Hetero-oligomerization between \( \beta_2 \) and \( \beta_3 \)-Adrenergic Receptors Generates a \( \beta \)-Adrenergic Signaling Unit with Distinct Functional Properties*

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The ability of the closely related \( \beta_2 \) and \( \beta_3 \)-adrenergic receptors (AR) to form hetero-oligomers was assessed by bioluminescence resonance energy transfer. Quantitative bioluminescence resonance energy transfer titration curves revealed that the \( \beta_2 \)AR has identical propensity to hetero-oligomerize with the \( \beta_3 \)AR than to form homo-oligomers. To determine the influence of hetero-oligomerization, a HEK293 cell line stably expressing an excess of \( \beta_2 \)AR over \( \beta_3 \)AR was generated so that all \( \beta_2 \)AR are engaged in hetero-oligomerization with \( \beta_3 \)AR, providing a tool to study the effect of hetero-oligomerization on \( \beta_3 \)AR function in the absence of any \( \beta_2 \)AR homo-oligomer. The hetero-oligomerization had no effect on the ligand binding properties of various \( \beta_2 \)AR ligands and did not affect the potency of isoproterenol to stimulate adenylyl cyclase. Despite the unaltered ligand binding properties of the \( \beta_3 \)AR hetero-oligomer, the stable association of the \( \beta_3 \)AR with the \( \beta_2 \)AR completely blocked agonist-stimulated internalization of the \( \beta_2 \)AR. Given that the \( \beta_3 \)AR is resistant to agonist-promoted endocytosis, the results indicate that the \( \beta_2 \)AR acted as a dominant negative of the \( \beta_3 \)AR endocytosis process. Consistent with this notion, the \( \beta_2 \)AR hetero-oligomer displayed a lower propensity to recruit \( \beta \)-arrestin-2 than the \( \beta_2 \)AR. The hetero-oligomerization also led to a change in G protein coupling selectivity. Indeed, in contrast to \( \beta_2 \)AR and \( \beta_3 \)AR, which regulate adenylyl cyclase and extracellular signal-regulated kinase activity through a coupling to \( G_i \) and \( G_{\text{out}} \), no \( G_{\text{out}} \) coupling was observed for the \( \beta_2 \beta_3 \)AR hetero-oligomer. Together, these results demonstrate that hetero-oligomerization between \( \beta_2 \)AR and \( \beta_3 \)AR forms a \( \beta \)-adrenergic signaling unit that possesses unique functional properties.

Cell surface receptors, which mediate their biological effects via coupling to G proteins, control major functions of eucaryotic organisms like neurotransmission, immune response, cell growth, and metabolism (1). Over the past few years, several lines of evidence have supported the notion that G protein-coupled receptors (GPCR) exist and act as homo- or hetero-oligomeric signaling units (2–5). Hetero-oligomerization among GPCR has been shown to modulate ligand binding, G protein-coupling, endocytosis, and desensitization of the receptors (6–19).

Because of their clear and distinct properties, the \( \beta_2 \) adrenergic receptor (\( \beta_2 \)AR) and \( \beta_3 \)AR offer an ideal receptor pair to investigate the pharmacological and functional consequences of hetero-oligomerization. Indeed, despite their high degree of sequence homology (49%), each receptor displays characteristic ligand binding properties that can be easily distinguished (20). Also, although the \( \beta_2 \)AR undergoes rapid and efficient agonist-promoted internalization (21–23), the \( \beta_3 \)AR is resistant to these regulatory processes (24–26). The fact that these two closely related receptor subtypes are naturally co-expressed in adipocytes (27–29) suggests the possibility of formation of hetero-oligomers in native tissues and makes the characterization of this hetero-oligomer of potential physiological interest.

One difficulty when investigating hetero-oligomerization of GPCR is the heterogeneity of ligand-binding sites and signaling units that can occur if two receptors are co-expressed in the same cell. To analyze the properties of a hetero-oligomer in the presence of two homo-oligomers, one must ensure that the hetero-oligomer represents the major subpopulation among the expressed receptors. Also, a good correlation between the extent of hetero-oligomerization and the functional changes attributed to the formation of hetero-oligomers should be established. Unfortunately, quantitative assessment of hetero-oligomers is not a trivial issue. The amount of hetero-oligomers formed will be a function of the affinity of the individual receptors for one another and of their relative expression levels. Because of the difficulty in getting good estimation for these parameters, studies investigating GPCR hetero-oligomerization remained largely qualitative.

In the present study, we established experimental conditions that ensured that the entire population of \( \beta_2 \)AR heterologously expressed in HEK293 cells is engaged in hetero-oligomerization with the \( \beta_3 \)AR, thus allowing the functional characterization of the \( \beta_3 \)AR within the \( \beta_2 \beta_3 \)AR hetero-oligomer in the absence of any \( \beta_2 \)AR homo-oligomer.

Our study reveals that in contrast to the robust agonist-promoted endocytosis characteristic of the \( \beta_2 \)AR, the \( \beta_2 \beta_3 \)AR
hetero-oligomer was not internalized upon agonist stimulation, suggesting that the β2AR-negative endocytotic phenotype pre-valied in the hetero-oligomer. This dominant-negative effect of the β2AR occurred without any change in the ligand binding properties of the receptors and most likely results from a diminished β-arrestin-2 recruitment to the hetero-oligomer. When considering the coupling properties of the hetero-oligomer, we noticed that unlike β2AR and β3AR, which can couple to Gαs (30, 31), the β2β3AR hetero-oligomer cannot engage this signaling pathway. Taken together, our results indicate that the β2β3AR hetero-oligomer is a β2AR-like signaling unit distinct from β2AR or β3AR expressed alone.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modiﬁed Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, l-glutamine, and gentamicin (G418) were obtained from Wisent Laboratories. Alprenolol, isoproteinol (ISO), fenoterol (FEN), IC118551, CGP12177A, 3-isobutyl-1-methylxanthine (IBMX), and methotrexate were purchased from Sigma. FuGENE 6 was from Roche Applied Science. Anti-rabbit-HP or anti-mouse-HP antibodies were from Amersham Biosciences, and anti-phospho-ERK1/2 (E-2, sc-7383) or anti-ERK1/2 (K-23, sc-94) was from Santa Cruz Biotechnology. ECL, DeepBlueC, 125I-cyanopindolol, and the rabbit-anti-human IgG from PerkinElmer Life Sciences, whereas coelenterazine H was from Molecular Probes.

