Agonist-induced sequestration, recycling, and resensitization of platelet-activating factor receptor

ROLE OF CYTOPLASMIC TAIL PHOSPHORYLATION IN EACH PROCESS

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Agonist-induced sequestration, recycling, and resensitization of platelet-activating factor (PAF) receptor were characterized in transfected Chinese hamster ovary cells. Exposure of the cells to PAF led to rapid sequestration of the receptors into the intracellular compartment and desensitization of the response to PAF. The sequestration was inhibited by pretreatments that perturbed the clathrin-mediated pathway. Subsequent removal of PAF by washing with receptor antagonists led to rapid recycling of the sequestered receptors to the cell surface accompanied by resensitization to PAF. To evaluate the potential role of phosphorylation in the receptor cytoplasmic tail during these processes, mutant receptors in which the tails were truncated or substituted, so as to lack serine/threonine residues, were created. PAF phosphorylated the wild-type receptor rapidly and strongly, but the mutants did not. The maximal extent of sequestration of each mutant was lower than that of the wild-type, and one of the substituted mutants showed no sequestration. Furthermore, the sequestration-defective mutant showed evidence of desensitization after agonist stimulation but not resensitization after agonist removal. Thus, agonist-induced phosphorylation of the cytoplasmic tail facilitates but is not essential for receptor sequestration, and sequestration/recycling appears important in receptor resensitization.

Exposure of G protein-coupled receptors (GPCRs)1 to their agonists is generally followed by a rapid desensitization of signaling. The mechanism of the desensitization involves a series of distinct steps including a functional uncoupling from G proteins and their effector systems, receptor sequestration into the intracellular compartment, and a net loss of receptors (receptor down-regulation). Among these steps, the receptor sequestration and following processes are considered to play an important role both in desensitization and resensitization of GPCRs (1–3).

The molecular mechanisms of GPCR sequestration have been extensively studied using a β2-adrenergic receptor (β2AR) as the main model (1–6). Functional uncoupling of the β2AR from Gs proteins is a consequence of agonist-induced phosphorylation of the receptor cytoplasmic tail by the following two types of protein kinases: cAMP-dependent protein kinase and G protein-coupled receptor kinases (GRKs) (7). Recent studies have shown that this phosphorylation promotes binding of β-arrestins to the β2AR and that β-arrestins subsequently act as adapters of clathrin, a major structural protein of coated pits, which suggests that β2AR sequestration is mediated by a physical interaction between receptor-associated β-arrestins and clathrin-coated pits (8–13). Despite the large amount of information concerning β2AR sequestration, much less is known of sequestration of other types of GPCRs, and available information is only fragmentary.

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent phospholipid mediator involved in the pathogenesis of allergic disorders, inflammation, anaphylactic shock, and various other physiological events (14, 15). PAF drives multiple signaling pathways through a single type of GPCR, PAF receptor (PAFR) (15). Repetitive or long lasting application of PAF leads to a rapid functional uncoupling of the PAFR from effector systems (16–18). We and other groups (18–22) suggested that agonist-induced phosphorylation of the PAFR by GRKs may be involved in the rapid uncoupling process. However, the information available on the subsequent sequestration process is limited (23, 24), and the fate of the sequestered PAFR has remained unknown. Furthermore, the potential role of receptor phosphorylation in the sequestration remains to be elucidated.

We expressed epitope-tagged guinea pig PAFRs with cytoplasmic tail mutations in Chinese hamster ovary (CHO) cells. Immunohistochemistry with monoclonal antibodies directed against the epitopes enabled us to quantitate cell surface expression of the PAFR, to visualize PAFR movement, and to immunoprecipitate PAFR proteins for receptor phosphorylation assays. We gained novel information on PAFR dynamics during agonist-induced sequestration, recycling, and resensitization. The role of phosphorylation of the receptor cytoplasmic tail in each process was also evaluated using the mutant receptors.

