Internalization and Sequestration of the Human Prostacyclin Receptor

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Prostacyclin (PGI₂), the major product of cyclooxygenase in macrovascular endothelium, mediates its biological effects through its cell surface G protein-coupled receptor, the IP. PKC-mediated phosphorylation of human (h) IP is a critical determinant of agonist-induced desensitization (Smyth, E. M., Hong Li, W., and FitzGerald, G. A. (1998) J. Biol. Chem. 273, 23258–23266). The regulatory events that follow desensitization are unclear. We have examined agonist-induced sequestration of hIP. Human IP, tagged at the N terminus with hemagglutinin (HA) and fused at the C terminus to the green fluorescent protein (GFP), was coupled to increased concentrations of iloprost that induce PKC-dependent desensitization. Neither the PKC inhibitor GF109203X nor mutation of Ser-328, the site for PKC phosphorylation, altered receptor sequestration indicating that, unlike desensitization, internalization is PKC-independent. Deletion of the C terminus prevented iloprost-induced internalization, demonstrating the critical nature of this region for sequestration. Internalization was unaltered by cotransfection of cells with G protein-coupled receptor kinases (GRK)-2, -3, -5, -6, arrestin-2, or an arrestin-2 dominant negative mutant, indicating that GRKs and arrestins do not play a role in hIP trafficking. The hIP is sequestered in response to agonist activation via a PKC-independent pathway that is distinct from desensitization. Trafficking is dependent on determinants located in the C terminus, is GRK/arrestin-independent, and proceeds in part via a dynamin-dependent clathrin-coated vesicular endocytotic pathway although other dynamin-independent pathways may also be involved.

Prostacyclin (PGI₂) is the major product of cyclooxygenase (COX) in macrovascular endothelium. In humans, the predominant source of prostacyclin biosynthesis is COX-2 (2, 3), probably reflecting induction of its expression in endothelium by physiological rates of shear (4). COX-2 is also up-regulated in vascular cells by cytokines and growth factors (5), and both COX isoforms are coexpressed in monocyte/macrophages infiltrating human atherosclerotic plaque (6). PGI₂ inhibits platelet activation, is a vasodilator, and possesses proinflammatory and antiproliferative properties in vitro (7–9). It is thought to function as a homeostatic regulator of platelet-vascular interactions in settings of plaque rupture, such as unstable angina, where biosynthesis of PGI₂ is increased during ischemic episodes (8, 10–12). Sustained overproduction of PGI₂ is evident physiologically in pregnancy (13) and in severe atherosclerosis (14). Interest in the importance of PGI₂ in vivo has increased recently with the observation that COX-2 inhibitors suppress PGI₂, without concomitant inhibition of COX-1-derived thromboxane formation by platelets (2, 3). PGI₂ activates a G protein-coupled membrane receptor (GPCR), the IP (15, 16). However, no antagonist of the IP exists, limiting our ability to probe the role of this eicosanoid in vivo. Nevertheless, directed overexpression of PGI₂ synthase (PGIS) reduces elevated pulmonary blood pressure (17), and the proliferative response to vascular injury (18) and polymorphism in the PGIS promoter has been related to the severity of hypertension (19). Deletion of the IP increases the response to thrombotic stimuli and both pain and inflammation in the periphery (20). On the other hand, PGIS and the IP are expressed widely in the central nervous system (21, 22), where the function of this eicosanoid is unknown.