**Phosphorylation**—The expression of the receptor-Rluc, receptor-FP, and receptor-FP/Rluc vectors were kindly provided by PerkinElmer Life Sciences. For pGFP-β2AR-GFP10, the β2AR coding sequence without its stop codon was amplified by PCR using primers harboring unique HindIII and AgeI restriction sites. This PCR fragment was subcloned into the pGFP10-C1 vector (obtained from PerkinElmer Life Sciences) in a way that fused the 3'-end of the β2AR-cDNA onto the 5'-end of the GFP10-DNA.

**Cell Culture and Transfection**—HEK293 or COS-1 cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin/streptomycin, and 2 mM l-glutamine. For transient expression of recombinant proteins, cells were seeded at a density of 2 × 10^5 cells per 100-mm dish, cultured for 24 h, and then transfected by the calcium phosphate precipitation method for HEK293 cells (33) or by using the 100-mm dish, cultured for 24 h, and then transfected by the calcium phosphate precipitation method for HEK293 cells (33) or by using the PolyEthylene Glycol (Fusion™ r-FM, PerkinElmer Life Sciences) was used as described before (33). Briefly, after the substrate coelenterazine H was added, light intensity was sequentially integrated in the 510⋅–590 nm and 440⋅–500 nm window. The BRET signal was defined as the ratio of the light intensity minus mean at 540⋅–590 nm over 440⋅–500 nm. The expression level of the energy donor (Rluc) or acceptor (GFP or YFP) was controlled by measuring luminescence and fluorescence for each BRET experiment as described before (33).

**Membrane Preparation**—HEK293 cells were homogenized in ice-cold buffer (5 mM Tris/HCl, pH 7.4, 2 mM EDTA, 5 mM methylglucoside, 10 mg/ml benzamidine, and 5 mg/ml soyan bean trypsin inhibitor) using a Polytron (Ultra-Turrax T24, IKA) for 5⋅–10 s at maximum speed. Lysates were centrifuged at 500 × g for 10 min at 4 °C. The resulting supernatant was centrifuged at 45,000 × g for 20 min (4 °C), and pellets were washed twice using the same buffer. Protein amount was determined (Bio-Rad Protein Assay), and membranes were stored at –80 °C.

**Radioligand Binding Assay**—For saturation binding with 125I-CYP on the plasma membrane preparation were incubated with increasing concentrations (0.002–10 nM) of the β2AR antagonist I21, labeled cyanopindolol (125I-CYP) in 5 mM Tris/HCl, pH 7.4, and 0.1% bovine serum albumin. Specific binding of 125I-CYP was determined as the amount of 125I-CYP binding inhibited by 10 μM alprenolol. Samples were incubated for 1 h at 22 °C and then isolated by rapid filtration through GF/C glass-fiber filters, and the retained radioactivity was measured (1271 RIAgamma Counter, LKB Wallac). Competition binding assays were performed under the same conditions using 25 pM 125I-CYP to occupy only β2AR (this concentration binds 1% or less of the β2AR sites) or 250 pM 125I-CYP to ensure the occupancy of β2AR and β3AR. Specific binding of 125I-CYP was determined in the presence of increasing concentrations of the competing ligands.

**Internalization Assay**—To induce receptor endocytosis, cells were stimulated for 30 min with 1 μM ISO or 50 nM FEN at 37 °C. After washing the cells twice with ice-cold PBS, receptor sequestration was measured by detecting total binding of 25 pM 125I-CYP (CYPtot) at 13 °C for 3 h. Specific binding of 125I-CYP on the plasma membrane was determined as the binding inhibited by 100 mM of the hydrophilic ligand CGP12177A (CYPmem), although total specific binding was defined as the binding inhibited by 10 μM of the hydrophilic ligand alprenolol (CYPmem). The internalization rate of the receptor in percentage was calculated based on the following equation: ([CYPtot] − [CYPlight]) / [CYPtot] × 100. Endocytosis was also assessed after cell fractionation as described previously (25). Briefly, after stimulation of whole cells for 30 min at 37 °C with 1 μM ISO, cells were detached and membranes prepared as described above. Membranes were then placed at the top of a sucrose cushion (35%) and centrifuged at 150,000 × g for 3 h at 4 °C. The light endosome (CYPlight) and the heavy plasma membrane fraction (CYPheavy) were isolated at the 35%–55% interface, whereas the heavy plasma membrane fraction (CYPheavy) was found at the 0–55% interface, whereas the heavy plasma membrane fraction (CYPheavy) was isolated at the 35%–55% interface.

**Adenylyl Cyclase Activity**—Adenylyl cyclase activity of membranes freshly prepared (see above) from HEK293 cells was measured using the cAMP AlphaScreen™ assay from PerkinElmer Life Sciences (for details see www.perkinelmer.com/assays) with slight modification. [3H]cAMP was measured in duplicate in [3H]adenine. Cells were stimulated for 30–45 min at 37 °C in DMEM containing 2.5 mM IBMX and different drugs at the indicated concentrations. The reaction was terminated by removing the DMEM/IBMX/ligand solution and the addition of ice-cold 5% trichloroacetic acid. The [3H]cAMP was separated by sequential chromatography (Dowex resin/aluminum oxide), and the accumulation of cAMP was expressed as the ratio of [3H]cAMP/[3H] ATP + [3H]AMP.

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and adding 200 μl of Laemmli buffer (35). Samples were then analyzed by SDS-PAGE and Western blotting by using anti-phospho-ERK1/2 antibodies from mouse (1:5000) and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies from sheep (1:10,000). The immunoactivity was then revealed by chemiluminescence using ECL. After stripping the membranes with 25 mM glycine, pH 2.2, 1% SDS for 1 h, the amount of total ERK1/2 proteins was detected using anti-ERK1/2 antibodies from rabbit (1:10,000) and anti-rabbit HRP-conjugated secondary antibodies from donkey (1:10,000). ERK1/2 phosphorylation was expressed as the ratio of the signal provided by the phospho-ERK1/2 antibody over the signal obtained with the total ERK1/2 antibody.