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1 The abbreviations used are: GPCR, G protein-coupled receptor; PAF, platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); β2AR, β2-adrenergic receptor; GRK, G protein-coupled receptor kinase; PAFR, PAF receptor; CHO, Chinese hamster ovary; mCPAF, 1-O-hexadecyl-2-(methylcarbamyl)-sn-glycero-3-phosphocholine; WT, wild-type; PBS, fetal bovine serum; IP, inositol 1,4,5-triphosphate; Ca2+; Mg2+-free Dulbecco’s phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; Bt2-cAMP, dibutyryl cAMP; HA, hemagglutinin.
EXPERIMENTAL PROCEDURES

Materials—[acetyl-3H]PAF C16 (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) (370 GBq/mmol), [3H]WEB 2086 (499.5 GBq/mmol), [3H]arachidonic acid (3.7 TBq/mmol), [35S]labeled sheep anti-mouse IgG Fab' fragment (300 kBq/µg), [3H]orthophosphate (330 TBq/mmol), and [3S]EXPRESS Methionine/Cysteine Protein Labeling Mix (37 TBq/mmol) were purchased from NEN Life Science Products. [3H]Folic acid (1.0 TBq/mmol) was from Amersham Pharmacia Biotech. PAF C16, 1-O-hexadecyl-2-(methylcarbamy1)-sn-glycero-3-phosphocholine (mcPAF), anti-FLAG M5 monoclonal antibody, and anti-HA 12CA5 monoclonal antibody were obtained from Cascade Biochem (Reading, UK), Cayman Chemical (Ann Arbor, MI), Kodak, and Boehringer Mannheim, respectively. PAFR antagonists, WEB 2086 and TCV-309, were generous gifts from Boehringer Ingelheim and Takeda (Osaka, Japan), respectively. Other reagents, unless otherwise stated, were of analytical grade and were from Wako (Osaka) or Sigma.

Plasmid Construction—All mutations were introduced into a pBluescript SK(-) vector (Stratagene) containing the entire coding region of guinea pig PAFR cDNA (25) using a Transformer Site-directed Mutagenesis Kit (CLONTECH). For construction of epitope-tagged receptors, nucleotide sequences that encoded 8- or 9-amino acid peptides (DYKDDDDK for FLAG and YPYDVPDYA for HA) were inserted between the amino-terminal initiator methionine and the second amino acid of the wild-type (WT) or mutant PAFR sequences by polymerase chain reaction using Ffu DNA polymerase (Stratagene), as described elsewhere (20, 26). The coding region of each construct was sequenced on both strands, using an ABI 373 DNA Sequencer (Perkin-Elmer) and was subcloned into a mammalian expression vector, pcDNA I Neo (Invitrogen) for stable expression in CHO cells.

Construction of Stable CHO Transformsants—CHO cells were maintained as monolayer cultures in Ham’s F12 medium (Nissui Pharmaceutical; Tokyo) supplemented with 10% fetal bovine serum (FBS) (Upstate Biotechnology, Lake Placid, NY). The cells plated on 12-well dishes were transfected with plasmid constructs using TRANSFECTAM (BioSseptra, Marlborough, MA) according to the manufacturer’s instructions. Cells resistant to 1 mg/ml G418 (Life Technologies, Inc.) were collected after 2 weeks of culture, cloned by limiting dilution, and screened for specific [3H]WEB 2086-binding activity (data not shown). The clones used in this study were selected based on PAFR expression level and were maintained in the presence of 0.3 mg/ml Geneticin.

Radioligand Binding Assay—CHO cells were seeded onto 12-well dishes at 48 h prior to assay. The cells were washed twice with Tyrode’s buffer (26) containing 10 mM Hepes (pH 7.4) and 10% fetal bovine serum (FBS) (Upstate Biotechnology, Lake Placid, NY). The cells plated on 12-well dishes were transfected with plasmid constructs using TRANSFECTAM (BioSseptra, Marlborough, MA) according to the manufacturer’s instructions. Cells resistant to 1 mg/ml G418 (Life Technologies, Inc.) were collected after 2 weeks of culture, cloned by limiting dilution, and screened for specific [3H]WEB 2086-binding activity (data not shown). The clones used in this study were selected based on PAFR expression level and were maintained in the presence of 0.3 mg/ml Geneticin.

