Agonist-independent Desensitization and Internalization of the Human Platelet-activating Factor Receptor by Coumermycin-Gyrase B-induced Dimerization*

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Platelet-activating factor (PAF)1 is a phospholipid with potent and diverse physiological actions, particularly as a mediator of inflammation. We have reported previously that mutant G protein-coupled receptors (GPCRs) affect the functional properties of coexpressed wild-type human PAF receptor (hPAFR) (Le Gouill, C., Parent, J. L., Caron, C. A., Gaudreau, R., Volkov, L., Rola-Pleszcynski, M., and Stankova, J. (1999) J. Biol. Chem. 274, 12548–12554). Increasing evidence suggests that dimerization of GPCRs may play an important role in the regulation of their biological activity. Additional data have also suggested that dimerization may be important in the subsequent internalization of the δ-opioid receptor. To investigate the specific role of dimerization in the internalization process of GPCRs, we generated a fusion protein of hPAFR and bacterial DNA gyrase B (GyrB), dimerized through the addition of coumermycin. We found that dimerization potentiates PAF-induced internalization of hPAFR-GyrB in Chinese hamster ovary cells stably expressing c-Myc-hPAFR-GyrB. Coumermycin-driven dimerization was also sufficient to induce an agonist-independent sequestration process in an arrestin- and clathrin-independent manner. Moreover, the protein kinase C inhibitors staurosporine and GF109203X blocked the coumermycin-induced desensitization of hPAFR-GyrB, suggesting the implication of protein kinase C in the molecular mechanism mediating the agonist-independent desensitization of the receptor. Taken together, these findings suggest a novel mechanism of GPCR desensitization and internalization triggered by dimerization.

Platelet-activating factor (PAF)1 is a phospholipid with potent chemoattractant and leukocyte activating properties (1, 2).

1 The abbreviations used are: PAF, platelet-activating factor; Ab(s), antibody(ies); arr, arrestin; BSA, bovine serum albumin; CHO, Chinese hamster ovary; GFP, green fluorescent protein; GPCR(s), G protein-coupled receptor(s); GyrB, bacterial DNA gyrase B; h, human; HA, hemagglutinin; IP, inositol phosphate(s); PAFR, PAF receptor; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate, 13-acetate.

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1A receptor, and the m2 muscarinic receptor can internalize by an unidentified mechanism that shows an atypical sensitivity to dynamin (18–21). In addition, further analysis revealed that the N-formyl peptide receptor and the C5a chemoattractant receptor are internalized via an arrestin- and dynamin-independent pathway, which leads to questions about an alternative mechanism involved in mediating internalization of these receptors (22).

An increasing number of studies have shown that many GPCRs form homodimers as well as heterodimers (23). Subsequent reports demonstrated homodimerization among the β2-adrenergic receptor (24), the δ-opioid receptor (25), the chemo- kinase receptors CCR2b, CCR4, and CCR5 (26, 27), the Ca2+/-sensing receptor (28), and the metabotropic glutamate receptor

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which may be correlated with the receptor susceptibility to kinase- and phospholipase C-independent mechanism. More of hPAFR-GyrB is regulated by PKC as well as a tyrosine kinase of hPAFR-GyrB led to the formation of dimers/oligomers, which is shown for the nontransfected cells were resuspended in ice-cold buffer containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The cells were then disrupted by sonication and pelleted by centrifuging at 12,500 rpm for 30 min at 4 °C. The supernatants were collected as total cell lysates, and protein concentration was determined by the Bio-Rad procedure with BSA as molecular weight markers are shown in the left margin. A representative of three independent experiments is shown. IB, immunoblotting.

**Fig. 1. Detection of human PAFR dimers via Western blotting analysis.** COS-7 were transiently transfected either with pcDNA3 (A, lane 1) as a negative control, c-Myc-tagged hPAFR (A and B, lane 2), or pcDNA3-HA-tagged hPAFR (B, lane 1). Cells were immunoprecipitated (IPP) with mouse anti-c-Myc (A) or anti-HA Abs (B) conjugated to protein A-Sepharose beads. Immunoprecipitated receptors were subjected to 10% SDS-PAGE and detected via Western blotting using the rabbit anti-c-Myc Ab. Positions of receptor bands are denoted by arrows, and molecular weight markers are shown in the left margin. A result representative of three independent experiments is shown. IB, immunoblotting.