**Data Analysis**—Data obtained in binding experiments were analyzed using Prism 3.0. Isotherms for saturation or competition binding assays were plotted for one or two binding sites, and the best fit was then used to calculate Kₐ, Kᵣ, or EC₅₀ values. Data obtained in ERK1/2 activity experiments were digitized on a flatbed scanner and analyzed using the Quantity One (Bio-Rad) software. Statistical significance of the differences was assessed by the two-tailed Student’s t test.

**RESULTS**

**Detection of β₂₃R/AR Hetero-oligomers by BRET**—To determine whether β₂R and β₂₃R can form hetero-oligomers, we took advantage of the BRET² technology and fused both receptors on their C terminus to Rluc or GFP10. After transient co-expression of β₂₃R-Rluc and β₂₃R-GFP in HEK293 cells, an energy transfer resulting in a BRET signal of 0.14 ± 0.02 was detected in the absence of ligand stimulation (Fig. 1A), consistent with the formation of constitutive β₂₃R homo-oligomers described previously (32, 33, 36, 37). Co-expression of β₂R-Rluc and β₂₃R-GFP also led to constitutive energy transfer (BRET signal, 0.16 ± 0.02), providing the first evidence that β₂R can form homo-oligomers in living cells (Fig. 1A). To assess whether β₂R and β₂₃R could also form hetero-oligomeric complexes, β₂₃R-Rluc/β₂R-GFP and β₂R-Rluc/β₂₃R-GFP pairs were co-expressed, and the resulting energy transfer was measured. Both combinations resulted in significant BRET signals (β₂₃R-Rluc/β₂R-GFP, 0.15 ± 0.02; β₂R-Rluc/β₂₃R-GFP, 0.17 ± 0.03) indicating that constitutive β₂₃R-AR hetero-oligomers can assemble (Fig. 1A). No significant energy transfer was detected between β₂R-Rluc and GABABR₂-GFP (BRET signal, 0.02 ± 0.005) when these receptors were expressed at levels comparable with those of β₂₃R-Rluc and β₂R-GFP, thus indicating that the energy transfer observed for the β₂R-Rluc/β₂₃R-GFP pair did not result from an overexpression of energy donor and acceptor.

To exclude further the possibility that the BRET signals obtained could result from nonspecific interaction or aggregation due to overexpression in a heterologous expression system, BRET experiments were carried out in HEK293 cells expressing a wide spectrum of receptor numbers (580–6570 fmol/mg) in an ~1:1 β₂R/β₂₃R ratio. If the BRET signal observed is the result of spurious interactions between the two receptors, it would be expected to increase as a function of the total receptors expressed even at constant energy acceptor/donor (β₂₃R-GFP/β₂R-Rluc) ratios. As shown in Fig. 1B, the BRET signals detected were constant over the entire range of receptor expression, indicating that overexpression did not drive the hetero-oligomerization. Most interesting, the lowest receptor density tested (580 fmol/mg) is similar to values reported previously (596 ± 263 fmol/mg) for fully differentiated human brown adipocytes (38), indicating that oligomerization could occur in native tissue.

To assess the propensity of β₂R to either homo- or hetero-oligomerize with β₂₃R, BRET titration curves were carried out in HEK293 cells stably expressing 410 fmol/mg of β₂R-Rluc by increasing the concentrations of β₂₃R-GFP or β₂₃R-GFP transiently expressed. As reported previously (33), increasing the GFP fusion proteins expressed led to a hyperbolic increase in the BRET signal that reached a maximum BRET signal once all β₂R-GFP molecules that can engage in oligomerization are in a complex with either β₂R- or β₂₃R-GFP. The apparent affinity of the β₂R-GFP and β₂₃R-GFP for the β₂R-Rluc can then be determined by estimating the GFP concentration required to attain 50% of the maximal BRET signal (BRET₅₀). Titration curves for the β₂R homo-oligomer and the β₂₃R-AR hetero-oligomer gave similar BRET₅₀ values of 2.30 ± 0.54 and 2.45 ± 0.67, respectively (Fig. 1C), indicating that β₂R had similar propensity to form homo-oligomers and hetero-oligomers with β₂₃R. In contrast, co-expression of GABABR₂-GFP in β₂₃R-GFP-expressing cells led to a marginal BRET signal.
that could not be fitted to a hyperbolic function, confirming the selectivity of interaction among the βAR subtypes (Fig. 1C).

To generate a cell system that would allow us to reliably assess the properties of the β2/3AR hetero-oligomer, we selected a HEK293 cell clone (HEK293-β2/3AR cells) that stably expressed a high ratio of β2AR-GFP/β3AR-RLuc. The high ratio observed for the HEK293-β2/3AR cells (3.6 ± 0.2) corresponds to a GFP/RLuc ratio that would be expected to cause near-saturation of the βAR-RLuc by β2AR-GFP (Fig. 1C). This prediction is further supported by the high level of constitutive BRET signal observed in these cells. Indeed, the BRET signal of 0.26 ± 0.02 observed was not significantly different from the maximum BRET signal (0.28 ± 0.00) obtained in the titration experiments (Fig. 1C). These data indicate that most if not all of the β2AR-RLucs, which can engage in oligomerization, are interacting with β2AR-GFP molecules, excluding the existence of a significant proportion of β2AR homo-oligomers in these cells. Since previous studies have suggested that if GPCR agonist-promoted endocytosis of the CGP12177 binding to fusion proteins into endosomes following cell fractionation. As described under “Experimental Procedures,” agonist-promoted receptor internalization was also determined by radioligand binding studies. Cell surface receptors were quantified by assessing the proportion of 125I-CYP-binding sites accessible to the hydrophilic ligand CGP12117. The extent of internalization is expressed as a percentage of the cell surface receptor lost following treatments with 1 μM ISO or 50 μM FEN for 30 min at 37°C. The binding experiments were carried out with 25 pm 125I-CYP, a concentration that selectively labels β2AR without binding to β3AR. Results are expressed as the mean ± S.E. of 3–5 independent experiments carried out in triplicate. Asterisk indicates a significant (p < 0.05) difference of the β2AR endocytosis observed in β2AR versus β3AR expressing cells.

