprecipitate from cells co-expressing either the α1b-HA/α1b-myc or the α1a-HA/α1b-myc couples (Fig. 4A, lanes 7 and 8). The migration pattern of the α1b-myc (one main band of ~67 kDa and a much fainter species of ~75 kDa) was different from that described previously for the wild type α1b-AR (one fully glycosylated band at ~95 kDa and one core-glycosylated band at ~67 kDa (22)). This suggests that tagging the C-tail of the receptor with a Myc epitope modifies the maturation process of the receptor. Similar bands were observed also in membranes from cells expressing the α1b-myc (results not shown).

To detect the presence of the various HA-tagged receptors in the immunoprecipitates, Western blots were also revealed using monoclonal anti-HA antibodies (Fig. 4B). Specific receptor bands were detected in all the immunoprecipitates from cells expressing different HA-tagged receptors. The immunoprecipitated α1b-HA (Fig. 4B, lanes 2, 6, 7, and 9) displayed two main bands at ~67 and ~95 kDa corresponding to those identified previously as the core-glycosylated and fully glycosylated forms of the non-tagged receptor, respectively (22). In contrast, the immunoprecipitated α1b-HA displayed two predominant bands of ~70 and ~150 kDa and a much fainter species of ~100 kDa (Fig. 4B, lanes 4 and 8). Because the glycosylation pattern of the wild type α1b-AR has not been fully characterized, the migration pattern of the HA-tagged receptor cannot be interpreted.

Altogether, these findings indicate that the α1b-myc can be co-immunoprecipitated with the α1b-HA as well as with the α1a-HA co-expressed in the same cells. No α1b-HA–α1b-myc complex was observed when a mixture of cells individually
expressing the tagged receptors was subjected to the same immunoprecipitation procedure (Fig. 4A, lane 6). These results seem to support the conclusions from the FRET experiments indicating that the $\alpha_{1b}$-AR could form homo-oligomers as well as hetero-oligomers with the $\alpha_{1c}$-AR. However, in cells co-expressing the two receptors immunoprecipitation of the $\alpha_{1b}$-HA resulted in the co-immunoprecipitation of a small portion of the Myc-tagged CCR5 chemokine receptor which was detected as an ~37-kDa band (Fig. 4A, lane 9). Similar results were also obtained with the NK1-myc which could be co-immunoprecipitated with the $\alpha_{1b}$-HA (data not shown). These findings are somehow in conflict with those from the FRET experiments that demonstrated a negligible interaction of the $\alpha_{1b}$-AR with the CCR5 and NK1 receptors.

Structural Determinants of the $\alpha_{1b}$-AR Potentially Involved in Homo-oligomerization—So far, the mechanism by which GPCRs form oligomers has been poorly elucidated at a molecular level. Several hypotheses have been suggested including transmembrane domain swap (9), disulfide links between N-terminal domains (26), or dimerization motifs within the transmembrane helices (14). Among the structural determinants of the $\alpha_{1b}$-AR potentially involved in receptor oligomerization, we initially investigated the role of its bulky C terminus, of the glycophorin dimerization motifs GXXXG in helix II and VI, as well as that of the N-linked glycosylation occurring at the N terminus.

To investigate whether the C terminus played a role in receptor oligomerization, we used a truncated $\alpha_{1b}$-AR mutant, Thr$^{369}$, lacking the last 146 amino acids. The fluorescent constructs Thr$^{369}$-CFP and Thr$^{369}$-GFP were transiently co-expressed in HEK-293 cells. Comparison of the fluorescence spectra from cells expressing the Thr$^{369}$-CFP or Thr$^{369}$-GFP individually with those from cells co-expressing the two constructs indicated that the truncated mutant was able to form oligomers. This indicates that the integrity of the C-tail is not required for $\alpha_{1b}$-AR homo-oligomerization. Moreover, the FRET efficiency measured between Thr$^{369}$-CFP and Thr$^{369}$-GFP was 44% higher than that between the $\alpha_{1b}$-CFP and $\alpha_{1b}$-GFP (Fig. 5). This could be explained by the fact that, in the absence of the flexible C terminus, the average donor-acceptor distance is decreased.