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Analysis of Receptor Sequestration—Quantification of cell surface expression of the PAFR was carried out as described by Gerard and Gerard (23), with some modifications. CHO cells were seeded onto CulturePlate 24 (Packard Instrument Co.) at 48 h prior to assay. The cells were washed twice with Hepes/Tyrode’s/BSA buffer, subjected to various treatments at 37 °C for the indicated time, and then fixed with 2% freshly prepared paraformaldehyde in Ca2+–Mg2+–free Dulbecco’s phosphate-buffered saline (PBS) (Nissui) for 10 min. After washing twice with PBS, the cells were incubated for 1 h in Tyrode’s buffer containing 10 mM Hepes (pH 7.4) and 20% (v/v) FBS (Hepes/Tyrode’s/BSA buffer) to reduce nonspecific binding, and for 30 min with or without 10 µg/ml anti-FLAG M5 or 5 µg/ml anti-HA 12CA5 antibody in the same buffer. After washing twice with PBS, the cells were incubated for 30 min with Hepes/Tyrode’s/BSA buffer and for 30 min with 33.3 kBq of [35S]labeled sheep anti-mouse IgG Fab’ fragment in the same buffer. The cells were then washed three times with PBS, dissolved in MicroScint 20 scintillator (Packard), and the radioactivity was counted in a TopCount Microplate Scintillation Counter (Packard). All procedures after fixing were carried out at room temperature. Antibody-dependent binding was used to show cell surface expression of the PAFR.

Immunofluorescence Microscopy—Microscopy was done basically as described by Trapaidze et al. (27). CHO cells were grown for 48 h in hand-prepared folic acid-free Dulbecco’s modified Eagle’s medium containing 5% FBS. Next, the cells were subjected to various treatments in folic acid-free Dulbecco’s modified Eagle’s medium to inhibit the clathrin- or caveolae-mediated pathways involving these treatment cells, the cell surface was washed twice with 10 µg/ml anti-FLAG antibody in PBS, washed three times with TBS, and incubated for 30 min with 5 µg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Life Technologies, Inc.) in TBS. Then, they were washed three times with TBS and mounted with SlowFade antifade reagents (Molecular Probes). The cells were viewed under a Nikon Diaphot TMD300 fluorescence microscope with a NCF Fluor 100 × oil-immersion objective lens (Ni-kon, Japan).

Measurement of Folic Acid Incorporation—Folic acid incorporation was measured as described (28, 29) but with some modifications. Cells were grown in 12-well dishes for 48 h in hand-prepared folic acid-free Dulbecco’s modified Eagle’s medium containing 5% FBS. Next, the cells were subjected to various treatments in folic acid-free Dulbecco’s modified Eagle’s medium to inhibit the clathrin- or caveolae-mediated pathways involving these treatment cells, the cell surface was washed twice with ice-cold Hepes/Tyrode’s/BSA buffer, chilled on ice for 20 min, and washed twice with ice-cold saline (150 mM NaCl adjusted to pH 3.0 with glacial acetic acid) to remove radioactive folic acid bound to the intrinsic cell surface folic acid receptors. The cells were then solubilized in 1% Triton X-100 and subjected to scintillation counting.

Inhibition of the Clathrin-mediated Pathway—CHO cells were subjected to hypertonic shock with 0.45 x sucrose, potassium depletion, and acidification with Dulbecco’s modified Eagle’s medium containing 10 mM acetic acid (pH 5.0), as described in detail elsewhere (30), followed by analysis of PAFR sequestration and folic acid incorporation.

Measurement of Protein Synthesis—CHO-FLAG-WT cells were seeded onto 6-well dishes at 48 h prior to assay. The cells were washed twice with methionine/cystine-free Dulbecco’s modified Eagle’s medium (Sigma) and incubated for 2 h in the same medium containing high concentrations of cycloheximide. The cells were then incubated for 90 min in medium containing each concentration of cycloheximide and 3.7 MBq of [35S]EXPRESS Methionine/Cysteine Protein Labeling Mix. Next, the cells were washed twice with ice-cold PBS, scraped into Eppendorf tubes with PBS, and precipitated in 10% (w/v) trichloroacetic acid for 20 min on ice. The samples were then washed with liquid scintillation counting. The specific binding activity (total binding minus nonspecific binding) was determined for Scatchard plot analysis, and ligand-binding parameters, Kd (nM) and Bmax (fmol/well) were calculated. A Bmax value of 100 fmol/well was roughly equal to 100,000 binding sites per single CHO cell (data not shown).