Pharmacological Characterization of β2/3AR Hetero-oligomers—Since previous studies (11, 12, 18) reported that hetero-oligomerization can inhibit ligand binding, alterations in the binding properties of the β2AR could be responsible for the lack of agonist-promoted endocytosis. Thus, to determine whether the ligand binding properties of the β2AR are affected by its hetero-oligomerization with β3AR, both saturation and competition radioligand binding studies were performed. 125I-CYP saturation isotherms carried out with membranes derived from HEK293-β2/3AR cells were found to be biphasic, revealing high (Kᵢ 1) and low (Kᵢ 2) affinity components that agreed well with the affinities of CYP determined in cells individually expressing the β2AR and β3AR, respectively (Table I). Based on the fact that all β2AR are engaged in oligomeric assembly with the β3AR in HEK293-β2/3AR cells, the data indicate that the β2AR component of the β2/3AR hetero-oligomer maintains β2AR-like affinity for CYP. The β3AR component of the hetero-oligomer also maintained β3AR-like affinities for the agonists ISO and norepinephrine. Indeed, in competition binding experiments performed with a concentration of 125I-CYP (25 pm) that
can only bind the β2AR component, ISO and norepinephrine showed \( K_v \) values that are not different from those observed in cells expressing the β2AR alone (Table I). Most interesting, both \( K_{H1} \) and \( K_{L1} \), which are characteristic of agonist affinities for the G protein-coupled and -uncoupled forms of the receptor (42), were similar, indicating that the β2AR component of the β2,3AR hetero-oligomer can engage G proteins. Next, to confirm that the selectivity of ligands toward β2AR was also preserved in the hetero-oligomer, competition binding experiments with the β2AR selective agonist, FEN, and inverse agonist, ICI118551, were carried out using a \( ^{125}\text{I}-\text{CYP} \) concentration (250 pm) that can detectably bind to both β2AR and β2AR. As shown in Table I, each compound recognized two binding sites on membranes derived from HEK293-β2,3AR cells that corresponded well with their affinities for the individually expressed β2AR and β2AR. Although we cannot exclude that the β2,3AR hetero-oligomer could have altered binding properties for other ligands, our results indicate that hetero-oligomerization with the β2AR does not radically affect the overall binding properties of the β2AR and clearly demonstrate that the lack of agonist-promoted β2AR endocytosis does not result from altered binding to ISO or FEN.

**cAMP Accumulation Induced by β2,3AR Hetero-oligomers—**

Hetero-oligomerization between AT₂ and AT₁ angiotensin receptors has been proposed to inhibit AT₁ receptor activation (14). Thus to determine whether the lack of internalization could result from a similar inhibition of the β2AR activity by β2AR, the functional characteristic of the β2,3AR hetero-oligomer was assessed. When expressed individually, β2AR and β2AR are well characterized positive regulators of adenyl cyclase (AC) through their coupling to \( G_s \) (43). To test whether the β2,3AR hetero-oligomeric complex is still able to stimulate AC activity, we examined ISO-induced cAMP production in HEK293-β2,3AR cells. As shown in Fig. 3A, the concentration-dependent increase in ISO-stimulated AC activity was very similar for β2AR, β2AR, and β2AR-expressing cells (EC\(_{50}\) 9.8 ± 2.6, 28.7 ± 5.8, and 13.9 ± 3.9 nm, respectively). Because the AC activity observed in β2AR cells could be entirely due to β2AR, the functional integrity of β2AR within the hetero-oligomer was assessed by using the β2AR-selective antagonist ICI118551. The EC\(_{50}\) for ISO in HEK293-β2AR cells increased about 65-fold in the presence of ICI118551 (894 ± 64 nm), whereas the response was not affected in HEK293-β2AR cells (Fig. 3A). For HEK293-β2,3AR cells, the presence of the β2AR-selective antagonist led to a biphasic dose-response curve. The first portion of the curve was insensitive to ICI118551, with an EC\(_{50}\) of 6.5 ± 2.2 nm, and thus represented the β2AR response. The second portion was significantly right-shifted by ICI118551, with an EC\(_{50}\) of 1254 ± 276 nm, and thus reflected a β2AR-mediated cAMP production. Considering the undetectable level of β2AR homo-oligomers in HEK293-β2,3AR cells, the ICI118551-sensitive response most likely resulted from the β2AR component of the β2,3AR hetero-oligomer. Taken with the detection of a high affinity ISO-binding site (Table I), these results indicate that β2,3AR can functionally interact with \( G_s \) and thus the lack of endocytosis does not reflect an inactive receptor. As in the case of the β2AR expressed alone (44), the spontaneous (agonist-independent) activity of the β2,3AR hetero-oligomer could be efficiently inhibited by the inverse agonist ICI118551 (Fig. 3B), confirming the unaltered functional properties of the β2AR within the hetero-oligomer.

| Binding site | \( K_D \) (pm) | \( B_{max} \) (pmol/mg) | \( K_D \) (pm) | \( B_{max} \) (pmol/mg) | \( K_D \) (pm) | \( B_{max} \) (pmol/mg) |
|-------------|----------------|------------------------|----------------|------------------------|----------------|------------------------|
| Binding site 1 | 0.09 ± 0.01 | 4.7 ± 0.3 | 2.7 ± 0.9 | 3.9 ± 0.5 | 0.03 ± 0.01 | 1.1 ± 0.2 |
| Binding site 2 | 2.2 nM | 28.76 | 0.3 | 2.76 | 2.4 ± 0.09 | 3.9 ± 0.8 |

**Table I.** Ligand binding properties of β2,3AR hetero-oligomers

Saturation binding competition experiments with the \( ^{125}\text{I}-\text{CYP} \) were carried out using a \( ^{125}\text{I}-\text{CYP} \) concentration (250 pm) (fenoterol/ICI118551) or \( ^{125}\text{I}-\text{CYP} \) were used for competition binding experiments. Binding curves were analyzed by using the program Prism 3.0 and the curves best-fitted to either one or two binding sites. \( K_v \) and \( K_i \) values are expressed in pm, and the \( B_{max} \) are given in pmol/mg. When two sites were identified in saturation binding experiments, they were defined as binding site 1 and 2. In competition binding experiments, high and affinity binding states for agonists were defined as \( K_{H1} \) and \( K_{L1} \), respectively. Results represent the mean ± S.D. of 2–3 independent experiments performed in triplicates. ND, not determined.
To address this question, we monitored the recruitment of β-arrestin-2 in the presence or absence of co-expressed β₂AR were stimulated or not with 1 μM (A) or with increasing concentrations (10⁻⁸ to 10⁻¹⁰ M) of ISO (B) for 30 min at room temperature, and the interaction between β₂AR-Rluc and β-arrestin-2-YFP was monitored by BRET following the addition of coelenterazine H. The β-arrestin-2-YFP/β₂AR-Rluc expression ratios are indicated below the bars (A). Results are expressed as the mean ± S.E. of three independent experiments performed in duplicate. Asterisk indicates a significant (p < 0.05) difference between ISO-treated and untreated cells.