Given the acute and chronic alterations in PGI₂ biosynthesis in disease (1), the tachyphylaxis that complicates administration of PGI₂ and its analogs (23, 24), and the interest in overexpression of its biosynthetic enzymes or receptor as a therapeutic strategy (18), a detailed understanding of the molecular mechanisms that regulate the response of the IP to ligation by agonist would seem desirable. We and others (25, 26) have previously provided evidence implicating both serine-threonine kinases, such as protein kinases A and C, and GPCR kinases (GRKs) in agonist-induced receptor phosphorylation and desensitization, consequent to the IP, and other eicosanoid receptors, being uncoupled from G proteins. However, the fate of the IP after those events is unclear. One possibility is that GRK-
mediated phosphorylation would target the IP for binding by arrestin-like adapter proteins, which in turn might direct it toward sequestration in clathrin-coated vesicles (CCVs), where dephosphorylation would prepare the IP for recycling to the plasma membrane (32, 33). Alternatively, it might be targeted for lysosomal degradation (32, 33). Arrestin/clathrin-dependent pathways of sequestration are followed by several GPCRs including the β2-adrenergic receptor (AR; 34) and m1, -3, and -4 muscarinic acetylcholine (35) receptors, although several GPCRs diverge from this paradigm. The preferred pathway for agonist-induced internalization of the m2 muscarinic receptor (36) and arrestin II type 1A receptor (37) is arrestin- and clathrin-independent. Similarly, internalization of the cholecystokinin receptor can occur via clathrin-dependent and -independent pathways (38). Furthermore, both GRK-dependent and -independent mechanisms may regulate the same receptor. The β2-AR (39), thrombin (40), arrestin II-1A (41), and m1 muscarinic acetylcholine (42) receptors are regulated by the action of both second messenger kinases and GRKs. In addition, a β2-AR Y326A mutant, which does not internalize, is unresponsive to phosphorylation by GRKs but is phosphorylated and desensitized in a PKA-dependent manner (43).

Whereas agonist-dependent phosphorylation of the human (h) IP primarily involves PKC in the desensitization process (25, 26), the events that may direct internalization and sequestration are unknown. We provide evidence that the IP is indeed sequestered following desensitization but that this process occurs independent of both PKC and GRKs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Iloprost, the camp radioimmunoassay, and enhanced chemiluminescence (ECL) kits, as well as all radiochemicals, were purchased from Amersham Pharma Biotech. Monoclonal anti-HA and anti-GFP antibodies were obtained from Convance (Princeton, NJ). Anti-clathrin and anti-caveolin-1 were from Transduction Laboratories and anti-GFP antibodies were obtained from Convance (Princeton, NJ).

**Chemiluminescence (ECL) kits, as well as all radiochemicals, were purchased from Roche Molecular Bio-

**cAMP Measurements**—Cells, grown to confluence in 24-well plates coated with lysine (0.1 mg/ml), were treated with iloprost (10 min at 37 °C). Reactions were terminated by aspiration, and cAMP was extracted with ice-cold 65% ethanol for 30 min. Samples were dried under vacuum and reconstituted in assay buffer, and cAMP was quantified by radioimmunoassay as described previously (25, 26).

**Inositol Phosphate Production**—Cells, grown to 70–80% confluence in 12-well plates coated with 0.1 mg/ml lysine, were labeled overnight with 2 μCi/ml [3H]inositol in DMEM (without inositol) containing 0.4% paraformaldehyde, 50 units/ml penicillin, and 50 μg/ml streptomycin. Thirty minutes prior to stimulation cells were treated with 25 mM LiCl at 37 °C. After stimulation for 10 min at 37 °C, the reactions were terminated by aspiration. Total inositol phosphates were extracted with 750 μl of 10 mM formic acid and recovered by anion exchange as described previously (25, 26).

**Adenylyl Cyclase Assay**—Adenylyl cyclase activity was assessed in cell membranes, as described previously (26). Briefly, assays were carried out in 50 mM Tris containing 3 mM MgCl2, 1.5 mM EDTA, 0.15 mM ATP, 0.05 mM GTP, 0.1 mM camp, 2.5 mM phosphoenolpyruvate, and 0.1 mM isobutylmethylxanthine. Each reaction contained 1 unit of myokinase, 1 unit of pyruvate kinase, and 2 μl of [33P]ATP (30 Ci/mmol). Reactions were started by the addition of membranes (5 μg per assay tube) and, after 30 min at 30 °C, were quenched by the addition of 1 ml of 5% trichloroacetic acid containing 30,000 cpm of [3H]cAMP (41 Ci/mmol). Samples were subjected to sequential chromatography through Dowex (AG W-X4, hydrogen form) and alumina (WN-6, neutral). [3P] and [3H] in the eluates were estimated by scintilla-