For glycophorin A, a protein with a single transmembrane segment forming SDS-resistant dimers, a specific motif GXXXG situated in the helical transmembrane domain is involved in its dimerization. The glycophorin motif GXXXG (27) can also be found in some GPCRs. In particular, it was reported previously (14) that in the $\beta_2$-AR replacing the glycines with alanines in the glycophorin motif in helix 6 destabilized receptor oligomerization. In the $\alpha_{1b}$-AR, two GXXXG motifs can be found in helices II and VI. To explore their role in receptor oligomerization, we constructed two mutants, G53L and G301L, in which Gly$^{53}$ and Gly$^{301}$ belonging to glycophorin motifs in helices II and VI, respectively, were mutated into leucines. For each mutant, the CFP- and GFP-tagged constructs were co-expressed in HEK-293 cells; the analysis of the emission spectra indicated that FRET occurred with an efficiency comparable with that between $\alpha_{1b}$-CFP and $\alpha_{1b}$-GFP (Fig. 5). Altogether these findings suggest that the glycophorin motifs identified in the $\alpha_{1b}$-AR are not involved in receptor homo-oligomerization.

To test if glycosylation of the receptor might contribute to $\alpha_{1b}$-AR oligomerization, we measured FRET efficiency between CFP- and GFP-tagged constructs of the receptor mutant, N4, carrying the mutation of the four glycosylation sites present in the N terminus of the receptor. As described previously, the N4 mutant lacked N-linked glycosylation but retained its binding and signaling properties (22). The FRET signal measured between the N4-CFP and N4-GFP was comparable with that between $\alpha_{1b}$-CFP and $\alpha_{1b}$-GFP (Fig. 5). These results seem to exclude a role of the N-linked glycosylation occurring at the N terminus of the $\alpha_{1b}$-AR in receptor homo-oligomerization.

Because of these negative results, we investigated whether distinct transmembrane helices were specifically involved in receptor oligomerization. The hypothesis that transmembrane helices are involved in GPCR oligomerization has become increasingly accepted even if it has been demonstrated only for few receptors.

As a first approach, we generated receptor fragments of the $\alpha_{1b}$-AR lacking different transmembrane helices and tested their ability to oligomerize with the receptor. The receptor fragments were constructed by progressively deleting groups of consecutive helices from the N or the C terminus of the receptor. In all receptor fragments the N terminus was preserved on the extracellular side and the fluorescent tag intracellularly. To improve the sensitivity of the FRET assay, in each fragment the YFP was fused in proximity of the C terminus of its last helix. In addition, to keep the fluorophores in greater proximity, FRET experiments were performed using the truncated receptors Thr$^{369}$-CFP and Thr$^{369}$-YFP lacking the C-tail. The energy transfer efficiency measured between the Thr$^{369}$-CFP and Thr$^{369}$-YFP receptors was 0.21 ± 0.02 (n = 5), which is significantly higher than that measured for Thr$^{369}$ tagged with the CFP/GFP fluorophore couple (results not shown).

Confocal microscopy indicated that the receptor fragments fused to YFP were expressed at a similar level, and their fluorescence was uniformly distributed in the cytosol and around the nucleus thus suggesting its localization in the en-
doplasmic reticulum (ER) (results not shown).

As shown in Fig. 6A, co-expression of the Thr369-CFP with the receptor fragments a1b(I–III) or a1b(I–V) including helices I–III and I–V, respectively, resulted in a strong FRET signal comparable with that obtained with the whole truncated receptor. In contrast, co-expression with the receptor fragments a1b(III–VII) or a1b(V–VII), lacking helices I and II, did not result in any FRET signal. These results strongly suggest that the structural determinants playing a major role in the homo-oligomerization of the a1b-AR are localized within helices I and/or II. In addition, given the ER localization of the receptor fragments, our results also indicate that FRET can efficiently occur in the ER. Altogether these findings suggest that replacement of those recently obtained on the Ste2 pheromone receptor homo-oligomers (28) or on the oxytocine/vasopressin hetero-oligomers (29).