**Table 1.** Listed are the specific characteristics of the native receptor, such as basal level of activity, affinity for the ligand, and cell surface expression (30). To determine whether artificially induced oligomerization of hPAFR could modulate the internalization process of the receptor, we applied the coumymycin-induced dimerization system (31), which is based on the binding of coumymycin to the amino-terminal 24-kDa subdomain of the B subunit of bacterial DNA gyrase (GyrB) (32). A hPAFR-GyrB fusion protein was thus generated, with the GyrB moiety fused to the COOH-terminal tail of hPAFR. We report here that coumymycin treatment on CHO cells stably expressing c-Myc-hPAFR-GyrB led to the formation of dimers/oligomers, which is sufficient to induce the sequestration process by an unknown endocytotic machinery. This coumymycin-driven desensitization of hPAFR-GyrB is regulated by PKC as well as a tyrosine kinase and phospholipase C-independent mechanism. More broadly, these data suggest that signals determined by secondary structure/conformation of the receptor involved in the conversion between the monomeric and the dimeric form of GPCRs may be implicated in the heterologous desensitization process, which may be correlated with the receptor susceptibility to phosphorylation by second messenger-dependent kinases.

**Materials and Methods**

**Chemical Reagents—Oligonucleotides, cell culture media, and Lipofectamine—**Oligonucleotides, cell culture media, and Lipofectamine were purchased from Invitrogen. Restriction endonucleases and T4 DNA ligase were from Promega and Amersham Biosciences. The hPAFR cDNA was a generous gift from Dr. R. Ye (University of Chicago), and pcDNA3-Raf-GyrB was kindly provided by Dr. Michael A. Farrar (Merek Research Laboratories, Rahway, NJ). Bovine serum albumin (BSA), typhostin 51, parafonadialdehyde, coumymycin, and novobiocin were from Sigma. Staurosporine and GF109203X were from BIOMOL Research Laboratories. Dimethyl sulfoxide from Fisher, and lipase-free BSA was from Calbiochem. AG 1-X8 resin was from Bio-Rad, and DEAE-dextran was purchased from Amersham Biosciences. PAF was from the Cayman Chemical Co. (Ann Arbor, MI). 1,2-Hydroxycyclopentyl-PAF and myo-[3H]Hinostiol were from Amersham Biosciences. WEB2086 and FuGENE 6 were from Roche Applied Science. WEB2086 was purchased from PerkinElmer Life Sciences. Antibodies (Abs) used were rabbit polyclonal anti-c-Myc (A-14) (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-c-Myc (9E10) (ATCC, Manassas, VA), and monoclonal anti-hemagglutinin (HA) (12CA5) (BabsCo, Richmond, CA). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit Abs were from Amersham Biosciences. Fluorescein isothiocyanate-conjugated goat anti-mouse and rhodamine-conjugated goat anti-mouse Abs were obtained from BioCan Scientific (Mississauga, ON, Canada).

**Construction of Epitope-tagged PAFR-GyrB—**Human PAF receptor was epitope tagged in the amino-terminal extracellular domain with either a c-Myc or a HA epitope, as described previously (12) and subcloned into the pcDNA3 vector. To construct PAFR-GyrB, NheI, NotI, and Aparf sites were introduced in the pcDNA3-c-Myc-PAFR by digestion using the oligonucleotides 5′-CTGGCAATTCCTCCTCAAAATGCTCT- 3′/CTGGCAATTCCTCCTCAAAATGCTCT-3′ corresponding to the last 23 nucleic acids of the hPAFR. The stop codon was then replaced at the NheI-NotI sites with the coding region of GyrB isolated from pcDNA3-Raf-GyrB at XbaI and NotI sites. Constructs were verified by restriction enzyme digestion.