**PKC Kinase Activity**—PKC activity in total cellular lysates was determined by phosphorylation of the α-casein substrate in the presence of 1 μM PKC activator, 100 μM CaCl2, 2 μM alanine, and 4-nitrophenyl phosphate were obtained from Roche Molecular Bio-

**Western Blotting**—Cells were lysed (RIPA, 50 mM Tris, 5 mM EDTA, pH 8.0, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 1 tablet/50 ml of Complete Protease Inhibitor mixture) drawn, then centrifuged at 14,000 rpm. Proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose. Re-

**Measurement of Surface HAhIP Expression**—Surface HAhIP expression was measured by ELISA. Cells were seeded on 24-well dishes coated with lysine (0.1 mg/ml) and, 48 h later, treated with the agonist of interest at 37 °C. Reactions were stopped by aspiration and fixation with 1% paraformaldehyde in PBS, 4 °C, 10–15 min. Following 3 washes with PBS, cell monolayers were blocked (2% BSA in PBS, room temperature, 30 min) and HA antibody was added at 1:5000 dilution. Antigen-antibody complexes were visualized by ECL.

**Agonist-induced Sequestration of Human Prostacyclin Receptor**

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surface alkaline phosphatase was detected, after four washes with PBS, by following the conversion of 4-nitrophenyl phosphate by measurement of absorbance at 405 nm. A background control in which anti-HA was not added was included in each plate and subtracted from the final absorbance measurements. Absorbance readings above background were negligible in vector control cells (data not shown) indicating that binding of the anti-HA antibody was specific for HAhIP.

Statistical Analysis—Data were compared by Student’s t test or analysis of variance, followed by Dunnet’s test, for multiple comparisons. A p value of <0.05 was considered significant.

RESULTS

Expression of HAhIP-GFP—We have described previously the generation of HEK cells lines that express a HA-tagged hIP (HAhIP-HEK, see Ref. 25). We generated a second HEK cell line expressing HAhIP to which GFP was fused at the C-terminal end (HAhIP-GFP-HEK) to follow the sequestration of hIP without the need for fixation of cells and treatment with antibodies. Lysates from transfected cells were resolved by SDS-PAGE and immunoblotted with an anti-GFP antibody. The HAhIP-GFP appeared as a broad complex with a molecular mass of 70–95 kDa (Fig. 1A), representing glycosylated HAhIP (44–60 kDa, see Ref. 25) plus the 27-kDa green fluorescent protein fused to the C-terminal end. The identity of this species as HAhIP-GFP was confirmed in parallel immunoblots with anti-HA (Fig. 1A). When living cells were observed in culture under a green fluorescent protein lamp, HAhIP-GFP was localized to the plasma membranes (Fig. 1B). GFP-vector control cells, in contrast, demonstrated the presence of a 27-kDa GFP band, by SDS-PAGE, and a diffuse cellular pattern of GFP expression in phase contrast micrographs. Treatment of HAhIP-GFP-HEK with the prostacyclin analog iloprost, for 10 min, induced a concentration-dependent increase in intracellular cAMP (EC_{50} = 0.39 ± 0.09 nM, n = 3; Fig. 1C) and inositol phosphate production (EC_{50} = 86.6 ± 18.3 nM, n = 4; Fig. 1C) indicating coupling to the two signaling systems similar to that...
seen for HAhIP and the non-tagged receptor (25). Thus, similar to other studies (27), addition of the GFP at the C-terminal end of HAhIP did not significantly alter the expression or signal transduction properties of the receptor.

**Agonist-induced Sequestration of HAhIP-GFP**—Treatment with iloprost induced a rapid sequestration of HAhIP-GFP from the cell membrane into the intracellular space. Internalization was evident after 5 min of agonist treatment and continued over a 45-min time course (Fig. 2A), as observed by confocal microscopy. Sequestered HAhIP-GFP was partially localized to early endosomes, preloaded with rhodamine-conjugated transferrin (Fig. 2B). These data indicate that early HAhIP sequestration events may occur, at least in part, via a clathrin-coated vesicular endocytotic pathway.

