Internalization and Recycling of the Human Prostacyclin Receptor Is Modulated through Its Isoprenylation-dependent Interaction with the δ Subunit of cGMP Phosphodiesterase 6*

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Prostacyclin (PGI2),2 the major cyclooxygenase (COX)-derived product of arachidonic acid formed in the vasculature, mediates its potent anti-thrombotic and anti-proliferative effects through its G protein-coupled receptor (GPCR) termed the IP. Unlike many GPCRs, agonist-induced internalization of the IP occurs in an arrestin/GPCR kinase-independent manner. However, deletion of the IP COOH-terminal region prevented internalization suggesting that protein interactions at this region are involved in IP regulation. Using the COOH-terminal region of IP as bait we identified the δ subunit of cGMP phosphodiesterase 6 (PDEδδ) as a novel IP-interacting protein in two independent yeast two-hybrid screens. Interaction of IP and PDEδδ was confirmed by co-immunoprecipitation in HEK293 cells, and in HEPG2 cells, which endogenously express neither IP nor PDEδδ. IP isoprenylation was critical for this interaction, as PDEδδ was unable to associate with an isoprenylation-deficient mutant IP (IPSSL). PDEδδ overexpression altered the temporal pattern of agonist-induced internalization of IP, but not IPSSL, in HEPG2 cells, increasing initial internalization but facilitating the return of IP to the cell surface despite the continued presence of agonist. Depletion of PDEδδ using short interfering RNA abolished cипростulsion-induced IP internalization in human aortic smooth muscle cells. Recycling of IP, but not IPSSL, upon agonist removal was facilitated by overexpression of PDEδδ. Thus PDEδδ interacts specifically with IP to modulate receptor trafficking.

PGI2 mediates its effects through the membrane-associated receptor IP, a member of the G protein-coupled receptor (GPCR) superfamily. Signaling by this family of receptors is tightly regulated. In the canonical regulatory pathway the activated receptor is phosphorylated by one or more GPCR kinases (GRKs) followed by recruitment of an adapter protein, arrestin. These events culminate in rapid uncoupling of the receptor from its G protein (desensitization) and subsequent receptor internalization (4). Once internalized the receptor may be dephosphorylated and recycled to the cell surface, restoring rapidly agonist activation, or undergo lysosomal degradation resulting in receptor down-regulation and a sustained loss of responsiveness (4). While the regulatory mechanisms employed by many GPCRs adhere, to a reasonable degree, to this pathway, we (5, 6) and others (7, 8) have reported that, similar to other GPCRs (9, 10), regulation of IP deviates from this norm. Thus rapid desensitization of activated IP occurs secondary to it’s phosphorylation, not by a GRK but by a second messenger activated kinase, PKC (6). Subsequently the IP is internalized through a phosphorylation- and arrestin-independent pathway (5, 7, 8). Rapid recycling of the sequestered IP upon agonist withdrawal, which restores the response to agonist without the need for de novo protein synthesis, is evident in platelets (8), fibroblasts (7), and transfected HEK293 cells (5).

The molecular pathways, therefore, that direct agonist-dependent trafficking of IP remain ill-defined but diverge substantially from the classical GPCR regulatory pathway. Deletion of the IP COOH-terminal tail abolished its rapid agonist-dependent receptor internalization (5) suggesting that key regulatory interactions occur along this portion of the protein. Using the COOH-terminal region of the IP as bait, we isolated a specific IP-interacting protein, in vascular smooth muscle cells, which was identified as the δ subunit of the phosphodiesterase (PDE) 6 enzyme complex. This protein was originally identified as the fourth subunit of rod-specific cGMP PDE6 (11). In that system the interaction of PDEδδ with the isoprenylated COOH termini of the enzymes α and β catalytic subunits leads to their solubilization from the plasma membrane and uncoupling of PDE6 from its effector, transducin (12), in addition to potentially regulating intracellular trafficking of newly synthesized PDE6 (13). The functional importance of PDEδδ appears, however, to extend beyond both retinal tissue and the PDE6 enzymatic complex with which it was first associated. Unlike the other subunits, widespread expression of PDEδδ in extraretinal tissue, including heart, placenta, lung, and brain (14), has been reported. This, along with its marked conservation throughout evolution (15), suggests a broader role in cellular signaling. Indeed, several groups have reported the interaction of PDEδδ with a established cell signaling molecules, including members of the Ras superfamily of guanine nucleotide-binding proteins (16) and the α subunit of at least one heterotrimeric G protein Gα, leading to their translocation into the cytosol (17). It is apparent that PDEδδ can act in a manner similar to guanine nucleotide dissociation inhibitors, to inhibit Rap and Ras signaling (16), while con-
versely acting as an effector for, or functional component of, signaling via Arl1, Arl2, and Arl3 (17, 18) and Rab13 (19), small G proteins involved in vesicular transport.