**Cell Culture and Transfections—**CHO and CHO cells were maintained at 37 °C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (high glucose) and Dulbecco’s modified Eagle’s medium F-12 (Ham’s medium, high glucose), respectively, supplemented with 5% fetal bovine serum. Cells were grown in 100-mm dishes to 70–80% confluence and transiently transfected the following day with 7 μl of a mixture of 100 μM chloroquine and 0.25 mg/ml DEAE-dextran containing 4 μg of plasmid DNA (pcDNA3-c-Myc-hPAFR, pcDNA3-HA-hPAFR, or pcDNA3-c-Myc-hPAFR-GyrB) in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. After 2 h at 37 °C, the solution was removed, and the cells were treated for 1 min at room temperature with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS), rinsed twice with PBS, and returned to the 37 °C incubator in growth medium supplemented with 5% fetal bovine serum. For confocal microscopy experiments, cells were seeded into 6-well plates (1–1.5 × 105 cells/ml) and transiently transfected with 1 μg of plasmid DNA/well by using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Stable CHO transfectants expressing the pcDNA3-c-Myc-hPAFR were generated as described previously (33). CHO stably transfected with the pcDNA3-c-Myc-hPAFR-GyrB were established as above except that positive cells were selected with a FACSVantage cell sorter (BD Biosciences) after labeling with anti-c-Myc monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse Ab.
standard. Samples containing 20 μg of protein were resolved using 6% Tris-glycine precast gels under nonreducing conditions. For Western blotting analysis, nitrocellulose membranes were blocked in PBS and 0.1% Tween containing 10% dried milk for 1 h and incubated with anti-HA or anti-Myc in PBS and 1% dried milk at room temperature. After washing with PBS-Tween and incubation with secondary Abs, a chemiluminescence detection system was used for protein detection (PerkinElmer Life Sciences).

Flow Cytometry Studies—Receptor internalization was determined as the level of receptor loss from the cell surface. Stably transfected CHO cells were seeded on 6-well plates (4 × 10^5 cells/well) 1 day prior to the assay. Cells were then washed with PBS and incubated in Dulbecco’s modified Eagle’s medium F-12 with the indicated concentration of coumarins for a specific time period. Cells were rinsed once with PBS and treated with 1 μM PAF for the appropriate time in medium containing 0.2% BSA. Cells were then washed once with PBS containing 2% BSA and harvested in ice-cold PBS. Cells were first labeled with or without anti-Myc antibody on ice for 1 h. After washing twice with ice-cold PBS, cells were incubated for an additional 45 min with fluorescein isothiocyanate-conjugated goat anti-mouse Ab on ice and washed as described above. Antibody-labeled cells were analyzed for fluorescence intensity on a FACSScan flow cytometer (BD Biosciences) with dead cells excluded by gating on forward and side scatter.

Ligand Internalization—The ligand internalization kinetics had been evaluated in CHO cells stably transfected with the c-Myc-hPAFR-GyrB in 12-well plates (2 × 10^5 cells/well). After 24 h, cells were pretreated or not with coumermycin (15 μM for 10, 45, 80, 120, 180, and 210 min). Cells were then incubated at 37 °C with 2 nM [3H]hexadecyl-PAF in a buffer containing 150 mM choline chloride, 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 0.2% lipid-free BSA for 45 min. After the incubation period, cells were washed twice with 1 ml of the same buffer but containing 2% BSA. Cells were then lysed in 0.1 N NaOH, and internalized radioactivity was measured by liquid scintillation.

Confocal Microscopy—COS-7 cells were grown on 25-mm coverslips and transiently transfected with pcDNA3-c-Myc-hPAFR-GyrB and green fluorescent protein (GFP)-conjugated arrestin 2 or arrestin 3 (kind gifts from Dr. J. Benovic, Philadelphia) using FuGENE 6 and processed as described previously (35). Cells were incubated at 37 °C in the presence or absence of coumermycin or novobiocin (15 μM, 180 min) and then treated or not with 1 μM PAF for 80 min. Cells were fixed with 4% paraformaldehyde (15 min at room temperature) and permeabilized in 0.1% saponin. Cells were then incubated with anti-Myc Ab followed by rhodamine-conjugated goat anti-mouse Ab. Cells were examined, as described (35), with a scanning confocal microscope (NORAN Instruments, Inc., Middleton, WI) equipped with a krypton/argon laser and coupled to an inverted microscope with a 40 × oil immersion objective (Nikon). Optical sections were collected at 1-μm intervals with a 10-μm pinhole aperture. Digitized images were obtained with 256 × line
averaging and enhanced with Intervision software (NORAN Instruments, Inc.) on a Silicon Graphics O2-work station.