Measurement of Arachidonic Acid Release and IP3 Production—CHO cells prelabeled with [3H]arachidonic acid were subjected to release assay, as described (26). Quantitation of inositol 1,4,5-triphosphate (IP3) production was performed as described (26), using an IP3 radioreceptor assay kit (NEN Life Science Products).

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Further sequestration (Fig. 2C). Immunofluorescence microscopy revealed bright staining of the receptors all over the cell surface, including numerous punctuate patterns (Fig. 2D, left). Following 60 min of incubation with 100 nM PAF, the fluorescence intensity of the cell surface was markedly reduced, and small areas of bright staining emerged in some intracellular compartments (Fig. 2D, middle).

**Effects of Pretreatments to Inhibit the Clathrin- or Caveolae-mediated Pathways on Sequestration**—To explore the mechanism of PAFR sequestration, CHO-FLAG-WT cells were subjected to pretreatments that inhibited the clathrin-mediated pathway such as hypertonic sucrose shock, potassium depletion, and acidification (30), followed by the sequestration assay (Table I). In parallel with these experiments, the pretreated cells were also assayed for folic acid incorporation, an event mediated by caveolae but not by clathrin (32). Folic acid incorporation was concentration-dependent up to 100 nM [3H]folic acid and was time-dependent within 4 h of incubation (data not shown), so incorporation was measured during 90 min of incubation with 50 nM [3H]folic acid.

Hypertonic shock with 0.45 M sucrose prevented about half of PAF-induced PAFR sequestration but had no effect on folic acid incorporation. Potassium depletion caused almost complete inhibition (97.5%) of sequestration but slightly inhibited folic acid incorporation (only 12.5%). Acidification inhibited sequestration by 90% but had no obvious effect on folic acid incorporation. In addition, effect of 100 nM phorbol 12-myristate 13-acetate (PMA) on both activities was examined, because protein kinase C activators such as PMA reduced the number of caveolae and thus inhibited folic acid incorporation (33, 34). Stimulation with PMA resulted in 35.6% loss of folate acid incorporation. Potassium depletion caused almost complete inhibition of sequestration but had no obvious effect on folic acid incorporation. No treatment caused marked interference with PAF binding to its receptor and PAF-induced signal transduction (data not shown). These data suggest that PAF-induced receptor sequestration is mostly through clathrin-coated pits.

**Phosphorylation and Sequestration of the PAF Receptor**—Agonist-induced phosphorylation of the PAFR at 10 min was compared among four kinds of CHO transformants (CHO-HA-WT, CHO-HA-Del, CHO-HA-Tail-1, and CHO-HA-Tail-2) (Fig. 3A). Exposure of CHO-HA-WT cells to 100 nM PAF for 10 min resulted in phosphorylation of both 40–50- and 80–100-kDa proteins. Because we detected no corresponding bands in untransfected CHO cells or CHO-WT cells (data not shown), the immunoprecipitated radioactive proteins were presumed to be the HA-WT receptors. The 80–100-kDa bands may represent further sequestration (Fig. 2C). Immunofluorescence microscopy revealed bright staining of the receptors all over the cell surface, including numerous punctuate patterns (Fig. 2D, left). Following 60 min of incubation with 100 nM PAF, the fluorescence intensity of the cell surface was markedly reduced, and small areas of bright staining emerged in some intracellular compartments (Fig. 2D, middle).

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receptor dimers, as suggested by Ali et al. (20). PAF induced phosphorylation of both bands in a concentration-dependent manner; it was detectable in 10 min at a concentration of 0.1 nM and reached a maximal level at 1 nM (data not shown). Phosphorylation was rapid and lasting; it was detectable 15 s after 100 nM PAF stimulation, reached a maximal level within 2.5 min, and lasted for at least 15 min (data not shown). In any case, both 40–50- and 80–100-kDa bands were phosphorylated in CHO transformants for each receptor was analyzed with all FLAG-WT cells. PAF-induced sequestration of the PAF receptor and folic acid incorporation were measured at 90 min, and both activities were expressed as percentages relative to the control as 100.