To investigate further the role of the transmembrane helices in a1b-AR oligomerization, we took advantage of the low energy transfer efficiency measured between fluorescent a1b- and beta2-AR (Fig. 3), and we selectively replaced each single helix of the a1b-GFP receptor with the corresponding helix of the beta2-AR. We decided to construct a1b-AR chimeras using portions of the beta2-AR instead of those from less closely related receptors to better preserve receptor function. Our hypothesis was that, if one helix was prominently involved in homo-oligomerization, its replacement would result in a decreased FRET signal. Co-expression of the beta2-AR with the a1b-CFP and a1b-GFP receptors did not decrease the energy transfer thus indicating that the beta2-AR did not compete for the homo-oligomerization of the a1b-AR (results not shown).

Confocal microscopy indicated that all the chimeric receptors were expressed at similar levels (Fig. 7). The a1b/beta2-hIII and a1b/beta2-hIV were mainly expressed at the plasma membrane, whereas the a1b/beta2-HI, a1b/beta2-HII, a1b/beta2-HVI, a1b/beta2-hV, and a1b/beta2-hVII were localized in the ER with a faint plasma membrane labeling.

The chimeric receptors a1b/beta2-hIII and a1b/beta2-hIV displayed a normal [125I]HEAT binding, but markedly decreased epinephrine-induced IP response (Table II). This suggests that replacement of helices III or IV, despite not perturbing the expression of the receptor at the plasma membrane, markedly decreases its ability to be activated. The a1b/beta2-hV chimera displayed normal expression, detected by [125I]HEAT binding, as well as normal agonist-induced IP response despite a large decrease of affinity for epinephrine. [125I]HEAT binding as well as receptor signaling were not measurable at the chimeric receptors a1b/beta2-hII and a1b/beta2-hVII. Only a small amount of [125I]HEAT and agonist-induced response could be detected at chimeric receptors a1b/beta2-hI and a1b/beta2-hVI. Altogether these findings suggest that replacement of helices I, II, VI, or VII had the most profound effect on receptor expression at the plasma membrane and/or on its ligand binding properties.

FRET was measured in cells co-expressing the wild type a1b-CFP and each of the six a1b/beta2 chimeric receptors fused to

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**Fig. 6.** FRET in cells expressing chimeric a1b/beta2 or receptor fragments. A, FRET efficiency was measured in HEK-293 cells co-expressing the truncated a1b-AR mutant, Thr369-CFP, and different a1b-AR fragments fused to YFP. Results are mean ± S.E. of three independent experiments and are expressed as % of the FRET efficiency obtained with Thr369-CFP/T39-YFP couple. ND, no FRET signal could be detected. B, energy transfer was measured in HEK-293 cells co-expressing the a1b-CFP and different a1b/beta2-AR chimeras fused to GFP. Results are mean ± S.E. of four to six independent experiments and are expressed as % of the FRET efficiency obtained with the a1b-CFP/a1b-GFP couple. *, unpaired Student’s t test, p < 0.05 compared with a1b-CFP/a1b-GFP. **, unpaired Student’s t test, p < 0.01 compared with a1b-CFP/a1b-GFP.