**Inositol Phosphate (IP) Determination**—Stably transfected CHO cells expressing c-Myc-hPAFR-GyrB were plated on 6-well dishes (4 × 10⁵ cells/well) and incubated at 37 °C in complete medium 12 h before the assay. Cells were then washed once in PBS and labeled for 18–24 h with myo-[³H]inositol at 3 μCi/ml in Dulbecco’s modified Eagle’s medium (high glucose, without inositol). After labeling, cells were washed once in PBS, preincubated at 37 °C in prewarmed modified medium containing 20 mM LiCl for 10 min. Medium was then removed, and cells were pretreated, or not, with 15 μM coumermycin or 15 μM novobiocin for the indicated periods of time in modified medium containing 20 mM LiCl. Cells were washed with PBS and incubated in prewarmed medium containing 2.2% BSA and 20 mM LiCl for 10 min with 1 μM PAF. The reactions were terminated with the addition of perchloric acid followed by a 30-min incubation on ice. IPs were extracted as described previously (30) and separated on Dowex AG 1-X8 columns. Total labeled IPs were then counted by liquid scintillation.

**Results**

**Dimerization of hPAFR**—To study whether hPAFR can form dimers, albeit under conditions of overexpression, we used COS-7 cells transiently expressing c-Myc epitope-tagged hPAFR. Immunoblotting of c-Myc epitope-tagged receptor with rabbit anti-c-Myc antibody consistently revealed the presence of molecular species corresponding to the expected positions of the monomeric receptor (38–45 kDa). Forms corresponding to molecular species corresponding to the expected positions of the monomeric receptor (38–45 kDa) were also observed, the monomeric receptor (38–45 kDa). Forms corresponding to molecular species corresponding to the expected positions of the monomeric receptor (38–45 kDa). The identity of the dimer was confirmed by immunoprecipitation experiments using differentially tagged receptors. In cells coexpressing both HA- and c-Myc-tagged hPAFR, blotting of the anti-c-Myc immunoprecipitate with the anti-HA antibody revealed the presence of hPAFR, indicating that dimerization occurred between the two different epitope-tagged receptors (Fig. 1B, lane 1). The specificity of the antibodies is illustrated by absence of cross-reactivity in cells expressing only one tagged receptor species (Fig. 1B, lane 2).

**Characterization of the hPAFR-GyrB**—To study the effect of artificially induced dimerization of hPAFR on cells, we constructed a chimeric cDNA encoding hPAFR and GyrB as a fusion protein (hPAFR-GyrB) and generated a stable c-Myc-hPAFR-GyrB-expressing CHO cell line. Coumermycin acts as a natural dimerizer of GyrB because it binds GyrB with a stoichiometry of 1:2; whereas a related coumarin antibiotic, novobiocin, binds GyrB as a 1:1 complex and thus serves as a nondimerizing control (36). We next determined the effect of fusing GyrB on hPAFR properties by comparing CHO cells stably expressing the c-Myc-wild-type hPAFR with CHO cells stably transfected with c-Myc-hPAFR-GyrB. Flow cytometry studies showed that wild-type hPAFR and hPAFR-GyrB had similar cell surface expression levels (Fig. 2, A and B), indicating that GyrB had no significant effect on cellular trafficking and distribution of the receptor. Binding characteristics were determined using the PAF receptor antagonist, [³H]WEB2086, by competition with WEB2086. Competition binding experiments showed that the affinity of WEB2086 for hPAFR-GyrB was the same as for the wild-type receptor (Fig. 2C). To investigate the signaling capacity of the chimeric receptor, we examined the ability of hPAFR-GyrB to mediate the stimulation of phosphatidylinositol hydrolysis. Agonist-independent basal IP production and agonist-induced increased IP production were very similar in both c-Myc-hPAFR and c-Myc-hPAFR-GyrB transfected cells (Fig. 2D). Thus, the addition of the GyrB moiety did not lead to detectable changes in hPAFR coupling.