In the present study, we examined the role of PDE6β, a specific hIP-interacting protein, in IP signaling internalization and recycling.

**MATERIALS AND METHODS**

**Yeast Two-hybrid Screen**—The COOH-terminal (CTER) region of hIP, from Va1 through Leu (sense oligonucleotide: 5′ GCC AGC GTC GCC ACG TCC CTC TGC TGA TGG ATC C), in the hIP, triple tagged (3x) at its amino terminus with the hemagglutinin (HA) epitope tag (obtained from the University of Missouri-Rolla cDNA Resource Center). Mutagenesis was carried out using the QuickChange II site-specific plasmid DNA mutagenesis kit (Stratagene). The mutated receptor was termed IP<sup>SLL</sup> reflecting the change in the amino acid sequence of the last four amino acids of hIP from CSLC to SSLC. Mutagenesis was confirmed by DNA sequencing.

To generate PDE6β tagged at its carboxyl terminus with a V5 (HKPiPNLLGLDST) epitope tag, the stop codon was removed from PDE6β by PCR using a sense oligonucleotide containing three miscellanous bases, followed by the sequence encoding the first five amino acids in the hPDE6β sequence (5′-ATG TCA GCC AAG GAC). The antisense oligonucleotide was complimentary to the coding sequence for the six amino acids preceding the stop codon (5′-AAC ATA GAA AAG TCT CAC). The resulting PCR product, encoding the full-length PDE6β absent its stop codon, was cloned directly in pCDNA3.1/V5-His, using the pCDNA3.1/V5-His TOPO TA expression cloning method, to generate V5-tagged or native PDE6β. Cells were incubated with siRNA-containing medium for 48 h, transfected to 12-well plates, and grown for a further 48 h prior to assay.

**Western Blotting**—Whole cell lysates were resolved (30 μg per lane) on NuPAGE (Invitrogen) 10% gels. HA-tagged receptors were visualized with anti-HA (Covance, 1:1000 dilution), in 5% nonfat milk in Tris-buffered saline (TBS; 50 mM Tris, 250 mM NaCl, pH 7.6), containing 1% Tween 20 (TBS-T), for 1 h at room temperature followed by horseradish peroxidase-conjugated anti-mouse IgG (1:5000 dilution; Jackson ImmunoResearch), after first blocking with 5% nonfat milk in TBS-T for 2 h at room temperature. Similarly, V5-tagged or native PDE6β was detected using anti-V5-HRP (Invitrogen, 1:5000) or anti-PDE6β (Affinity Bioreagents, Golden, CO). Antibody-antibody complexes were visualized by enhanced chemiluminescence.

**Co-immunoprecipitation**—Cells were treated with 2 mM dithiothreitoldisuccinimidyl propionate (Pierce) for 30 min, to cross-link covalently surface proteins, and lysed with radioimmune precipitation buffer (50 mM Tris, 5 mM EDTA, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, and a mixture of protease inhibitors) for 10 min at 4 °C prior to centrifugation at 14,000 rpm for 10 min. The resulting supernatants were pre-cleared by 1-h rotation with 50 μl of 10% (w/v) protein G-Sepharose (Amersham Biosciences) to each tube. Anti-HA-protein G-Sepharose was prepared by adding 5 μg anti-HA acetics per lysate to 10% protein G-Sepharose followed by 1-h rotation. HAILP or HAILP was immunoprecipitated, from pre-cleared lysates, by adding 100 μl of anti-HA-protein G-Sepharose to each lysate and rotating for 16 h at 4 °C. Protein G was precipitated at 14,000 rpm for 1 h, washed three times with radioimmune precipitation buffer, and resuspended in 10 μl of sample buffer (NuPAGE). Immunoblotting for co-immunoprecipitated V5-PDE6β was carried out, as described above.