**β-Arrestin Recruitment by β₂/₃AR Hetero-oligomers**—Because agonist-induced β-arrestin recruitment is a crucial step in the β₂AR internalization process (45), the lack of agonist-promoted internalization of β₂AR raises the question of whether β₂/₃AR hetero-oligomers are able to recruit β-arrestin. To address this question, we monitored the recruitment of β-arrestin-2-YFP by β₂AR-Rluc in the absence or presence of co-expressed β₂AR using the BRET technology (32, 46, 47) after transient expression in HEK293 cells. As shown in Fig. 4A, stimulation with 1 μM ISO promoted a significant BRET signal between β₂AR-Rluc and β-arrestin-2-YFP, reflecting the translocation of β-arrestin-2 to the receptor. In contrast, no BRET signal could be observed between β₂AR-Rluc and β-arrestin-2-YFP under the same conditions (data not shown), an observation consistent with the lack of agonist-promoted internalization of this receptor. Co-expression of the β₂AR with β₂AR-Rluc and β₂/₃AR-Rluc significantly reduced the ISO-promoted BRET signal (Fig. 4A). Given that the β-arrestin-2-YFP/β₂AR-Rluc co-expression, the decreased BRET signal most likely reflects a decreased ability of the hetero-oligomer to recruit β-arrestin-2. However, because the extent of energy transfer does not only reflect the number of BRET pairs generated but also the distance and orientation between the energy donor and acceptor within the pairs, one cannot exclude the possibility that the reduced BRET signal could reflect a different conformational arrangement of the β₂AR-Rluc/β₂/₃AR-Rluc complex within the hetero-oligomer.

To determine whether the decreased BRET signal results from a reduced ability of the hetero-oligomer to recruit β-arrestin-2 or from an altered conformation of the complex, the potency of ISO to recruit β-arrestin-2-YFP to β₂AR-Rluc was assessed in the presence or absence of co-expressed β₂AR. Indeed, given that the hetero-oligomerization with β₂AR did not affect the affinity of the β₂AR for ISO (Table 1), the potency of ISO to promote the BRET signal will reflect the relative affinity of β-arrestin-2 for the receptor. In the absence of β₂AR, the potency of ISO to promote β-arrestin-2 recruitment was 17.6 ± 4.1 nM (Fig. 4B), a value consistent with the potency of ISO to stimulate AC (13.9 ± 3.9 nM; Fig. 3A) and with its high affinity binding site (15.6 ± 3.6 nM; Table 1). In cells co-expressing β₂AR, the ISO-promoted BRET signal between β₂AR-Rluc and β₂/₃AR-Rluc was found to be biphasic, with a high potency close to the one revealed in the absence of β₂AR (35.6 ± 5.6 nM) and a second potency that was significantly right-shifted (EC₅₀ = 26,680 ± 3192 nM). The high potency component is not statistically different from the one observed in cells expressing the β₂AR alone and thus most likely reflects the potency of the β₂AR homo-oligomer to recruit β-arrestin-2. The presence of a β₂AR homo-oligomer component indicates that the amount of β₂AR expressed in this transient expression system used was not sufficient to saturate all the β₂AR. When expressed alone, the β₂AR cannot recruit β-arrestin-2 (48) (data not shown).

Thus, the low potency component of the β₂/₃AR hetero-oligomer complex dose-response that was best fitted to a two component model (Fig. 4B). The correlation between the reduced β₂AR endocytosis and the reduced ability of the β₂/₃AR hetero-oligomer to recruit β-arrestin was further investigated in an additional cell system. For this purpose, COS-1 cells expressing β₂AR-Rluc and β₂/₃AR-Rluc were stimulated with 1 μM ISO and the β-arrestin recruitment detected in the biphasic curve probably corresponds to the recruitment of β-arrestin-2 to the β₂/₃AR hetero-oligomer, consistent with the hypothesis that the hetero-oligomer has a decreased ability to recruit β-arrestin-2.

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**ISO-induced Desensitization of Agonist-promoted AC Activity in HEK293-β₂/₃AR Cells**—Because of the central role played by β-arrestin in the agonist-promoted desensitization of the β₂AR, we next examined the ability of the β₂/₃AR hetero-oligomer to undergo ISOP-promoted desensitization. For this purpose, ISO-promoted AC activity was assessed in cell membranes derived from HEK293-β₂AR or HEK293-β₂/₃AR cells pretreated or not with ISO for 1 h. As shown in Fig. 6, ISO stimulated AC with almost identical potency in membranes derived from naive HEK293-β₂AR and HEK-β₂/₃AR cells (EC₅₀: β₂AR, 32.5 ± 4.2 nM; β₂/₃AR, 29.2 ± 3.5 nM). The agonist-promoted desensitization of the β₂AR can be readily seen by the significant decrease in ISO potency observed in membranes derived from HEK293-β₂AR cells pretreated with ISO (EC₅₀ = 7560 ± 137 nM). In the case of the HEK293-β₂/₃AR cells, ISO pretreatment led to a complex dose-response that was best fitted to a two component curve. The first component displayed an EC₅₀ (39.4 ± 4.8 nM) almost identical to that obtained in membranes derived from untreated cells, whereas the EC₅₀ of the second component was significantly right-shifted to a value similar to that obtained in...
Fig. 5. ISO-promoted β-arrestin-2 recruitment and β2,3AR hetero-oligomer endocytosis in COS-1 cells. COS-1 cells transiently expressing β2AR-RLuc and β-arrestin-2-YFP in the presence or absence of co-expressed β2AR were stimulated or not for 30 min with ISO (1 μM). A, ISO-promoted endocytosis of the β2AR-RLuc was measured by assessing the proportion of 125I-CYP-binding sites accessible to the hydrophilic ligand CGP12117 and expressed as % internalization, as described under “Experimental Procedures.” Results are expressed as the mean ± S.E. of three independent experiments performed in triplicate. Asterisk indicates a significant (p < 0.05) difference. B, ISO-induced β-arrestin-2-YFP recruitment by β2AR-RLuc was monitored by BRET following the addition of coelenterazine H to cells derived from the transfection experiments described in A. The β-arrestin-2-YFP/β2AR-RLuc expression ratios are indicated below the bars. Results are expressed as the mean ± S.E. of three independent experiments performed in triplicate. Asterisk indicates a significant (p < 0.05) difference between ISO-treated and -untreated cells. The transfection experiments were carried out to favor an excess of β2AR. The expression of the receptors was determined by radioligand binding assays and found to be 1.4 ± 0.4 pmol/mg for β2AR-RLuc and 6.2 ± 1.6 pmol/mg for β2AR.