| Treatment          | PAF-induced sequestration of PAF receptor (% control) | Folic acid incorporation (% control) |
|--------------------|-------------------------------------------------------|-------------------------------------|
| 0.45 M sucrose     | 50.9 ± 6.2                                            | 100.6 ± 3.7                         |
| Potassium depletion| 2.5 ± 5.3                                             | 87.5 ± 6.0                          |
| Acidification (pH 5.0) | 19.7 ± 15.4                                          | 114.6 ± 8.8                         |
| 100 nM PMA         | 96.7 ± 3.9                                            | 64.4 ± 0.6                          |

TABLE I
PAF-induced sequestration of the PAF receptor but not folic acid incorporation was suppressed by treatments inhibiting the clathrin-mediated pathway

CHO-FLAG-WT cells were subjected to hypertonic sucrose shock, potassium depletion, acidification, and 100 nM PMA treatment. PAF (100 nM)-induced sequestration of the PAF receptor and folic acid incorporation were measured at 90 min, and both activities were expressed as percentages relative to the control as 100.

PAF-induced phosphorylation of the guinea pig receptor (Fig. 2A) was about half that caused by PAF, whereas Bt$_2$-cAMP was much less effective than PAF even though its effect was obvious by radioactive counting and reproducible. PAF, PMA, and Bt$_2$-cAMP induced much less phosphorylation in the three cytoplasmic tail mutant receptors as in the HA-WT receptor. Thus, most of the phosphorylation targets in the PAF receptor for these different stimuli exist in the cytoplasmic tail Ser/Thr residues (especially the carboxyl-terminal six Ser/Thr residues).

These four transformants were also examined for receptor sequestration. PAF induced sequestration of HA-Del and HA-Tail2 receptors, although the extent of sequestration was much less than that of the HA-WT receptor (Fig. 2B). The HA-Tail-1 receptor did not show sequestration. Six different CHO transformants for each receptor were analyzed with almost identical results (data not shown). Furthermore, treatment of the cells with PMA or Bt$_2$-cAMP neither induced sequestration nor affected PAF-induced sequestration in CHO-FLAG-WT cells (Fig. 2C). Thus, PAF-induced phosphorylation of the receptors through enzymes other than protein kinase C or cAMP-dependent protein kinase, and it is related to receptor sequestration.

**PAF Receptor Recycling and Resensitization**—In some GPCRs, sequestered receptors return to the cell surface after agonist removal in the process called “recycling.” To observe PAF recycling, PAF (100 nM)-treated CHO-FLAG-WT cells were quickly washed twice with the Hepes/Tyrode’s/BSA buffer containing an excess (10 $\mu$M) of the hydrophobic PAFR antagonist WEB 2086 at 10, 60, or 90 min after PAF application, followed by incubation in the same buffer containing 10 $\mu$M WEB 2086 (Fig. 4A). The sequestered receptors after 60 or 90 min of incubation with 100 nM PAF began to recycle rapidly (<10 min) after agonist removal by antagonist washing, but receptors sequestered after 10 min of incubation needed a longer time (>50 min) to begin recycling. We obtained almost the same results using a highly hydrophilic PAFR antagonist, TCV-309 (Fig. 4B), which has a similar IC$_{50}$ value to that of WEB 2086, as seen in experiments on [3H]PAF replacement (data not shown). In immunofluorescence microscopy, the se-
Sequestration, Recycling, and Resensitization of PAF Receptor

Fig. 3. PAF-, PMA-, or Bt₂-cAMP-induced phosphorylation and sequestration of the PAF receptor in CHO transformants. A, phosphorylation of mutant PAF receptors in the four CHO transformants induced by 10 min of stimulation with 100 nM PAF, 100 nM PMA, or 1 nM Bt₂-cAMP. The numbers indicate the estimated molecular masses (kDa) on 10% SDS-polyacrylamide gel electrophoresis. B, time course for PAF (100 nM)-induced sequestration of the HA-tagged mutant PAF receptors. C, effect of PMA or dibutyryl cAMP on PAF concentration-dependent sequestration of the PAFR at 90 min in CHO-FLAG-WT cells. Data are representative of three independent experiments (A) or the mean of duplicate samples and representative of two independent experiments (B and C).

questered receptors were seen to return to the cell surface which included numerous punctuate patterns (Fig. 2D, right). Neither of the PAFR antagonists had any effect on receptor distribution in the absence of PAF (Fig. 4, A and B, open triangles). Such recycling was also observed in experiments with CHO-HA-Tail-2 cells (data not shown). In sequestration-defective CHO-HA-Tail-1 cells, agonist removal did not increase cell surface expression of the receptor (data not shown).