**Agonist-induced Sequestration of HAhIP**—Iloprost-induced sequestration of HAhIP was quantified by ELISA. Treatment of HAhIP-HEK with iloprost induced a time- and dose-dependent loss of HA expression at the cell surface indicating internalization of the receptor. The time course for iloprost-induced HAhIP sequestration was similar to that seen in the confocal experiments; HAhIP sequestration was evident within the first 5–10 min of agonist (1 μM) treatment and reached a plateau within 30 min (Fig. 3A). Minimal sequestration was evident following treatment for 60 min with low (<10 nM) concentrations of iloprost (Fig. 3B). In contrast, substantial (up to 50%) sequestration of HAhIP was evident at higher agonist concentrations (EC50 = 27.6 ± 5.7 nM, n = 7). These concentrations increase inositol phosphate generation and induce PKC-dependent phosphorylation and desensitization of the HAhIP (25, 26).

**Effect of PKC and PKA Inhibitors on Agonist-induced Sequestration of HAhIP**—Pretreatment of cells for 30 min with GF109203X inhibited iloprost-stimulated total PKC activity by approximately 60% (Fig. 4A, inset). The residual kinase activity may be due to GF109203X-insensitive PKC isoforms or non-PKC-mediated phosphorylation of the α-pseudosubstrate peptide. We have demonstrated previously that the majority of rapid, iloprost-induced phosphorylation of HAhIP is inhibited by pretreatment of cells with GF109203X but not the PKA inhibitor H89 (25). However, sequestration of the HAhIP in response to iloprost was not altered by inhibition of PKC (Fig. 4A). As expected, similar treatment of cells with H89 was also without effect (Fig. 4B).

**Agonist-induced Sequestration of Mutant HAhIPs**—There are two consensus sites for PKC phosphorylation located in the C-terminal region of the hIP, serine 328 and serine 374 (15). We have demonstrated previously that mutant HAhIPs, in which serine 328 was replaced with an alanine, either alone (Ser-328 → Ala) or in combination with serine 374 (Ser-328 → Ala/Ser-374 → Ala), were not substrates for PKC-mediated phosphorylation and demonstrated significantly blunted desensitization responses to iloprost (26). Similarly, a C-terminal deletion mutant (C-DEL), in which most of the C-terminal region of the receptor was deleted, did not undergo agonist-induced phosphorylation or desensitization. In contrast, when serine 374 alone was replaced with an alanine (Ser-374 → Ala),
phosphorylation and desensitization proceeded as for the non-mutated receptor. Iloprost-induced sequestration of HAhIP was examined with these mutant receptors. As demonstrated in Fig. 5, Ser-374 → Ala, Ser-328 → Ala, and Ser-328 → Ala/Ser-374 → Ala were sequestered in a similar manner to wild type HAhIP, regardless of the potential for PKC-dependent phosphorylation. In contrast, C-DEL did not sequester in response to agonist. Thus, sequestration was independent of agonist-induced PKC-mediated phosphorylation of the hIP but was dependent on the presence of the C-terminal region of the receptor.

Effect of Inhibitors of Clathrin-mediated Endocytosis on Agonist-induced Sequestration of HAhIP—Clathrin-coated vesicular pathways of endocytosis can be pharmacologically inhibited by pretreating cells with concanavalin A or sucrose. Pretreatment of HAhIP-HEK with either of these agents significantly reduced iloprost-induced sequestration (Fig. 6A). Furthermore inhibition of clathrin-coated vesicular endocytosis by coexpression of a dominant-negative mutant (K44A) also detected a role for CCV-mediated endocytosis in sequestration of the HAhIP (Fig. 6B). Endocytosis via caveolae is also dependent on dynamin (25), raising the possibility that HAhIP is sequestered via this pathway and not CCV. The absence of caveolin-1, a marker for caveolae in many commonly used cell lines (30), including HEK 293 cells (31), has been reported. In agreement with these studies, we did not detect caveolin-1 in HEK 293 cells (Fig. 7) suggesting strongly that this pathway of receptor internalization is not involved in HAhIP trafficking, at least in this cellular model. In contrast, clathrin expression was readily detectable (Fig. 7), further supporting a role for CCVs in HAhIP internalization.