**Measurement of Cell Surface HAIP**—Surface expression was measured by ELISA, as described previously (5). Briefly, 48 h after transfection, cells seeded on 24-well dishes were treated with the agent of interest at 37 °C and reactions stopped by aspiration and fixation (4% paraformaldehyde in PBS, 4 °C, 10–15 min). HA expression was quantified by incubation with monoclonal anti-HA antibody (1:1000 dilution in PBS) followed by alkaline peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch, 1:10,000 dilution in PBS) for 30 min. Cell surface alkaline phosphatase was detected, after four washes with PBS, following conversion of 4-nitrophenyl phosphate by measurement of absorbance at 405 nm.

**Binding Studies**—Cell surface IP was examined in hASM C by radioligand binding to intact cells. Indomethacin (3 μM)-pretreated hASM C were incubated with a saturating concentration (50 nM) of [H]Iloprost, an IP agonist, in binding buffer (Hanks’ balanced salt solution containing 2 mg/ml bovine serum albumin) in the presence or absence of excess (10 μM) unlabeled iloprost for 18 h at 4 °C. Cells were washed three times in ice-cold buffer, treated with 1 mM NaOH for 30 min at 37 °C and cell-associated radioactivity quantified.
**Prostacyclin Receptor Interaction with Phosphodiesterase 6δ**

**FIGURE 1. Detection of IP-PDE6δ interaction by co-immunoprecipitation.** Cell lysates, derived from HEK293 cells co-transfected with V5-PDE6δ plus either empty vector (pcDNA3), HA1TPα or HAhIP, were resolved by 10% reducing SDS-PAGE and expression of HA1TPα and HAhIP (A) or V5-PDE6δ (B) confirmed by immunoblotting for the appropriate epitope tag. Lysates were subjected to immunoprecipitation with an anti-HA antibody and co-immunoprecipitated V5-PDE6δ detected by immunoblotting with an anti-V5-HRP antibody. Co-immunoprecipitation of V5-PDE6δ was only observed HAhIP and V5-PDE6δ were co-expressed (arrow). Data are representative of three independent experiments.

CAMP Measurements—HEPG2 cells grown to confluence in 12-well plates were pretreated with isobutylmethylxanthine (0.01 M) 30 min prior to agonist addition. Cells were treated and reactions were terminated by aspiration. 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 (22).

**RESULTS**

**Yeast Two-hybrid Screen**—The COOH terminus of hIP (hIP-CTER) was used as bait in a hASMC cDNA library yeast two-hybrid screen. Eleven β-galactosidase-positive clones, which grew in the absence of His/Leu/Tryp and presence of 5 mM 3-AT, were identified. Analysis of the DNA sequences revealed that each encoded the same 150 amino acid protein; two independent clones were identified, 9 were identical. BLAST analysis revealed an exact match with a 17-kDa protein identified as the δ subunit of PDE6, a cGMP PDE (23). These findings were confirmed in a second independent screen, carried out at lower stringency (2.5 mM 3-AT). The coding sequence of PDE6δ was cloned, minus its stop codon, into the expression vector pcDNA3.1/V5-His to generate the PDE6δ-V5 fusion protein.

The interaction of hIP with PDE6δ was confirmed using HEK293 transiently with PDE6δ-V5 and co-transfected with or without HAhIP. Lysates, subjected to immunoprecipitation with anti-HA, were examined for the presence of PDE6δ by immunoblotting with anti-V5. PDE6δ was co-immunoprecipitated only when both proteins were co-expressed (Fig. 1C, lane 5). In contrast, PDE6δ-V5 was not evident in anti-HA precipitated lysates prepared from HAhTPα/PDE6δ-V5 co-transfected cells (Fig. 1C, lane 6) confirming that the interaction was specific to hIP and was not due to the presence of HA or V5 epitope tags.

Screening for Endogenous PDE6δ Expression—Widespread expression of PDE6δ has been reported (14). To elucidate the role of PDE6δ in modulating IP function, we first sought to identify a suitable cell model for ectopic PDE6δ expression. Of the several commonly used cell types screened by Western blot for endogenous PDE6δ expression, HEPG2 cells were found to express negligible levels of protein (Fig. 2A). Furthermore IP expression was also absent in HEPG2 cells, as determined by CAMP accumulation in response to treatment with the IP agonist cica-

**Isoprenylation as a Requirement for IP-PDE6δ Interaction**—Interaction between PDE6δ and the majority of its binding partners is dependent on their isoprenylation (11, 16). The IP is one of the very few GPCRs that contains a conserved “CAAX” motif, Cys^{383}-Ser-Leu-Cys^{386}, for isoprenylation in its COOH-terminal tail. Indeed addition of C15-farnesyl isoprenoid groups to the IP has been reported. Moreover this modification was appeared critical for both IP signaling and efficient agonist-dependent sequestration (24). To determine whether the IP-PDE6δ interaction was dependent on IP isoprenylation, we generated an isoprenylation-deficient IP receptor (IP^{SSL.C}) in which Cys^{383}, a critical residue within the isoprenylation motif, was mutated to Ser (24).