**Coumermycin Treatment Induces the Dimerization of hPAFR-GyrB**—To verify that coumermycin modulates dimerization of hPAFR-GyrB as predicted by the system, CHO cells stably expressing c-Myc-hPAFR-GyrB were treated with coumarins for the indicated periods of time with cells and cells were lysed by sonication. Lysate preparation were immunoblotted with the anti-c-Myc polyclonal A-14 antibody after a 6% Tris-glycine gel electrophoresis under nonreducing conditions. The blot clearly shows that the relative abundance of dimer is increased by coumermycin. B, densitometric analysis of two experiments similar to that shown in A. The relative intensity of the dimer is expressed as a dimer:monomer ratio.
mers as well as dimers of hPAFR-GyrB. Exposure of cells to coumermycin induced a rapid increase in the relative abundance of the dimeric form, up to a 5-fold increase of the dimer to monomer ratio after a 60-min treatment (Fig. 3B). As expected, no change was observed after addition of the monomeric coumarin antibiotic novobiocin.

**Ligand-independent Internalization of hPAFR-GyrB Mediated by Coumermycin-induced Dimerization**—Internalization of receptors in response to coumermycin was assayed by flow cytometry, measuring the level of depletion of epitope-tagged cell surface receptors. Internalization was defined as the fraction of total cell surface receptors that, after exposure to agonist, were removed from the plasma membrane and thus not accessible to antibodies. CHO cells stably expressing the c-Myc-tagged-hPAFR or -hPAFR-GyrB receptors were first pretreated with dimethyl sulfoxide (vehicle) for 100 min at 37 °C and exposed to PAF for 0–80 min. After an 80-min stimulation with the agonist, cell surface expression of hPAFR-GyrB was assayed by flow cytometry using anti-c-Myc antibody. The data represent means ± S.E. of three independent experiments each performed in triplicate.

**Fig. 4.** Flow cytometric analysis of coumermycin-induced sequestration of hPAFR-GyrB in CHO cells. Time course of agonist-induced internalization of c-Myc-tagged-hPAFR-GyrB (A) or wild-type hPAFR (B) receptors in stably transfected CHO cells. After a 100 min-incubation at 37 °C with or without the indicated coumarin (15 μM), cells were exposed to PAF for 0–80 min. C, agonist response of hPAFR-GyrB-transfected cells to graded concentrations of coumermycin. Cells were pretreated at 37 °C for 100 min with coumermycin concentrations ranging from 150 nM to 15 μM and then incubated with PAF at 37 °C for 80 min. DMSO, dimethyl sulfoxide. D, cells were exposed to coumermycin for the times indicated, and cell-surface hPAFR-GyrB receptor expression levels were measured by flow cytometry using anti-c-Myc antibody. The data represent means ± S.E. of three independent experiments each performed in triplicate.
bly expressing c-Myc-hPAFR-GyrB were subjected to 15 μM coumermycin alone in the absence of agonist. As shown in Fig. 4D, a significant decrease in the level of cell surface receptors by 30 ± 1% was observed after the coumermycin treatment. The agonist-independent and coumermycin-mediated internalization was a very slow process that required more than 100 min compared with agonist-mediated sequestration, which was observed already at 20 min poststimulation (Fig. 4A). This indicates that coumermycin may activate an alternative endo-
cytic mechanism(s) in addition to agonist-induced, clathrin-de-
independent endocytosis.

**Coumermycin-mediated Sequestration of the hPAFR-GyrB in CHO Cells**—To demonstrate directly the effect of coumermy-
cin-induced dimerization of hPAFR-GyrB on the internalization process, binding experiments using [3H]PAF were carried out. CHO cells stably expressing c-Myc-hPAFR-GyrB were thus exposed to coumermycin, for various times, at 37 °C, prior to the addition of 2 nM [3H]PAF for 40 min. Cells were then washed with 2% BSA to remove surface-bound [3H]PAF and lysed to allow the detection of internalized ligand. As shown in Fig. 5, coumermycin induced a time-dependent decrease in [3H]PAF uptake with a nadir of 61 ± 1% of the values obtained for untreated control cells, at 180 min of treatment. These results strongly suggest that the coumermycin-induced disappearance of cell surface receptors (Fig. 4A) is responsible for the loss of ligand binding sites in c-Myc-hPAFR-GyrB-expressing cells.