**Fig. 7.** Co-expression of the a1b-AR and chimeric a1b/beta2 receptors. Confocal images were taken on fixed HEK-293 cells expressing a1b-AR and different a1b/beta2 chimeras fused to GFP. Anti-HA polyclonal antibodies, followed by anti-rabbit antibodies labeled with rhodamine, were used to detect the a1b-AR receptor. Images were taken after excitation at 488 nm for GFP or 543 nm for rhodamine. Merge represents the superimposition of the GFP and rhodamine images.
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GFP. As shown in Fig. 6B, a FRET signal could be measured under each condition. In particular, the energy transfer efficiency in cells expressing the fluorescent α1b/β2-hII, α1b/β2-hIII, α1b/β2-hIV, and α1b/β2-hVII was similar to that of cells expressing the wild type α1b-AR/GFP couple. In contrast, the FRET signals measured in cells expressing the fluorescent α1b/β2-hII and α1b/β2-hVII were 40 and 25% lower, respectively. This indicates that the integrity of helix I and, to a lesser extent, that of helix VII are required for α1b-AR homo-oligomerization. The results obtained on the receptor fragments indicated that only those including helix I (α1b-I-III) or α1b(I-VII) could associate with the receptor. Therefore, we suggest that in the whole α1b-AR helices I and VII, which are close to each other within the helical bundle, act in concert in favoring receptor homo-oligomerization, with helix I being the prominent interface. The symmetrical or asymmetrical nature of the inter-helical interactions involved in receptor oligomerization will require additional studies.

To support the results from the FRET experiments, we used confocal microscopy to investigate the localization of the chimeric receptors fused to GFP in cells co-expressing the wild type α1b-AR. As shown in Fig. 7, the chimeric receptors that did not display a significant decrease of the FRET signal (α1b/β2-hII, α1b/β2-hIII, α1b/β2-hIV, α1b/β2-hV, and α1b/β2-hVII) co-localize with the wild type receptor as indicated by the overlapping of both green and red fluorescence both at the plasma membrane and in the cytosol (Fig. 7, merge). This supports the hypothesis that these chimeric receptors can oligomerize with the α1b-AR. In contrast, in cells co-expressing the α1b/β2-hII and the wild type receptor, the green and red fluorescence did not overlap at all, thus suggesting that the receptors do not co-segregate within the same cellular localization. This supports the hypothesis that the α1b/β2-hII chimeras to oligomerize is significantly reduced as indicated by the FRET results (Fig. 6).

For those chimeric receptors that were mainly expressed at the plasma membrane (α1b/β2-hIII and α1b/β2-hIV), the co-localization with the wild type receptor could be clearly appreciated at the cell surface. For those found in the ER and, to a lesser extent, in the plasma membrane (α1b/β2-hII, α1b/β2-hV, and α1b/β2-hVI), the co-localization was evident at both locations suggesting that the wild type receptor was partially retained inside the cell when co-expressed with the chimeric receptors.

The Effect of α1b-AR Activation on Homo-oligomerization—To investigate whether oligomerization was dependent on the activation state of the α1b-AR, we followed two different approaches. In the first approach, we took advantage of two receptor mutants previously characterized in other studies: the R143E which is signaling-deficient (20) and the A293E which is constitutively active (21). These receptor mutants were fused to CFP and GFP, and FRET was measured in HEK-293 cells co-expressing the CFP- and GFP-tagged constructs of each receptor mutant. As shown in Fig. 8, both the R143E and A293E mutants proved able to form homo-oligomers, and the FRET efficiency was not significantly different from that measured for the wild type α1b-AR homo-oligomers.

As a second approach, we tested whether receptor ligands could have an effect on receptor homo-oligomerization. Cells expressing different fluorescent receptor constructs were incubated for 15 min at 37 °C with the agonist epinephrine or the inverse agonist prazosin. As shown in Fig. 8, no significant ligand-induced effect on the energy transfer efficiency could be detected under our experimental conditions. Altogether these findings suggest that the α1b-AR homo-oligomers are constitutively formed and independent from the activation state of the receptor as well as from ligand binding. Nevertheless, we cannot discredit the hypotheses that a putative ligand effect fell beyond the sensitivity range of our measurement or that the conditions under which the experiments were carried out were too distant from physiological parameters.