Interaction of IP^{SSL.C} with PDE6δ was examined in HEPG2 cells transfected transiently with PDE6δ-V5 and co-transfected with 3xHAhIP or IP^{SSL.C}. PDE6δ was co-immunoprecipitated from HEPG2 cells co-expressing 3xHAhIP but not from cells co-expressing the isoprenylation-deficient IP mutant or those expressing PDE6δ alone (Fig. 2D), demonstrating that similar to many other PDE6δ interactors, IP isoprenylation was required for its interaction with PDE6δ. Concordantly, treatment of cells with lovastatin (20 μM), which inhibits IP isoprenylation (25), also prevented IP-PDE6δ interaction (data not shown).
were co-transfected with PDE6IP-cAMP signaling was unaltered when IP-transfected HEK293 cells were subjected to immunoprecipitation with anti-HA antibody and co-immunoprecipitated V5-PDE6. Western blots are representative of three independent experiments.

**Effect of PDE6δ Overexpression on IP-mediated cAMP Generation**—Impaired effector activation via IPSSLC has been demonstrated (24) leading those authors to conclude that isoprenylation of IP was critical for its coupling to downstream G proteins. The dependence of the IP-PDE6δ interaction on IP isoprenylation led us to examine whether PDE6δ modulates IP signaling. HEPG2 cells, transfected with 3xHAhIP with or without PDE6δ, were stimulated with the prostacyclin analogue cicaprost for 5 min. A robust concentration-dependent increase in cAMP was observed in response to IP activation (EC50 = 0.11 ± 0.06 mM, n = 3). Overexpression of PDE6δ did not alter significantly IP-mediated cAMP generation (EC50 = 0.1 ± 0.06 mM, n = 3). In a similar manner IP-cAMP signaling was unaltered when IP-transfected HEK293 cells were co-transfected with PDE6δ-V5 (data not shown). Thus, the interaction with PDE6δ was not responsible for the signaling deficit associated with IP isoprenylation (24).

**Role of PDE6δ in IP Sequestration**—Although the precise function of PDE6δ remains unknown, its interaction with proteins such as the α and β subunits of PDE6, and the small G protein Rab13, resulted in their translocation from the membrane to the cytosol. We (5) and others (7) have reported that sequestration of activated IP does not operate through the classical phosphorylation/arrestin-dependent pathway associated with GPCRs. Indeed, while the pathways that direct IP trafficking remain ill-defined, a COOH-terminal truncated IP was not internalized following activation with agonist (5). Moreover, in contrast to a phosphorylation-deficient IP mutant (6), sequestration of the isoprenylation deficient IPSSLC mutant markedly was impaired (24), suggesting a role for isoprenylation in IP trafficking. The involvement of PDE6δ in IP internalization was assessed in HEPG2 cells transfected with 3xHAhIP with or without PDE6δ-V5. IP internalization in response to cicaprost (1 μM) occurred more rapidly in HEPG2 cells overexpressing PDE6δ; 15 min after agonist activation cell surface expression of 3xHAhIP was reduced to only 87.6 ± 7.5% in cells co-transfected with 3xHAhIP and PDE6δ (Fig. 3A; p < 0.01). Furthermore, PDE6δ overexpression altered the temporal pattern of IP internalization. In 3xHAhIP transfected HEPG2 cells, surface expression of IP declined steadily over a 2-h treatment period. In contrast, and similar to IP transfected HEK293 cells (5), which express endogenously PDE6δ (Fig. 2A), substantial IP internalization was evident after only 15 min of cicaprost treatment in HEPG2 cells co-transfected with PDE6δ. Indeed, in stark contrast to the PDE6δ-null HEPG2 cells, sequestration of IP was maximal at the 15-min time point and appeared reversible, with a significant return of surface IP as agonist exposure was extended (Fig. 3A; 82.9 ± 4.9% after 2 h versus 64.2 ± 7.5% after 15 min; p < 0.05).