Fig. 6. ISO-promoted desensitization of the β2,3AR hetero-oligomer. HEK293-β2AR (triangle) or HEK293-β2,3AR cells (circle) were pretreated (open symbols) or not (closed symbols) with 10 μM ISO for 1 h at 37 °C. Receptor desensitization was then assessed by measuring the ISO-promoted AC activity in membranes derived from naive or ISO-pre-stimulated cells, as described under “Experimental Procedures.” Data represent the mean ± S.E. of three independent experiments carried out in duplicate.

the desensitized β2AR-expressing membranes (EC50, 12.470 ± 1730 nM). Because β2AR does not undergo agonist-promoted desensitization (24, 26), and no β2AR homo-oligomer exists in the HEK293-β2AR cells, we conclude that the first component of the curve represents the non-desensitized β2AR, whereas the second component corresponds to the desensitized β2AR hetero-oligomer. These data therefore suggest that despite its inefficient recruitment of β-arrestin, the β2AR hetero-oligomer can undergo agonist-promoted desensitization, indicating the contribution of β-arrestin-independent mechanism(s).

β2,3AR-mediated Activation of ERK1/2—Several studies (49–52) have suggested that the activation of the ERK1/2 signaling pathway by β2AR involves β-arrestin recruitment and β2AR endocytosis. Particularly relevant to the present study is the observation that hetero-oligomerization between β2AR and either β1AR or κ-opioid receptors blocked both β2AR endocytosis and β2AR-mediated ERK1/2 activation (15, 16). To determine whether the inhibition of β2AR endocytosis resulting from its hetero-oligomerization with β2AR also blocked its ability to activate ERK1/2, the agonist-stimulated ERK1/2 activity was assessed. As shown in Fig. 7, ISO promoted ERK1/2 phosphorylation in HEK293-β2,3AR cells as well as in cells expressing β2AR or β3AR alone. To determine whether the β2,3AR hetero-oligomer contributed to the response or if it could be entirely attributed to the β2AR, cells were stimulated with the β2AR-selective agonist FEN. Fifty nM FEN activated ERK1/2 in HEK293-β2,3AR cells but not in cells expressing β2AR alone, indicating that β2,3AR hetero-oligomers are able to activate ERK1/2.

β2AR and β2,3AR-stimulated ERK1/2 activity was previously shown to be PTX-sensitive, suggesting a role for Gαi/o in this pathway (30, 31). To determine whether this is also a property of the β2,3AR hetero-oligomer, the effect of PTX was investigated. In cells expressing the β2AR or β2AR alone, PTX significantly decreased the agonist-stimulated ERK1/2 activation (Fig. 7), confirming previous reports. In contrast, ERK1/2 activity stimulated by the selective β2AR agonist FEN was found to be resistant to PTX treatment in HEK293-β2,3AR cells (Fig. 7), indicating that the β2,3AR activates ERK1/2 in a Gαi/o-independent manner. This PTX resistance cannot be attributed to a cell-specific difference such as those recently found among various HEK293 cell clones (53) because the ISO-stimulated ERK1/2 activity (resulting from the β2AR homo-oligomers) was found to be PTX-sensitive in the same HEK293-β2,3AR cell clone (Fig. 7). Together, these results indicate that the β2,3AR hetero-oligomer activates the ERK1/2 through a signaling cas-
Figu re 8. PTX sensitivity of receptor-stimulated cAMP accumulation. HEK293-β2AR, HEK293-β3AR, and HEK293-β2AR cells pre-treated or not with PTX were stimulated for 30 min at 37 °C with 50 nM or 1 μM FEN. The agonist-stimulated cAMP production was assessed by measuring the accumulation of [3H]cAMP in cells prelabeled with [3H]adenine and expressed as the ratio of [3H]cAMP/([3H]cAMP + [3H]ATP). Data represent the mean ± S.E. of five independent experiments carried out in triplicate. Asterisk indicates a significant (p < 0.05) difference between PTX-treated and -untreated cells.

**β2,3AR Hetero-oligomers**

**Effect of PTX on FEN-mediated cAMP Accumulation in Cells Expressing β2AR, β3AR, or β2,3AR.—**To determine whether the lack of G<sub>io</sub> dependence for the β2,3AR-stimulated ERK1/2 activation could also be observed in the AC signaling, we tested the effect of PTX on agonist-induced cAMP accumulation. As shown in Fig. 8, PTX treatment increased the net agonist-stimulated cAMP production generated by a nonselective concentration of FEN (1 μM) in β2AR-, β3AR-, and β2,3AR-expressing cells, indicating that it alleviated an inhibitory influence of G<sub>io</sub> on the receptor-mediated responses. However, cAMP production induced by a concentration of FEN (50 nM), which activates only the β2AR, was PTX-sensitive in HEK293-β2AR but not in HEK293-β2,3AR cells, whereas β3AR-expressing cells did not respond at all (Fig. 8). Thus, in contrast to β2AR or β3AR, β2,3AR-stimulated AC activity is not limited by a concomitant activation of G<sub>io</sub> proteins, confirming that the hetero-oligomer has a distinct G protein coupling pattern.