To examine the roles of sequestration and recycling in desensitization/resensitization to PAF, we carried out the following experiments. CHO-FLAG-WT cells were stimulated by 100 nM PAF for 1 h followed by washing and 1 h of incubation with buffer containing 10 μM TCV-309, and then were exposed again to 100 nM PAF. The recycled receptors regained the potential to undergo sequestration (Fig. 5A). We next examined PAFR desensitization and resensitization based on the IP₃ response to PAF. PAF-dependent IP₃ production was measured in CHO-FLAG-WT cells at each step indicated by the arrows in Fig. 5A (Fig. 5B). Incubation for 1 h with 100 nM PAF shifted the concentration-dependent curve to the right by about 2 orders of magnitude, thereby indicating desensitization. Subsequent incubation for 1 h with 10 μM TCV-309 led to a 1 order curve shift to the left, indicating restoration of the PAF-induced IP₃ response. We obtained similar results with CHO-HA-Tail-2 cells (Fig. 5D) but not with CHO-HA-Tail-1 cells (Fig. 5C). Sequestration-defective CHO-HA-Tail-1 cells displayed desensitization but not resensitization.

DISCUSSION

Recently, Le Gouill et al. (24) reported the agonist-induced sequestration of the human PAFR expressed in COS-7 and CHO cells. They measured the activity of [³H]PAF incorporation as an indicator of PAFR sequestration. In this present study, we used immunochemical approaches with antibodies to directly detect PAFR, and this action facilitated a quantitative analysis of the dynamic PAFR sequestration/recycling process. Since suitable anti-PAFR antibodies for this purpose were not available, FLAG or HA epitope was introduced into the amino-terminal extracellular domain of PAFR. Because CHO cells have endogenous proteins recognized by anti-HA antibody (data not shown), we utilized FLAG tagging for precise characterization of PAFR sequestration. No detectable endogenous proteins on the CHO cell surface were recognized by anti-FLAG antibody (data not shown). However, as anti-FLAG antibody did not efficiently immunoprecipitate the FLAG-tagged receptor in the presence of solubilizing detergents (0.5% Triton X-100 and 0.05% SDS), we used HA tagging in some experiments. The anti-HA 12CA5 antibody binds to its epitope with an extremely high affinity, and interactions were stable under the immunoprecipitation conditions we used (data not shown).

Sequestration and Recycling of PAF Receptors—In CHO-FLAG-WT cells, PAF induced PAFR sequestration in both a time-dependent and a concentration-dependent manner (Fig. 2, A and B). Thrombin, which activates CHO cells via endogenous GPCR, did not induce PAFR sequestration, and pretreatment with PAFR antagonists completely inhibited PAF sequestration (data not shown). Thus, this phenomenon is agonist-specific. Initiation of sequestration was rapid after agonist stimulation (<5 min). Fig. 2A shows no apparent lag period between agonist (PAF or mcPAF) addition and the onset of sequestration. The PAF concentration-dependent profile of sequestration over 90 min was similar to that observed in the assay of arachidonic acid release in 6 min (data not shown). Not all of the cell surface receptors were sequestered even after stimulation with a high concentration of PAF (Fig. 2B) or a second application of 100 nM PAF (Fig. 2C), and some (30–40% of the total) remained on the cell surface. PAF might be metabolized by PAF acetylhydrolases (35) after receptor-mediated incorporation (23, 24). However, such metabolism did not affect sequestration because mcPAF, a synthetic analog of PAF resistant to the PAF acetylhydrolases, had a time-dependent or a concentration-dependent effect similar to that of PAF (Fig. 2, A and B). The cell surface-remaining receptors could transmit signals in response to PAF, even though some desensitization was observed (Fig. 5B).