**PDE6δ Has No Effect on IPSSLC Internalization**—We investigated next whether the effect of PDE6δ on IP internalization was mediated...
IP sequestration in its native setting.

when PDE6 resulted in almost complete abolition of PDE6 IP sequestration in hASMC, which endogenously express both proteins.

transfected cells (Fig. 4). Hence, PDE6 siRNA were treated with cicaprost (100 nM; 1 h) and cell surface expression of IP determined by binding of [3H]iloprost to intact cells. Data are presented as mean percent ± S.E. from three experiments, each performed in duplicate. **, p < 0.01 with reference to control unless otherwise indicated. Western blots are representative of three independent experiments.

through its direct interaction with the isoprenylated receptor. Cicaprost-induced internalization of the IPSSLCL mutant was observed in HEPG2 cells and occurred more rapidly than previously reported in HEK293 cells (24), likely reflecting cell type-specific differences in the relative importance of receptor trafficking pathways present in these cells. Importantly, however, and in direct contrast to the wild type IP, co-expression of PDE6δ did not modify internalization of the isoprenylation- and PDE6δ interacting, deficient IP mutant (IPSSLCL; Fig. 3B). Moreover, cicaprost-induced sequestration of IPSSLCL was indistinguishable from the wild type IP in the absence (open bars; Fig. 3, A and B) but not in the presence of PDE6δ (closed bars). Thus, divergence in the sequestration of the wild type and mutant receptors was evident only when PDE6δ was co-expressed, either endogenously (24) or ectopically (closed bars; Fig. 3, A and B). Taken together these data suggest strongly that interaction with PDE6δ was responsible for the defect in sequestration reported previously for the isoprenylation deficient mutant IP (24).

Effect of siRNA-mediated Knockdown of PDE6δ on IP Internalization in hASMC—We next wished to determine whether PDE6δ modulated IP sequestration in hASMC, which endogenously express both proteins (Fig. 4A and Ref. 21). siRNA targeted against PDE6δ (PDE6δ-siRNA) resulted in almost complete abolition of PDE6δ protein expression in hASMC, when compared with either untransfected or control siRNA transfected cells (Fig. 4A). Substantial IP internalization was observed in control siRNA-transfected hASMC following a 1-h treatment with 100 nM cicaprost (Fig. 4B; 79.3 ± 2.6%; p < 0.001 compared with cells without cicaprost treatment), with a similar response observed in untransfected cells (data not shown). Strikingly, cicaprost-induced IP internalization was abolished in hASMC transfected with PDE6δ-siRNA (Fig. 4B), demonstrating the importance of this novel pathway in facilitating IP sequestration in its native setting.

Effect of PDE6δ Overexpression on IP Recycling—A substantial number of sequestered GPCRs, including the IP (5, 7, 8), are recycled to the plasma membrane following agonist withdrawal. Having observed a biphasic change in surface expression of IP, in which the initial rapid decline was reversed with extended treatment in PDE6δ co-expressing HEPG2 cells, we wished to determine its contribution to IP recycling. As the rate of IP internalization differed significantly between PDE6δ-null and PDE6δ transfected cells, we employed those treatment conditions that evoked maximal and equivalent levels of sequestration in the two settings, namely 1 μM cicaprost for 1 h or 15 min, respectively, prior to agonist removal. Surface expression of IP was restored to control levels within 60 min of agonist withdrawal only in cells co-expressing IP with PDE6δ (Fig. 5), with levels remaining significantly depressed in PDE6δ-null cells (84.8 ± 6.2% of control, p < 0.05). In contrast, co-expression of PDE6δ did not induce recycling of IPSSLCL (Fig. 5), indicating that the effect of PDE6δ on IP recycling is a consequence of its interaction with the isoprenylated receptor.

DISCUSSION

We (5, 6) and others (7, 8) demonstrated that, similar to other GPCRs, IP undergoes rapid desensitization and internalization in response to agonist activation. However, IP does not utilize the classical GRK/arrestin-dependent pathways that are implicated commonly in regulation of this superfamily (4). Instead, desensitization is mediated through PKC-dependent phosphorylation (6), while IP sequestration occurred, in part, through a dynamin-dependent clathrin-coated vesicular pathway that did not employ arrestin as an adapter molecule (5). Determinants for both regulatory steps are located in the COOH-terminal tail region of the IP, although only in the case of PKC-dependent phosphorylation has the critical residue, Ser328, been identified (6). Although this particular site was not required for agonist-dependent sequestration of the IP, the COOH-terminal tail of the receptor proved indispensable for this regulatory step to proceed (5), suggesting that this region may provide a docking site for proteins involved in IP regulation (5). Indeed, novel GPCR interacting proteins, including some that contribute to receptor trafficking, have been reported previously (26, 27). Thus, we sought to identify proteins interacting with the IP COOH-terminal region and characterize their role in IP regulation.
Using the COOH-terminal region of IP as bait in a yeast two-hybrid screen of a hASMC cDNA library, we identified the δ subunit of PDE6 enzyme as a novel IP-interacting protein. The identity of this IP interactor was verified in a second independent screen, and the interaction was confirmed in two distinct mammalian cell lines, HEK293 and HEPG2 cells, transfected with HA- and V5- epitope-tagged versions of the hIP and PDE6δ, respectively. Co-immunoprecipitation PDE6δ-V5 was specific to the IP receptor and not an artifact either of its forced overexpression or the presence of the HA- or V5-tag, since under similar transfection conditions, in HEK293 cells, PDE6δ-V5 was not co-immunoprecipitated with HAHTPα (Fig. 1C).