**DISCUSSION**

Resonance energy transfer approaches similar to the BRET assay used in the present study are becoming tools of choice to monitor protein-protein interactions in living cells (32, 54). Because BRET between Rluc and GFP can occur only if the two molecules are within ~100 Å from one another, the occurrence of BRET between Rluc- and GFP-bearing proteins indicates a molecular proximity that is consistent with oligomerization. Although dimerization is the simplest form of oligomerization, the detection of BRET does not provide any direct information about the stoichiometric arrangement of the protomers and the proportion of molecules involved in the oligomeric assembly. Thus, the term oligomer rather than dimer, which is often used to offer a simple model, is used throughout this article. Despite these limitations, proper quantitative analysis can provide useful information about the oligomerization process. For instance, BRET<sub>50</sub> values obtained from titration assays can provide information about the relative affinity of the partners involved (33). Also, by assuming that all the considered molecules are free to oligomerize, the maximal BRET values can be used to estimate the proportion of Rluc-bearing molecules that are engaged in oligomerization with the GFP-bearing molecules. Here, we took advantage of some of these features of BRET to quantitatively assess the formation of β2,3AR hetero-oligomers.

The similar BRET<sub>50</sub> observed in the present study for the β2AR homo-oligomer and the β2,3AR hetero-oligomer indicate that the β2AR has similar propensity to homo- and hetero-oligomerize with the β3AR. This is not unique to this receptor pair because similar high affinity hetero-oligomerization has been reported for several closely related GPCR subtypes (33, 55–57). The similar propensity of the β2AR to form homo and β2,3AR hetero-oligomers suggests that rather small changes in the relative expression levels of the two subtypes could significantly affect the homohetero-oligomer ratio. We took advantage of this characteristic to generate a cell line where the overexpression of the β2AR would favor the formation of β2,3AR hetero-oligomers so that the properties of the hetero-oligomer could be studied in the absence of significant amounts of β2AR homo-oligomers or monomers. One line of evidence suggesting that the HEK293-β2AR cells fulfill this criterion was provided by the quantitative analysis of the BRET data. Indeed, the BRET signals observed between β2AR-Rluc and β3AR-GFP in HEK293-β2AR cells correspond to the theoretical value expected for the hetero-oligomerization of all β2AR-Rluc with β3AR-GFP. Although one cannot exclude the possibility that a proportion of β2AR could be incapable of forming homo- or hetero-oligomers (leading to the persistence of monomer despite a saturation of the BRET signal), the observation that the excess β2AR expressed in HEK293-β3AR cells completely inhibited the agonist-promoted endocytosis and G<sub>io</sub> coupling of the β2AR strongly indicates that all functional β2AR were engaged in hetero-oligomerization. Thus, HEK293-β3AR cells provided a tool to characterize selectively the property of β2,3AR hetero-oligomers without the confounding effects of the β2AR homo-oligomers, thus solving a problem often encountered in studies assessing the functional outcome of GPCR hetero-oligomerization.

The analysis of HEK293-β2,3AR cells revealed that the β2,3AR hetero-oligomeric complex maintains some of the properties of the individual protomers but acquired distinct ones. The identical properties includes unchanged ligand binding profiles and a preserved positive coupling to both AC and ERK signaling pathways. The hetero-oligomer differs from the β2AR homo-oligomers in its inability to functionally interact with G<sub>io</sub>. Although several studies (7, 11, 12, 18, 58) reported that hetero-oligomerization changes the ligand binding properties of one or both protomers, no such effect was observed for the β2,3AR hetero-oligomer. Indeed, neither the binding affinity, potency, nor efficacy of any of the β3AR ligands tested was found to be affected by β2,3AR hetero-oligomerization. This lack of changes in the pharmacological properties suggests that each protomer maintains its cognate ligand binding pocket. Given the role of the transmembrane domains in defining binding affinity and selectivity among β-AR subtypes, the preservation of a β2AR-like binding profile argues against a hetero-oligomerization model involving swapping of transmembrane segments between β2AR and β3AR and would be more compatible with a contact type hetero-oligomer, where each protomer maintains its original transmembrane domain organization (59, 60).

Despite the apparent lack of effect on ligand binding, hetero-oligomerization had a significant impact on receptor endocytosis. The association of the endocytosis-prone β2AR with the endocytosis-resistant β3AR led to a complex that could not be internalized upon agonist stimulation, indicating that the β2AR acted as a dominant-negative of the β3AR endocytosis. Changes in the internalization profile of a given receptor upon co-expression of a distinct receptor have often been reported and attributed to hetero-oligomerization (10, 12, 13, 17–19, 61).
In the case of the \( \beta_2 \)AR, co-expression with \( \beta_1 \)AR (16), \( \kappa \)-opioid receptors (15), or \( V_2 \)-vasopressin receptors (62) has been shown to inhibit its internalization. In the latter case, the inhibition required the concomitant activation of the \( \beta_2 \)AR and \( V_2 \)-vasopressin receptors and was attributed to the sequestration of \( \beta_2 \)-arrestin by the \( V_2 \)-vasopressin receptors resulting in a decreased concentration of \( \beta_2 \)-arrestins available for the \( \beta_2 \)AR (62). Such a mechanism cannot be invoked to explain the inhibitory effect of the \( \beta_2 \)AR on the \( \beta_2 \)AR internalization because: 1) the \( \beta_2 \)AR blocked \( \beta_2 \)AR internalization promoted by a concentration of FEN that does not activate the \( \beta_2 \)AR, and 2) even when activated, \( \beta_2 \)AR is not able to recruit \( \beta_2 \)-arrestin (48). The observation that endocytosis was blocked in the absence of \( \beta_2 \)AR activation also rules out a possible involvement of downstream signaling cross-talk in the dominant-negative effect of the \( \beta_2 \)AR. Thus, as in the case of the \( \beta_2 \)AR/\( \kappa \)-opioid receptor co-expression, the inhibition of the \( \beta_2 \)AR internalization by the \( \beta_2 \)AR most likely results from their hetero-oligomerization.