**Mechanisms of hPAFR-GyrB Internalization**—It is well es-
tablished that nonvisual arrestins bind to agonist-activated GPCRs and serve as adaptor proteins during internalization of GPCRs through clathrin-coated pits. Arrestin (arr)2-GFP has been demonstrated to redistribute from the cytosol to the plasma membrane upon receptor stimulation (37) and colocalize with selective internalized GPCRs (38, 39). To evaluate the arrestin dependence of the coumermycin-mediated sequestration pathway, c-Myc-hPAFR-GyrB was cotransfected with the arr2-GFP or arr3-GFP fusion protein in COS-7 cells, and sub-
cellular distribution was analyzed by confocal microscopy. In unstimulated cells, hPAFR-GyrB was found predominantly on the cellular membrane, and arr2-GFP was distributed evenly throughout the cytoplasm (data not shown). After an 80-min stimulation with PAF, a punctate pattern of arrestin fluores-
cence (green) at the plasma membrane was observed, in prox-
imity to the receptor (red). Extensive colocalization (yellow) of

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**Fig. 5. Effect of coumermycin on the internalization of hPAFR-GyrB-ligand.** After an incubation at 37 °C in the presence ([■]) or absence ([□]) of coumermycin for the times shown, CHO cells stably expressing c-Myc-hPAFR-GyrB were further exposed to 2 nM [3H]PAF for 40 min at 37 °C. Nonspecific binding values were deter-
mined in the presence of 2 μM PAF. The results are the mean of three independents experiments, each done in triplicate.

**Fig. 6. Distribution of arr2-GFP and hPAFR-GyrB after stimulation with coumermycin and/or agonist.** Confocal microscopy visualization of in-
tracellular distribution and colocalization (yellow) of arr2-GFP (green) and c-Myc epitope-tagged hPAFR-GyrB (red), la-
belled with rhodamine-conjugated Ab in COS-7 cells. After a 180-min incubation at 37 °C in the presence (B and C) or absence (A) of 15 μM coumermycin, COS-7 cells were incubated further with (A and B) or without (C) 1 μM PAF for 80 min. The images are representative of three separate experiments.
hPAFR-GyrB and arr2-GFP fluorescence was also observed in endocytic vesicles (Fig. 6A). We next evaluated the internalization behavior of the receptor under PAF stimulation after coumermycin treatment. Under these conditions, arr2-GFP redistributed from a homogeneous cytosolic localization to a membrane-associated pattern, and hPAFR-GyrB appeared in endocytic vesicles distributed randomly throughout the cytoplasm (Fig. 6B). The overall distribution pattern of arr2-GFP appeared similar to the agonist-mediated internalization of hPAFR-GyrB observed above. In contrast, arr2-GFP fluorescence did not colocalize with hPAFR-GyrB immunofluorescence emanating from endocytic vesicles which were observed after the coumermycin treatment (Fig. 6C). Arrestins remained exclusively in the cytoplasm, suggesting an arrestin-independent internalization pathway. Novobiocin also failed to induce any change in the cellular distribution of hPAFR-GyrB and arr2-GFP, which remained on the cell surface and cytoplasm, respectively (data not shown). Similar results were found using arr3-GFP (data not illustrated). NH4Cl pretreatment did not inhibit the coumermycin-induced internalization, indicating that this process is also independent of clathrin-coated pits (data not shown).

Functional Characterization of the Coumermycin-induced Internalization of hPAFR-GyrB in CHO Cells—To determine whether the coumermycin-mediated internalization of hPAFR-GyrB was associated with receptor signaling, phospholipase C activation was assessed in intact, stably transfected CHO cells by measuring IP accumulation. As shown in Fig. 7, no IPs were produced by hPAFR-GyrB-transfected cells over the entire duration of stimulation with coumermycin. In contrast, however, a further 10-min incubation with PAF at 40, 100, or 120 min did result in significant IP accumulation, showing that coumermycin treatment did not impair a functional response of the receptor to its ligand. These data suggested that phospholipase C activation does not participate in the coumermycin-induced internalization of hPAFR-GyrB and that coumermycin stimulates the internalization of a nonsignaling subset of receptors, in the absence of ligand.