Role of GRKs and Arrestins in HAhIP Internalization—HAhIP-HEKs were cotransfected with cDNAs for GRK2, GRK3, GRK5, or GRK6. Expression of these GRKs did not increase HAhIP sequestration (Table I). Similarly coexpression of arrestin-2 or a dominant negative arrestin-2-(319–418) did not alter the pattern of sequestration (Table I). Successful transfection of cells with GRK2, arrestin-2, or arrestin-2-(319–418) was verified by Western blotting (Fig. 8) and was assumed for the other constructs. As expected, immunoblots with the anti-human arrestin-2 antibody revealed the presence of endogenous protein (Fig. 8, lower panel), whereas anti-bovine GRK-2 did not (Fig. 8, upper panel). These data indicate that GRKs and arrestins are unlikely to play a primary role in this pathway of HAhIP regulation.

Recycling of HAhIP to the Cell Surface—Cells were returned to agonist-free conditions, following treatment with iloprost to induce HAhIP sequestration. In the absence of agonist for 30 or 60 min, substantial recycling of the HAhIP to the cell surface was apparent (Table II). Iloprost-stimulated adenylyl cyclase activity was reduced from 2.4 ± 0.7 (n = 3) to 0.9 ± 0.2-fold over basal in membranes prepared from cells pretreated with iloprost (1 μM, 60 min) to induce sequestration. Adenylyl cyclase activation was restored to 3.1 ± 1.0 (n = 3)-fold over basal when cells were subjected to the 60-min recovery period indicating that a significant amount of the recycled receptor was functionally coupled to adenylyl cyclase activation. The phosphatase inhibitor okadaic acid, which prevented β2-AR dephosphorylation, a critical step for subsequent recycling to the plasma membrane (44), did not alter the recovery of cell surface HAhIP expression. Thus, HAhIP sequestration induced by iloprost is reversible, upon agonist withdrawal. Furthermore, recycling of the HAhIP was not dependent on dephosphorylation of the receptor by an okadaic acid-sensitive phosphatase.

DISCUSSION

Given the increasing interest in the role of PGI2 in human disease and the prospects of modulating its formation and/or action as a therapeutic strategy, it would seem judicious to understand the mechanisms that regulate agonist-IP interactions. We have previously demonstrated that agonist-induced receptor phosphorylation, predominantly mediated by PKC (25), is critical to uncoupling of the hIP from its attendant G proteins and results in desensitization of the response to agonist (26). We now extend these observations to clarify the subsequent fate of the receptor.

Agonist-induced regulation of GPCR is a multistep process (32–33). In the “classical” pathway of GPCR regulation, agonist-induced receptor phosphorylation is mediated by the second messenger-activated kinases, PKC or PKA and/or GRKs. Binding of an adapter protein, arrestin, leads to uncoupling of the receptor from the G protein and receptor sequestration through CCVs. The internalization step is dependent on dynamin, a GTPase that drives pinching off of the endocytotic vesicles. The sequestered receptor may be recycled to the cell surface to undergo another round of signal transduction, be
down-regulated via lysosomal degradation, or may direct activation of additional signaling systems. In the present study, we demonstrate that the hIP was indeed sequestered in response to treatment with the PGI2 analog iloprost. However, unlike desensitization, sequestration was independent of PKC. Furthermore, several of the classical molecular determinants of GPCR sequestration appeared not to be involved in sequestration of the hIP.