Two main hypotheses could be proposed to explain how hetero-oligomerization with an endocytosis-resistant receptor can block \( \beta_2 \)AR internalization: 1) the hetero-oligomerization leads to a complex that cannot efficiently interact with \( \beta_2 \)-arrestin, or 2) the interaction of the complex with \( \beta_2 \)-arrestin occurs but does not result in the targeting of the hetero-oligomer to the clathrin-coated pits and endosomes. The results obtained in the BRET-based \( \beta_2 \)-arrestin recruitment studies clearly favor the first hypothesis. Indeed, the very low propensity of the \( \beta_2 \)AR to recruit \( \beta_2 \)-arrestin indicates that the \( \beta_2 \)AR efficiently blocks the interaction between \( \beta_2 \)-arrestin and \( \beta_2 \)AR via hetero-oligomerization, hence inhibiting \( \beta_2 \)-arrestin-mediated endocytosis of the \( \beta_2 \)AR. The difference in the potency of ISO to promote \( \beta_2 \)-arrestin recruitment in face of an unaltered receptor binding and AC stimulation potency suggests that the hetero-oligomerization selectively affected the ISO-promoted interaction with \( \beta_2 \)-arrestin but not with \( G_s \). Because G protein-coupled receptor kinase-mediated phosphorylation of the \( \beta_2 \)AR has been shown to increase its affinity for \( \beta_2 \)-arrestin, it could be hypothesized that hetero-oligomerization prevents efficient G protein-coupled receptor kinase-promoted phosphorylation.

Despite the reduced recruitment of \( \beta_2 \)-arrestin by the \( \beta_2 \)AR hetero-oligomer, this oligomeric complex was found to undergo agonist-promoted desensitization. This is somewhat surprising when considering the important role played by \( \beta_2 \)-arrestin in this regulatory process. However, several \( \beta_2 \)-arrestin-independent processes such as CAMP-dependent protein kinase-mediated receptor phosphorylation (63, 64) and endocytosis-independent receptor degradation (65) have been suggested previously. Additional studies will be needed to determine the precise mechanism underlying the hetero-oligomer desensitization.

It has been suggested previously that receptor endocytosis and/or \( \beta_2 \)-arrestin recruitment contributes to the activation of ERK1/2 by \( \beta_2 \)AR (49–52). Consistent with this notion, the inhibition of \( \beta_2 \)AR endocytosis upon hetero-oligomerization with either \( \beta_2 \)AR or \( \kappa \)-opioid receptors was accompanied by an abolition of the \( \beta_2 \)AR-stimulated ERK1/2 activation (15, 16). In contrast with these studies, the \( \beta_2 \)AR, which failed to undergo endocytosis, still evoked ERK1/2 activation in response to the \( \beta_2 \)AR-selective agonist FEN, indicating that endocytosis is not required for the activation of ERK1/2 by the \( \beta_2 \)AR complex. The low apparent affinity of the \( \beta_2 \)AR for \( \beta_2 \)-arrestin also suggests that the ERK1/2 activation promoted by the hetero-oligomer occurs in a \( \beta_2 \)-arrestin independent manner. These characteristics are reminiscent of the \( \beta_2 \)AR-stimulated ERK1/2 that also takes place in the absence of \( \beta_2 \)-arrestin recruitment and receptor endocytosis (31, 66). These results raise the intriguing possibility that agonist binding to the \( \beta_2 \)AR could transactivate the \( \beta_2 \)AR within the \( \beta_2 \)AR hetero-oligomer. Such transactivation within receptor oligomers has been proposed recently for GPCR of the family A (67, 68) and family C (69). However, as reported previously (31, 66, 70), the \( \beta_2 \)AR-mediated ERK1/2 activation was found to be PTX-sensitive, whereas the \( \beta_2 \)AR-promoted activation was not. Although this does not exclude the possibility of transactivation within the \( \beta_2 \)AR, it clearly indicates that the \( \beta_2 \)AR hetero-oligomer induces ERK1/2 activity by a mechanism that differs from the \( \beta_2 \)AR.

The lack of PTX sensitivity of the \( \beta_2 \)AR-stimulated ERK1/2 also clearly distinguishes the response of the hetero-oligomer from that observed for the \( \beta_2 \)AR expressed alone. Indeed, in the present study as well as in numerous previous studies (71–78), \( \beta_2 \)AR was found to couple to both \( G_s \) and \( G_i \). This dual coupling of the \( \beta_2 \)AR, observed in both ERK1/2 and AC pathways, was entirely lost for the \( \beta_2 \)AR hetero-oligomer. Similar loss of \( G_i \) coupling upon hetero-oligomerization has been suggested previously for the CCR2/CCR5 (79) and \( \delta \)-opioid receptor (10) pairs. In these two studies, coupling of the hetero-oligomer to a G protein that is not classically engaged by each of the protomers, \( G_{i/1} \), in the case of the CCR2/CCR5 and not identified for the \( \delta \)-opioid receptors, was invoked to explain the maintained signaling. In the present study, no coupling to an additional G protein was observed. Rather, all the signaling properties of the \( \beta_2 \)AR could be explained by its maintained coupling to \( G_s \).

The data presented here demonstrate that upon heterologous co-expression, human \( \beta_2 \)AR and \( \beta_2 \)AR can associate to form a hetero-oligomeric complex that maintains some of the properties of each of the protomers but acquires distinct ones. The distinct signaling characteristics could have important functional consequences on the catecholamine signaling efficacy in cells endogenously co-expressing these two receptors. Most interesting, mammalian adipocytes co-express \( \beta_2 \)AR and \( \beta_2 \)AR with a high \( \beta_2 \)/\( \beta_2 \)AR expression ratio that resembles the one observed in HEK293-\( \beta_2 \)AR cells established in the present study (27–29, 80). Moreover, the hetero-oligomerization between human \( \beta_2 \)AR and \( \beta_2 \)AR could be observed at expression levels that are similar to those reported for human adipocytes (38). If, as in the case of HEK293-\( \beta_2 \)AR cells, hetero-oligomerization with the \( \beta_2 \)AR blocks the \( G_s \) coupling of the \( \beta_2 \)AR in adipocytes, it would undoubtedly lead to a more efficient activation of AC and ensuing lipolysis (27, 28, 38). The hetero-oligomerization would thus contribute to increase the AC responsiveness of this tissue to the endogenous catecholamines. The inhibition of both \( \beta_2 \)AR recruitment and \( \beta_2 \)AR endocytosis, resulting from the hetero-oligomerization with the \( \beta_2 \)AR, would also be predicted to increase the adipocyte responsiveness to catecholamine stimulation. Although the experiments were not performed in the adipocytes, the present work carried out in heterologous expression systems allowed us to make predictions about the potential functional consequences of hetero-oligomerization in cells endogenously co-expressing \( \beta_2 \)AR and \( \beta_2 \)AR. The testing of these predictions in adipocytes derived from knock-out mice lacking either one of the two receptors should now help to determine the physiological importance of hetero-oligomerization for these receptor subtypes.

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