Pharmacological Investigation of the Receptor Oligomers—It has been reported previously (30–32) that co-expression of different GPCRs in cells can modify the binding properties of ligands resulting either in binding cooperativity or in a new pharmacological profile. Therefore, we compared the binding

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**Table II**

| Receptor Expression | Epi-stimulated IP | IC50 Epi |
|---------------------|------------------|---------|
| α1b-AR | 614 ± 106 | 1138 ± 211 | 5.6 ± 0.4 |
| α1b/β2-hI | 63 ± 17 | 201 ± 20 | |
| α1b/β2-hII | ND | 102 ± 3 | |
| α1b/β2-hIII | 545 ± 60 | 111 ± 30 | 140 |
| α1b/β2-hIV | 685 ± 240 | 104 ± 4 | 240 |
| α1b/β2-hV | 650 ± 90 | 1308 ± 9 | >1000 |
| α1b/β2-hVI | 80 ± 3 | 499 ± 106 | |
| α1b/β2-hVII | ND | 131 ± 15 | |

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**Fig. 8. Effect of ligands and activation on oligomerization of the α1b-AR.** Energy transfer was measured in HEK-293 cells co-expressing the CFP- and GFP-tagged forms of the α1b-AR or of the Thr463 (truncated), A293E (constitutively active), and R143E (signaling-deficient) mutants. Emission spectra were monitored before (no ligand) or after the incubation for 15 min with the agonist (10−6 M epinephrine) or the inverse agonist (10−6 M prazosin) at 37 °C. The results are the mean ± S.E. of five to nine independent experiments.
properties of a number of ligands in membranes derived from cells expressing the \( \alpha_{1a} \)- and \( \alpha_{1b} \)-AR individually or in combination. In addition, the ligand binding curves on membranes from cells co-expressing the two receptor subtypes were compared with those measured on a 1:1 mixture of membranes from cells individually expressing the receptors. Competition binding curves using the non-selective radioligand \( [125\text{I}] \)HEAT were measured for the non-selective agonists epinephrine and norepinephrine, for the \( \alpha_{1a} \)-selective agonists oxymetazoline and cirazoline, for the non-selective antagonist prazosin, as well as for the \( \alpha_{1b} \)-selective antagonists 5-methylurapidil and phenotolamine. The \( K_I \) values of the non-selective ligands were similar at the receptors expressed individually or in combination, whereas the \( \alpha_{1a} \)-selective ligands displayed biphasic competition curves in membranes from cells co-expressing the \( \alpha_{1a} \) - and \( \alpha_{1b} \)-AR (results not shown). Similar competition curves were obtained on a 1:1 mixture of membranes derived from cells individually expressing the \( \alpha_{1a} \) and \( \alpha_{1b} \)-AR (results not shown). In conclusion, the ligand binding curves did not detect any additional binding sites or change in the Hill coefficient that could be attributed to receptor oligomers. Two interpretations can be given for these results. One possibility is that \( \alpha_{1b} \)-AR hetero-oligomerization does not influence the pharmacological properties of the receptors within the oligomer. Another possibility is that the amount of receptor hetero-oligomers is too small to result in any detectable pharmacological change.

Internalization of the Receptor Oligomers—It has been reported previously that receptor oligomerization can play a role in the trafficking of GPCRs (17, 18, 33, 34). We therefore used confocal imaging to investigate the internalization properties of differently tagged \( \alpha_{1b} \)-AR subtypes, CCR5 and NK1 receptors expressed alone or in combination in HEK-293 cells. In particular, we made the hypothesis that only those receptors predicted to form oligomers based on the FRET experiments would mutually influence their internalization properties.

Confocal imaging of HEK-293 cells transfected with different DNAs using the calcium-phosphate method revealed that 30–50% of cells were routinely transfected and that receptors were co-localized in about 50% of the transfected cells. As shown in Fig. 9, exposure of cells expressing the \( \alpha_{1b} \)-GFP to epinephrine for 1 h resulted in receptor internalization, as demonstrated by the marked increase in the intracellular green fluorescence. To explore the role of hetero-oligomerization on receptor endocytosis, the \( \alpha_{1b} \)-GFP and the \( \alpha_{1a} \)-HA were co-expressed in HEK-293 cells and exposed to \( 10^{-5} \) M oxymetazoline that can selectively activate the \( \alpha_{1b} \)-AR but not the \( \alpha_{1a} \)-AR subtype. As shown in Fig. 9, oxymetazoline was unable to induce the internalization of the \( \alpha_{1b} \)-GFP, whereas it triggered the internalization of the \( \alpha_{1a} \)-HA. Interestingly, in cells co-expressing the two receptors, oxymetazoline-induced internalization of the \( \alpha_{1a} \)-HA drove the endocytosis of the \( \alpha_{1b} \)-GFP as demonstrated by the co-localization of the green and red fluorescence within the cell (Fig. 9, merge).