Agonist-independent and PKC-dependent Desensitization of the hPAFR-GyrB—To examine the possible role of coumermycin-driven dimerization in the desensitization process, CHO cells stably expressing the c-Myc-epitope-tagged hPAFR or hPAFR-GyrB receptors were treated with coumarins for the indicated times, and the number of antagonist binding sites on the cell surface was measured using a saturating concentration of the membrane-impermeant ligand [3H]WEB2086. Surprisingly, exposure to coumermycin alone initiated a rapid, time-dependent loss of receptor binding sites which reached 37 ± 2% of controls (Fig. 8A). The phenomenon occurred within 5 min after stimulation and reached a plateau after 1 h. The coumermycin-induced desensitization of hPAFR-GyrB, which is independent of the agonist, did not prevent the further loss of binding sites induced by PAF, the two effects being additive (data not shown). In addition to agonist-specific receptor desensitization, functions of GPCRs can be regulated by second messenger-dependent protein kinases, leading to heterologous desensitization (14). To investigate further the molecular mechanism mediating the coumermycin-induced ligand-independent desensitization of hPAFR-GyrB, we determined whether the effect of coumermycin occurred through activation of PKC. We preincubated CHO cells stably expressing c-Myc-hPAFR-GyrB in the presence or absence of the PKC inhibitors staurosporine and GF109203X prior to a 40-min incubation with coumermycin at 37 °C (Fig. 8B). Under these conditions, staurosporine and GF109203X inhibited the coumermycin-induced loss of [3H]WEB2086 binding sites by 75 and 50%, respectively. On the other hand, preincubation with the PKC activator PMA or the tyrosine kinase inhibitor tyrphostin 51 had no significant effect on desensitization of hPAFR-GyrB. Exogenous PKC activation by PMA did, however, significantly increase multimerization of hPAFR-GyrB after 60 min of incubation (Fig. 8, C and D). These data suggest that PKC plays a role in coumermycin-mediated desensitization of hPAFR-GyrB, possibly by enhancing receptor multimerization.

In this study, we found that hPAFR can exist as multimers in transfected COS-7 cells using coexpression and immunoprecipitation of receptors bearing different epitope tags. Receptor dimerization has been shown to be important for the activities of many receptor families, including growth factor receptors with tyrosine kinase activity, bacterial sensory receptors, and GPCRs such as the growth hormone receptor family and cytokine receptors (40, 41). In the receptor kinase family, exposure to ligand induces receptor dimerization, leading to autophosphorylation, which is necessary for the subsequent intracellular signaling. However, the role of dimerization in GPCR function and subsequent regulatory events is still not well understood.

Coumermycin-induced Internalization of hPAFR-GyrB—Receptor dimerization has been linked to GPCR sequestration in a number of instances. Thus, the coexpression of an endocytosis-defective mutant of the yeast α-factor receptor with the wild-type receptor leads to efficient endocytosis of the mutant receptor, indicating that these receptors are internalized as dimers/oligomers (42). Dimerization, however, is not necessarily associated with internalization in all GPCRs. In the case of the δ-opioid receptor, it was shown that the reduction in the level of dimers was faster than internalization, suggesting that monomerization precedes internalization (25). Moreover, dimerization of the m3 receptor and the calcium-sensing receptor has been shown to be agonist-insensitive (43, 44), indicating that agonist stimulation neither promotes nor destabilizes dimer formation. It thus remains to be clarified whether the presence of receptor dimers or oligomers in the absence of ligand is sufficient to drive internalization, or whether this process requires ligand-occupied receptors. Here we investigated this question for the hPAFR, using the coumermycin-GyrB dimerization strategy. As for other GPCRs, we quantified cell surface epitopes by flow cytometry, which is a nonradioactive method of monitoring receptor sequestration (45–47). We found that the pattern and overall level of expression as well as agonist-mediated internalization of hPAFR-GyrB were almost indistinguishable from the wild-type receptor, indicating that
the pharmacological properties of the receptor were not affected by the GyrB. We first demonstrated that coumermycin pretreatment substantially increases the rate and extent of agonist-mediated internalization of c-Myc-tagged hPAFR-GyrB by flow cytometry. We also found that exposure to coumermycin alone results in a concentration-dependent internalization of hPAFR-GyrB, indicating that this process is dimerization-dependent. Similarly, binding experiments using [3H]PAF demonstrated a time-dependent coumermycin-driven attenuation in radioligand uptake, resulting from the disappearance of cell surface receptors. Because novobiocin or dimethyl sulfoxide did not induce any change in the surface expression level of receptors, these results suggest that by inducing dimerization of hPAFR-GyrB, coumermycin specifically induced a PAF-independent conformational change leading to the sequestration process. These data thus demonstrate that dimerization of hPAFR-GyrB is sufficient to induce its sequestration.