The fusion protein HAhIP-GFP displayed characteristics similar to the wild type hIP, when stably overexpressed in HEK 293 cells (25); HAhIP-GFP expression was localized to plasma membranes, and the receptor was coupled both to adenylyl cyclase and phospholipase C (Fig. 1). GPCR-GFP fusion proteins have been generated for at least 20 different receptors in which ligand binding and signaling were reported to be normal (27). Furthermore, when GPCR phosphorylation was examined specifically, it was found to be normal, despite the addition of the GFP tag (28, 29). We did not examine directly whether the GFP tag altered receptor HAhIP phosphorylation. However, given that signaling and membrane localization of HAhIP-GFP appeared normal, a change in receptor phosphorylation is extremely unlikely.

Treatment with iloprost induced a time- and concentration-dependent sequestration of receptor from the plasma membrane to the intracellular space. Similar to desensitization (26), this phase of hIP regulation was only evident at those concentrations of iloprost that increase inositol phosphate generation but not cellular cAMP (Figs. 1C and 3B). However, the time course of desensitization and sequestration were quite different. Iloprost-induced phosphorylation and desensitization of HAhIP in HEK 293 cells was evident within the first 15 s and reached a maximum level within 5 min (25, 26). Sequestration, on the other hand, was minimal within the first 5 min of iloprost treatment, and up to 30 min were required for the response to maximize. Thus, sequestration appears to occur consequent to desensitization.

A second point of distinction was that PKC-mediated phosphorylation seemed unimportant in sequestration. Unlike phosphorylation (25), sequestration was not altered by pretreatment of cells with the PKC inhibitor, GF109203X. Sequestration was similarly unaltered when the site for PKC-mediated phosphorylation was mutated. Thus, similar to other GPCR (45, 46), desensitization and sequestration of hIP are
Cells expressing HAhIP were cotransfected with empty vector (control) or vector containing the cDNA for GRK2, -3, -5, -6, arrestin-2 or an arrestin-2 dominant negative mutant (arrestin-2-(319–418)). Iloprost (60 min)-induced sequestration of HAhIP was quantified 48 h later by ELISA as indicated under “Experimental Procedures” and calculated as the percentage of absorbance in the absence of iloprost for each transfection condition. Data are the mean ± S.E. of three experiments each performed in triplicate.

| Treatment          | % surface HA | Control | GRK2 | GRK3 | GRK5 | GRK6 |
|--------------------|--------------|---------|------|------|------|------|
| Iloprost = 0.1 μM | 57.0 ± 4.4   | 59.6 ± 5.4 |
| Iloprost = 1.0 μM | 56.7 ± 2.7   | 53.5 ± 5.2 |

**TABLE I**
Effect of GRKs or arrestins on iloprost-induced sequestration of HAhIP

## FIG. 7.
Expression of clathrin or caveolin-1 in HEK 293 cells. Lysates from untransfected HEK 293 cells (lane 1) or HAhIP-HEK (lane 2) were immunoblotted, alongside positive controls for clathrin (HELA cell lysate; lane H) or caveolin (endothelial cell lysate, EC), with anti-clathrin or anti-caveolin. The presence of clathrin or caveolin-1 is indicated. Molecular masses are in kilodaltons.

## FIG. 8.
Expression of GRK2, arrestin-2, or arrestin-2-(19–418). Cells expressing HAhIP were cotransfected with empty vector (lane 1) or vector containing the cDNA for GRK2 (lane 2), arrestin-2 (lane 3), or arrestin-2-(319–418) (lane 4). The expression of GRK2 or arrestin-2 was examined by Western blotting with a monoclonal anti-GRK2 or polyclonal anti-arrestin-2. The presence of arrestin-2 or arrestin-2-(319–418) is indicated. Molecular masses are in kilodaltons.

**TABLE II**
Recovery time of sequestered HAhIP to the plasma membrane

| Recovery time | % surface HA |
|--------------|-------------|
| 0 min        | 57.4 ± 6.6  |
| 30, control  | 79.7 ± 10.5 |
| 60, control  | 88.7 ± 7.4  |
| 60, ethanol  | 86.4 ± 1.5  |
| 60 okadaic acid 5 μM | 98.0 ± 4.9 |

distinct processes that are mediated by distinct molecular determinants.