The NK1-GFP expressed in HEK-293 cells could also undergo endocytosis in response to substance P, but not to epinephrine (Fig. 10). In cells co-expressing the NK1-GFP and \( \alpha_{1b} \)-HA, exposure to epinephrine did not induce internalization of the NK1-GFP, and vice versa, treatment with substance P did not trigger the internalization of the \( \alpha_{1b} \)-HA, as demonstrated by the clear segregation of the green and red fluorescent markers (Fig. 10, merge).

Similar results were obtained in cells co-expressing the \( \alpha_{1b} \)-HA and myc-CCR5 (Fig. 11). In cells co-expressing the myc-CCR5 and \( \alpha_{1b} \)-HA, exposure to epinephrine did not induce internalization of the myc-CCR5, and vice versa, treatment with RANTES did not trigger the internalization of the \( \alpha_{1b} \)-HA, as shown by the fact that the green and red fluorescent markers did not overlap (Fig. 11, merge).

Altogether these findings strongly support the hypothesis that co-internalization is related to receptor oligomerization, because only those receptors forming oligomers in FRET experiments, like the \( \alpha_{1a} \)- and \( \alpha_{1b} \)-AR subtypes, were able to co-internalize.

**DISCUSSION**

The present study provides evidence that both the \( \alpha_{1a} \)- and \( \alpha_{1b} \)-AR subtypes can form homo- as well as hetero-oligomers
and that oligomerization correlates with the agonist-induced co-internalization of the receptors. In addition, it suggests a role of helices I and VII in homo-oligomerization of the 1b-AR. Oligomerization was mainly demonstrated monitoring FRET between receptors fused to donor CFP and acceptor GFP or YFP co-expressed in a transient expression system. As mentioned above, FRET only occurs when the average distance between the donor-acceptor pair falls below 100 Å (10). However, whether a FRET between two fluorescent receptors involves intermolecular interactions among receptors versus their increased proximity within the cell membrane without direct contact cannot be unequivocally demonstrated by FRET. Another limitation of the energy transfer measurements is that, while detecting proximity between the fluorescent receptors, neither number of molecules per complex nor the fraction of the total receptor population existing in the oligomeric state can be assessed.

Despite these limits, our FRET show a selective pattern of receptor oligomerization. In fact, our results indicate that both the 1a- and 1b-AR form homo-oligomers with similar transfer efficiency of 0.10 (Fig. 2). Hetero-oligomers between the 1a- and 1b-AR could also be observed, the FRET efficiency being in this case of -0.05. These results suggest that, when the 1-AR subtypes are expressed at comparable levels, the trend to oligomerize is greater for the homo-oligomers than for the hetero-oligomers. In contrast, almost no FRET signal could be measured between the 1b-AR and the 2-AR, NK1 tachykinin, or the CCR5 chemokine receptors expressed at similar levels (Fig. 3). This strongly suggests that for the 1b-AR the ability of forming hetero-oligomers with other GPCRs is related to the degree of sequence homology. This is also in agreement with the results from a recent study (33) showing that homo-oligomerization of the opioid receptors occurred more efficiently than their hetero-oligomerization with the 2-AR.

An important observation of our study is that the results obtained from the co-immunoprecipitation experiments were not entirely consistent with those from the FRET measurements. In fact, when differently tagged receptors were co-expressed in HEK-293 cells the 1b-AR could co-immunoprecipitate with the 1a-AR as well as with a small amount of the CCR5 chemokine or NK1 tachykinin receptors (Fig. 4 and results not shown). Therefore, the conditions used in our experiments resulted in a certain promiscuity in the ability of the receptors to be co-immunoprecipitated. This suggests that the
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results obtained using co-immunoprecipitation should be interpreted with some caution and, possibly, be confirmed using other independent approaches.