Using a membrane-impermeant antagonist radioligand to estimate changes in the binding sites on the cell surface, we then observed that coumermycin induced a rapid loss of [3H]WEB2086 binding sites in CHO cells stably expressing the hPAFR-GyrB. Coumermycin-mediated dimerization of hPAFR-GyrB might thus induce a conformational change of the receptor which leads initially to a loss of ligand binding capacity and later to a disappearance of the unoccupied hPAFR-GyrB from the cell surface. Coumermycin-induced internalization was not preceded by receptor activation because coumermycin treatment did not increase phosphoinositide hydrolysis, to which the hPAFR is coupled via the G protein Gαq. Both the rapid desensitization and the long term sequestration may explain the reduced intracellular accumulation of receptor-bound [3H]PAF in coumermycin-pre treated cells compared with cells treated only with agonist.

**Dimerization-induced Heterologous Desensitization**—Previous results suggest that GPCR kinases are the primary protein
kinases involved in agonist-induced phosphorylation of hPAFR, but it has also been shown that desensitization of the PAFR is accompanied by PKC-dependent phosphorylation as well (10). Here, we found that the two PKC inhibitors, staurosporine and GF109203X, greatly attenuated the agonist-independent binding site-depleting actions of coumermycin, whereas the tyrosine kinase inhibitor tyrphostin 51 had no effect. In contrast to receptor phosphorylation by GPCR kinases, phosphorylation by second messenger-activated kinases does not require receptor occupancy by agonist. This kind of desensitization, termed heterologous desensitization, has been implicated in the regulation of a number of GPCRs (48, 49).

Dimerization-induced β-Arrestin-and Clathrin-independent Internalization of hPAFR-GyrB—β-Arrestins have been shown to be involved in the internalization and signaling of many GPCRs (14). After agonist-promoted recruitment to the plasma membrane, they serve as clathrin adaptors, which help to target agonist-occupied GPCRs to clathrin-coated pits for internalization. Indeed, agonist activation of overexpressed wild-type hPAFR receptors has been shown to trigger the redistribution of arrestins from intracellular pools to the plasma membrane where they colocalize with internalized receptors to discrete intracytoplasmic vesicles (11). To characterize whether coumermycin-driven hPAFR-GyrB endocytosis follows the same pathway as agonist-induced internalization, we investigated the cellular trafficking of GFP-conjugated arrestin-2 in COS-7 cells. However, activation with coumermycin failed to induce a translocation of arrestins from the cytosol to the plasma membrane, suggesting a mechanism distinct from PAF-induced hPAFR-GyrB endocytosis. Recent studies have suggested that in addition to the clathrin-mediated sequestration pathway utilized by a large number of GPCRs, alternative mechanisms of GPCR internalization exist (13). Indeed, m2 muscarinic and angiotensin 1A receptors have been shown to internalize in an arrestin- and clathrin-independent manner via an unknown machinery (19, 21). The potential involvement of phospholipase C activation in the internalization process was considered after the report that the AP-2 complex could bind to phosphatidylinositol 4,5-bisphosphate (53) and inositol 1,4,5-trisphosphate (54). However, the agonist-independent sequestration induced by coumermycin did not induce accumulation of IP, suggesting that phospholipase C activation does not participate in this alternative sequestration pathway. Considering that some PKC isoforms and GPCRs can associate with caveolae (55), it is conceivable that the coumermycin-driven internalization of hPAFR-GyrB occurred through this mechanism, as observed for the bradykinin 2 receptor (56). Alternatively, a novel mechanism of GPCR sequestration would have to be postulated.

In conclusion, we have shown that coumermycin-induced dimerization of hPAFR-GyrB is sufficient to induce the desensitization/internalization of hPAFR-GyrB via a mechanism that is independent of arrestins, clathrin, or phospholipase C activation, but requires PKC activity. These data represent the first demonstration that dimerization of a GPCR can induce receptor desensitization and internalization without agonist stimulation. They may, therefore, provide insight into a novel mechanism governing cellular responsiveness to stimuli targeting GPCRs. If agonist-independent GPCR dimerization occurs in vivo, it is conceivable that potency and efficacy of an agonist of a given receptor may be shifted through dimerization-dependent desensitization, providing as well as concerns for drug development.

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Agonist-independent Desensitization and Internalization of the Human Platelet-activating Factor Receptor by Coumermycin-Gyrase B-induced Dimerization
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