A link between GPCR phosphorylation and sequestration, although frequently reported, has not been established for all receptors. GRK-mediated phosphorylation generally directs arrestin-dynamin-mediated trafficking via CCVs (32, 33). However, phosphorylation-dependent but arrestin/clathrin-independent sequestration has been described (36). In addition, trafficking that does not depend on receptor phosphorylation has been reported (47, 48). In the current study, several lines of evidence support the conclusion that sequestration of HAhIP is phosphorylation-independent. Pretreatment of cells with GF109203X did not alter HAhIP internalization. We have previously demonstrated that these conditions, which result in approximately 60% inhibition of total iloprost-induced PKC activity (Fig. 4A), reduce the large majority of iloprost-mediated HAhIP phosphorylation (25). However, negative data with kinase inhibitors must always be viewed with caution, since we cannot exclude the possibility that a GF109203X-insensitive PKC isoform may phosphorylate HAhIP and direct its internalization. Therefore, to investigate further the role of phosphorylation in HAhIP trafficking, we examined agonist-induced internalization of a series of mutant HAhIPs in which the recognized consensus sites for PKC phosphorylation (Ser-328 and Ser-374) have been disrupted (26). Two mutants (Ser-328 → Ala and Ser-328 → Ala/Ser-374 → Ala) sequester normally (Fig. 5) but are devoid of PKC-mediated phosphorylation whether activated directly with phorbol 12-myristate 13-acetate or indirectly with thrombin (26). These observations are consistent with the data obtained with GF109203X and support the conclusion that PKC-mediated HAhIP phosphorylation does not play a role in sequestration. We have observed that both the HAhIP and its Ser-328 mutants undergo a minor component of PKC-independent phosphorylation (26) which is likely due to activation of GRKs. However, cotransfection of HAhIP with GRKs did not increase sequestration of the receptor in response to agonist activation, suggesting that HAhIP internalization is independent of GRK-mediated phosphorylation. Finally, recycling of HAhIP to the plasma membrane upon agonist withdrawal (Table II) was not altered by pretreatment of cells with okadaic acid, a phosphatase inhibitor that prevents dephosphorylation of the internalized β2-AR (44). Taken together, the internal consistency of these data suggest that sequestration of the HAhIP is independent of phosphorylation.

The HAhIP traffics, at least in part, via a CCV-mediated pathway. Sequestered HAhIP-GFP partially colocalized with rhodamine-conjugated transferrin, which is constitutively internalized by CCVs. In addition, sequestration was markedly inhibited by both concanavalin A and sucrose, pharmacological inhibitors of CCV-mediated trafficking (Fig. 6). These data, taken together with the weight of evidence from other GPCR studies (32, 33), initially suggested that HIP sequestration was likely to be mediated through a GRK-arrestin-dynamin-dependent pathway of CCV trafficking. Indeed, although the majority of HAhIP phosphorylation in response to iloprost is PKC-dependent, a small but consistent PKC-independent phosphorylation is evident in both the wild type receptor in the
presence of GP109203X (25) and in PKC-phosphorylation-deficient HAhIP mutants (26), suggesting that GRK-dependent pathways of receptor phosphorylation may be of relevance to hIP regulation. However, several lines of evidence suggested that hIP sequestration may not primarily proceed via this pathway. First, cotransfection of HAhIP-expressing cells with the cDNAs for GRK2, -3, -5, or -6 did not increase sequestration response to iloprost. Expression of these kinases was similarly without effect on ioprost-induced phosphorylation of HAhIP (data not shown). Second, cotransfection of HAhIP-expressing HEK 293 cells with the cDNA for arrestin-2 did not increase ioprost-induced sequestration. Third, expression of an arrestin-2 dominant-negative mutant did not reduce HAhIP sequestration. Finally, inhibition of dynamin-dependent sequestration with a dominant-negative mutant did not completely inhibit HAhIP internalization.