Initiation of recycling also began rapidly after agonist removal (Fig. 4, A and B). The rapid onset of recycling of the
FLAG-WT receptor may exclude the possibility that PAFR protein synthesis is accelerated after agonist removal. In agreement with this, recycling was not blocked by 2 h of preincubation with a high concentration of cycloheximide, which effectively inhibits protein synthesis (85, 94, and 97% inhibition at concentrations of 10, 30, and 60 μg/ml cycloheximide, respectively). It is important to note here that shut-down of the signals from cell surface PAFR was needed to trigger the recycling after receptor sequestration. Incubation with a hydrophobic PAFR antagonist (WEB 2086) or a hydrophilic antagonist (TCV-309) could induce recycling, suggesting that some signals triggering recycling may be transmitted through the cell surface PAFR detached from agonists. Thus, sequestered receptors may be sorted into early endosomal compartments and prepared to recycle back to the cell surface waiting for signal shut-down.

Microscopic analysis revealed PAFR movement into some intracellular compartments after PAF stimulation (Fig. 2D, middle). Interestingly, recycled receptors regained their cell surface distribution patterns, including numerous dots (Fig. 2D, right). Characterization and identification of these intracellular compartments and dots is an ongoing study.

Sequestration via the Clathrin-coated Pits-mediated Pathway—Many plasma membrane proteins have been shown to internalize via the clathrin-mediated pathway or other pathways such as the caveolae (36, 37). It has long been controversial whether or not GPCRs are sequestered via clathrin-coated pits. Most but not all of the data suggest the involvement of clathrin in GPCR sequestration (9, 38–42). It should also be noted that heterotrimeric G proteins are concentrated within the caveolar fraction (43, 44) and interact with caveolin in a regulated form (45). It seems reasonable to assume that sequestration mechanisms may vary among GPCRs and cell types in which they are expressed.

In our experiments, cells were subjected to hypertonic sucrose shock, potassium depletion, and acidification to inhibit the clathrin-mediated pathway in a specific manner (30, 46). Sucrose shock and potassium depletion prevent the interaction of clathrin and adapter proteins, whereas acidification interferes with the budding of clathrin-coated vesicles from the cell membrane (30). After these pretreatments, we measured PAF-induced PAFR sequestration and folic acid incorporation. Intrinsic folic acid receptors in the plasma membrane belong to the family of glycosyl phosphatidylinositol-anchored proteins localized in caveolae (47–50), and folic acid is known to be incorporated via the caveolae (36). We used incorporation of folic acid as a control for a clathrin-independent pathway. Each pretreatment inhibited PAF-induced sequestration but not folic acid incorporation (Table I). In contrast, PMA treatment, which inhibits folic acid incorporation by inactivating caveolar internalization (33, 34), did not affect PAFR sequestration (Fig. 3C and Table I). Thus, PAFR sequestration seems to be mediated by clathrin rather than by caveolae.

Resensitization of the PAF Receptor—Recently, Krueger et al. (3) reported that phosphorylated β2ARs are dephosphorylated by some GPCR-specific protein Ser/Thr phosphatases in acidic environments like the endosomal compartments, events which lead to receptor recycling and resensitization. As shown in Fig. 5, A and B, the recycled receptors regained the potential to respond efficiently to PAF. In contrast, sequestration-defective CHO-HA-Tail-1 cells (Fig. 3B) showed desensitization but not resensitization of the IP₃ response to PAF (Fig. 5C), suggesting that sequestration/recycling may play a role in the resensitization.

Role of Receptor Cytoplasmic Tail Phosphorylation—In the cytoplasmic domain of guinea pig PAFR, there are 14 Ser/Thr residues in intracellular portions (25). Among them, nine cluster in the cytoplasmic tail. We reported that a synthetic 18-amino acid peptide corresponding to the cytoplasmic tail of guinea pig PAFR containing the six Ser/Thr residues (Ser³¹⁸-Thr³⁵⁸) was phosphorylated in vitro by recombinant GRK2 (18). Moreover, other investigators reported that PAF induced the translocation of transfected GRK2 from the cytosol to the cell membrane in human leukocytes (19). In the present study, we used cytoplasmic tail mutant PAFRs (HA-Del, HA-Tail-1, and HA-Tail-2) to examine the role of receptor cytoplasmic tail phosphorylation.