For some GPCRs the structural determinants involved in receptor oligomerization have been investigated. Whereas the C terminus is implicated in the GABAA-R1/GABAA-R2 receptor oligomerization (11), the glyrophosphoinositide motif seems to be involved in the formation of β2-AR homo-oligomers (14), and a role of the N terminus was reported for the oligomerization of the bradykinin and calcium receptors (36, 37). Our findings indicate that the α1b-AR homo-oligomerization does not require the integrity of either the C-tail, the N-linked glycosylation sites in the N terminus, or the glyrophosphoinositide motifs in helices II and VI (Fig. 5).

Recent crystallographic data from native membranes of mouse retina showed that rhodopsin, a prototypical GPCR, was arranged in the membrane as a paracrystalline lattice of dimers as structural units. The intradimeric contacts seem to involve helices IV and V, whereas helices I and II, as well as the cytoplasmic loop 3β, might be involved in the formation of dimer rows (38). So far, based on mutagenesis and cross-linking experiments, helix VI for β-AR (14), helix IV for dopamine D2 (39), or helices I and II for Ste2 pheromone receptor homo-oligomers (28) have been proposed to provide inter-monomeric contacts. A theoretical study on the opioid receptors based on the subtractive correlated mutation method predicted that in receptor homo-oligomerization the most likely interfaces are helices IV and V for the δ, helix V for the κ, and helix I for the μ receptors (40). Our findings provide the evidence that for the α3-AR helices I and VII act in concert favoring receptor homo-oligomerization with helix I representing a prominent interface. Altogether these results support the notion that GPCR oligomerization involves the participation of different transmembrane helices whose role might differ among receptors. In addition, our findings do not support a domain swapping oligomerization model because we were unable to rescue the functional properties of binding- and signaling-deficient α1b-AR mutants co-expressed in the same cells (data not shown).

The ability of ligands to modulate GPCR oligomerization has been investigated by a large number of studies in which a wide range of possibilities can be found. In several cases agonists promoted association of receptors, e.g., the β2-AR, the receptors for GnRH and TRH, as well as the chemokine CXCR4, the bradykinin B2 and SSR5 somatostatin receptors (16, 34, 41–45). However, in an equally large number of cases, oligomers appear to be constitutive, e.g., for the somatostatin receptors the m3 muscarinic receptor, and for the yeast pheromone the Ste2 receptor (25, 46, 47), or to dissociate upon treatment with the agonist, like the δ-opioid or cholecystokinin receptor oligomers (48, 49). The basis for these discrepancies remains obscure and might, at least in part, be related to the different experimental approaches used to monitor receptor oligomerization. In our study, the α1b-AR oligomers were found to be constitutive because neither the agonist epinephrine nor the inverse agonist prazosin had any effect on the FRET signal (Fig. 8). In addition, the oligomerization of the α1b-AR did not seem to be related to its activation state because a constitutively active (A293E) as well as a signaling-deficient (R143E) mutant were equally able to associate with an energy transfer efficiency comparable with that of the wild type receptor (Fig. 8). Co-expression of the α1a- and α1b-AR did not result in any pharmacological changes that could be attributed to a novel receptor species resulting from receptor hetero-oligomerization (results not shown).

Despite the lack of ligand-induced regulation, our findings suggest that oligomerization of the α1-AR subtypes might have functional implications in receptor endocytosis. In fact, we found that oligomerization of the α1-AR subtypes correlated with their ability to co-internalize upon exposure to the agonist. Although the α1b-AR could co-internalize with the α1b-AR subtype (Fig. 9), it did not co-internalize with the NK1 tachykinin or CCR5 chemokine receptors (Figs. 10 and 11). Therefore, only the α1a- and α1b-AR subtypes, which could form hetero-oligomers, were able to co-internalize. The ability of the α1a- and α1b-AR subtypes to co-internalize might have physiological consequences on receptor regulation. The two receptors have an overlapping distribution in several tissues (e.g., heart, brain, prostate, etc.). One can expect that hetero-oligomerization might provide yet another means to fine-tune the responses mediated by the α1a- and α1b-AR subtypes like, for example, coordinating their internalization properties.