It may be argued that, since HEK 293 cells have a high level of endogenous GRK and arrestin expression (49), increasing the cellular level of this proteins may not produce any further increases in GRK/arrestin-dependent receptor trafficking. However, this approach has been used successfully by other investigators to study GPCR trafficking. For example, coexpression of GRKs and/or arrestins, in HEK 293 cells, increases agonist-induced sequestration of the α isoform of the thromboxane receptor (TP; see Ref. 50). Furthermore, inhibition of the GRK-arrestin-dynamin pathway of sequestration through coexpression of dominant negative mutants for both arrestin-2 and dynamin, which would be expected to inhibit the endogenous cellular proteins, were either ineffectual or partially effective in reducing sequestration of the HAhIP. Indeed, the demonstration that a component of HAhIP trafficking is resistant to inhibition of dynamin further strengthens the argument that a GRK-arrestin pathway of regulation is not primarily involved in HAhIP sequestration, since dynamin is the downstream common denominator for GRK-arrestin-regulated receptors that traffic through CCVs (32, 33).

Another consideration is that the ineffectiveness of GRKs or arrestins, and the partial effectiveness of dynamin K44A, was due the transient expression of these proteins in a cell line stably expressing HAhIP. However, this is not likely to be the case since, when similar experiments were carried out with a second HAhIP-HEK cell line, in which substantially less HAhIP was expressed, dynamin K44A expression produced a similar partial effect on HAhIP sequestration in response to iloprost (data not shown). Similarly, when both HAhIP and K44A were transiently coexpressed, K44A-mediated inhibition of HAhIP sequestration was not enhanced (data not shown). It is therefore likely that the HAhIP is sequestered from the plasma membrane in part via a dynamin-dependent, but GRK/arrestin-independent, CCV-mediated pathway in addition to a dynamin-independent pathway of receptor trafficking. Indeed it has emerged that, in addition to the classical trafficking pathway, at least two other modes of GPCR endocytosis exist, namely arrestin-independent, dynamin-dependent internalization (51) and arrestin-independent, dynamin-independent internalization (36, 37), both may prove important for regulation of the hIP.

Truncation of the C-terminal region of the HAhIP completely prevented receptor trafficking in response to iloprost (Fig. 5), indicating its critical role in sequestration. Although it may be that deletion of the C terminus alters the tertiary structure of the receptor in a manner that changes its cellular trafficking, it is more likely that specific determinants of HAhIP sequestration are located in this region. Several investigators have attempted to determine sequence motifs that may be important for GPCR sequestration. For example, mutation of a dileucine motif located in the C-terminal tail of the β3-AR inhibited agonist-induced sequestration (52) but did not alter agonist-independent internalization of the TPβ receptor (50), whereas a C-terminal NPYβ1 receptor sequestration motif is important for regulation of some, but not all, GPCRs in which it is found (53, 55, 54). Although a universal consensus sequence for GPCR sequestration has not been identified, many reports point to the importance of the C-terminal region of the receptor for trafficking. For example, truncation of the C-terminal region of, among others, the CCK type A (55), δ- opioid (56), and histamine H2 receptors (45) reduced or prevented agonist-mediated sequestration. These observations extend to the eicosanoid receptors. The importance of the C-terminal region of the EP-4 receptor for PGE2 (57) in sequestration has been reported. Furthermore, α and β isoforms of the TP receptor that differ only in their C-terminal regions demonstrate strikingly different rates of agonist-induced internalization (50).

In summary, our findings demonstrate several important features of the response of hIP to agonist activation. First, the agonist-induced hIP sequestration occurs subsequent to desensitization. Second, hIP trafficking in response to agonist activation appears to be independent of receptor phosphorylation and is distinct from desensitization. Finally, although agonist activation directs the HAhIP in part to CCVs, other endocytic pathways may play a role in its cellular trafficking. Given the agonist-induced loss of the native hIP from cell membranes (58), including platelets (59), sequestration is likely to be a key step in regulating the response to PGI2.

Acknowledgments—We thank Dr. Jeffery Benovic, Jefferson University, Philadelphia, for the gifts of GRK, arrestin, and dynamin cDNAs and antibodies. We are also grateful to Ekaterina Kosteska for technical assistance.

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