PAF as well as PMA and Bt₂-cAMP induced phosphorylation of the wild-type receptor but not those of the mutants. Guinea pig PAFR possesses only two consensus motifs (RX/S/T) for phosphorylation by CAMP-dependent protein kinase (51) in the intracellular domain, which may reflect the low extent of phosphorylation by Bt₂-cAMP. Most of the phosphorylation sites related to these stimuli existed in the cytoplasmic tail Ser/Thr...
residues (Fig. 3A). Both PMA and Bt2-cAMP failed to induce PAFR sequestration (Fig. 3C). All cytoplasmic tail mutations created in this study more or less impaired the ability of the receptor to undergo sequestration (Fig. 3B). We speculate that agonist-induced phosphorylation of the receptor cytoplasmic tail by some GRKs rather than protein kinase C or cAMP-dependent protein kinase may facilitate but not be essential for PAFR sequestration. The phosphorylated Ser/Thr residues should vary between different kinases. Ferguson et al. (6) reported that a sequestration-defective mutant of the b2AR was rescued in sequestration by GRK2 overexpression. In that case, receptor phosphorylation by GRK2 facilitated sequestration of the mutant b2AR. The HA-Tail-1 receptor were not sequestered after PAF stimulation (Fig. 3B) even though it did transmit signals (Fig. 5C), a result which agrees well with a report that human PAFR with a cytoplasmic tail deletion (Cys5317 → termination) did not show PAF-induced sequestration (24). As described for other GPCRs (52), PAFR sequestration may be independent from signal transduction. Phosphorylation of six Ser/Thr residues following Cys5317 may play a role to facilitate sequestration. Additional substitution of three Ser residues in the cytoplasmic tail (in HA-Tail-2 receptor) or a large deletion of the cytoplasmic tail (in HA-Del receptor) rescued sequestration. Additional substitution of three Ser residues (Fig. 3A). Both PMA and Bt2-cAMP failed to induce PAFR sequestration (Fig. 3C). All cytoplasmic tail mutations created in this study more or less impaired the ability of the receptor to undergo sequestration (Fig. 3B). We speculate that agonist-induced phosphorylation of the receptor cytoplasmic tail by some GRKs rather than protein kinase C or cAMP-dependent protein kinase may facilitate but not be essential for PAFR sequestration. The phosphorylated Ser/Thr residues should vary between different kinases. Ferguson et al. (6) reported that a sequestration-defective mutant of the b2AR was rescued in sequestration by GRK2 overexpression. In that case, receptor phosphorylation by GRK2 facilitated sequestration of the mutant b2AR. The HA-Tail-1 receptor were not sequestered after PAF stimulation (Fig. 3B) even though it did transmit signals (Fig. 5C), a result which agrees well with a report that human PAFR with a cytoplasmic tail deletion (Cys5317 → termination) did not show PAF-induced sequestration (24). As described for other GPCRs (52), PAFR sequestration may be independent from signal transduction. Phosphorylation of six Ser/Thr residues following Cys5317 may play a role to facilitate sequestration. Additional substitution of three Ser residues in the cytoplasmic tail (in HA-Tail-2 receptor) or a large deletion of the cytoplasmic tail (in HA-Del receptor) rescued sequestration activity, indicating that there may be both positive and negative structural determinants regulating sequestration in the cytoplasmic tail, as described for other GPCRs (53). Finally, at least in the PAFR, it seems that the receptor phosphorylation/dephosphorylation cycle is not the sole or critical determinant governing receptor sequestration/recycling/resensitization processes, because HA-Tail-2 receptor, which lacked all phosphorylation targets in its cytoplasmic tail, still underwent all these processes (Fig. 3B and Fig. 5D).

Conclusion—In this paper, we extensively examined the dynamics (sequestration, recycling, and resensitization) of the PAFR. We found that phosphorylation facilitates but is not essential for receptor sequestration and that receptor sequestration/recycling is important in resensitization. For a better understanding of the molecular mechanisms that govern GPCR desensitization and dynamic redistribution, more information is needed on GPCR protein modification such as receptor dimerization and receptor palmitoylation in addition to current knowledge of receptor phosphorylation.

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Agonist-induced Sequestration, Recycling, and Resensitization of Platelet-activating Factor Receptor: ROLE OF CYTOPLASMIC TAIL PHOSPHORYLATION IN EACH PROCESS
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