The literature on GPCR hetero-oligomerization harbors several examples of modified trafficking of one receptor upon association with another. For example, the rate of internalization of the β2-AR (17) was markedly decreased when co-expressed with the κ-opioid receptor that does not undergo agonist-induced internalization. In contrast, the internalization of the δ-opioid receptor could trigger the co-internalization of the β2-AR (17). As a consequence of its co-expression with mGluR1, the trafficking of the calcium receptor became susceptible to modulation by Homer, a protein known to interact with glutamate mGluRs (34). In the same study, internalization of the calcium receptor in response to glutamate was demonstrated to occur (34). Inhibition of the internalization of the β2-AR was observed upon co-expression with β1-AR, which by itself internalizes to a lesser extent (18). The µ-opioid receptor could be internalized by exposure to a sst2A-selective ligand in cells co-expressing the two receptors (33).

These examples indicate that the receptor partners associated in putative oligomers can mutually influence their internalization properties. However, a causal relationship between receptor oligomerization and internalization properties cannot be easily drawn. Co-internalization of two GPCRs could result from the recruitment to the plasma membrane of proteins belonging to the endocytic machinery following the agonist-induced activation of one of the receptors. However, in our study the co-internalization was selective for the α1a- and α1b-AR subtypes, whereas the recruitment of the endocytic machinery at the plasma membrane could be expected to enhance the internalization also of other GPCRs like the NK1 and CCR5 receptors. Based on this observation, we propose that the co-internalization of the α1a- and α1b-AR subtypes is directly related either to the co-segregation of the receptors in membrane micro-domains or to their intermolecular interaction. An important issue concerning GPCR oligomerization is whether this phenomenon is due to the high levels of receptor expression in transient systems that might lead to receptor aggregation in the membrane. So far, the vast majority of the studies reporting oligomerization of GPCRs have been performed in such transient expression systems because of the free choice and convenient labeling of the target receptors. A recent study using bioluminescence resonance energy transfer reported that the number of β2-AR oligomers could be consistently measured throughout a wide range of receptor expression levels (50). Because of the low sensitivity of the FRET measurements, in our study the expression of the fluorescent receptors could not be decreased below 1–2 pmol/mg of proteins. However, even if high levels of receptor expression might favor protein aggregation, the selectivity of the FRET signals observed for the α1-AR subtypes strongly suggest that the energy transfer signals measured were not a mere consequence of receptor overexpression.

The results of our study as well as those from others (28, 29)
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clearly indicate that receptor oligomerization can occur at the plasma membrane as well as in the ER. In fact, some of our findings suggest that α₂-AR oligomers exist at the plasma membrane, where epinephrine is acting to trigger receptor endocytosis, and that they do not dissociate during this process. However, given the ER localization of the receptor fragments and of some α₁/β₃ chimeric receptors, their association with the α₁b-AR must occur at large extent in the ER. Altogether, these findings support the notion that oligomerization of the GPCRs is part of their maturation process playing a role in their export from the ER.

In conclusion, despite the growing number of studies on GPCR oligomerization, a number of aspects remain to be elucidated. In particular, the molecular mechanisms underlying GPCR oligomerization (e.g., intermolecular interaction versus co-segregation of receptors in microdomains; structural determinants of the receptor involved; number of receptor molecules per oligomer) cannot be unequivocally established. It is possible that, despite some common features, the oligomerization mechanisms might differ among GPCRs thus increasing the complexity of receptor signaling and regulation. Future studies should aim at investigating the role of receptor oligomerization in physiological systems as well as at further unraveling its molecular basis.

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