Although first identified in the retina as a subunit of PDE6, a cGMP phosphodiesterase (28), its broad expression (14), marked conservation (19), and the relative promiscuity of its protein interactions (17–19) have led to recharacterization of PDE6δ, not as an PDE6 subunit but rather an associated protein with a more general role in cell signaling (29). Having no catalytic activity of its own, PDE6δ interacts with the isoprenylated, COOH termini of PDE6 α and δ subunits, resulting in the solubilization of the holoenzyme from the membrane to the cytosol. Similarly, PDE6δ interacts members of the Ras, Rap, Rhö, and Rab families of small G proteins resulting in their membrane extraction and fulfilling a role reminiscent of a guanine nucleotide dissociation inhibitor (16, 17). In contrast to these inhibitory roles, PDE6δ reportedly acted as an effector for two other small G proteins, Arl2 and Arl3 (17), both of which appear to play a role in vesicular transport, interaction specifically with their GTP bound form.

To assess the biological function of IP-PDE6δ interaction we identified a cell model suitable for ectopic expression of both proteins. Most cell types examined expressed PDE6δ at a high level (Fig. 2A), concurrent with the widespread expression of the protein (14); however, HEPG2 cells demonstrated negligible expression (Fig. 2A). Thus HEPG2 cells, which also lack functional IP expression (data not shown), were selected as an ideal model to study the impact of PDE6δ on IP function.

The majority of the protein partners of PDE6δ (11, 17), including the IP (24), identified to date are modified post-translationally by the addition of an isoprenoid group at the so-called “CAAX” motif where A stands for an aliphatic amino acid and X stands for any amino acid. Mutation of IPs “CAAX” motif, from Cys383-Ser-Leu-Cys386 to Ser383-Ser-Leu-C386 rendered the receptor insensitive to isoprenylation (24) and ablated its interaction with PDE6δ (Fig. 2D), linking the two processes. It should be noted that although a frequent requirement, isoprenylation is not always necessary for interaction with PDE6δ; interaction with the Arl proteins can proceed without their isoprenylation (17), while, though isoprenylated, neither Rab4 nor Rab6 are capable of interaction with PDE6δ (16). Thus PDE6δ, though promiscuous, is not indiscriminate in its associations and likely realizes consequences relevant to the function of its specific partners including the IP.

Isoprenylation appears to be a critical post-translational modification in the biosynthesis of IP; the isoprenylation-deficient IPSSLΔ mutant receptor does not couple efficiently to its requisite G protein (24), making the IP unusual among GPCRs. At least one heterotrimeric G protein subunit, Gδ (17), has been added to PDE6δ’s repertoire of associated proteins, raising the possibility of its involvement in potentially complex interactions within the microenvironment of a GPCR. Signaling of IP to activation of adenylyl cyclase was unaltered, however, by co-expression of PDE6δ in either HEK293 cells (data not shown) or HEPG2 cells, suggesting that the interaction with PDE6δ does not contribute directly to signaling events immediately downstream of IP activation, at least in relation to the Gδ-adenyl cyclase cascade. Thus, although IP isoprenylation is necessary for its interaction with both Gα (24) and PDE6δ (Fig. 2D), the two processes are not functionally linked. We have not examined the role PDE6δ in modulating IP signal transduction through additional signaling pathways with which it has been associated, namely Gq (22) and, in the case of the mouse receptor, Gβ (30). However, the biological relevance of IP signaling via pathways other than cAMP generation is not apparent.

Although the precise role of PDE6δ remains unclear, it’s ability to solubilize proteins from the membrane into the cytosol has led to the suggestion that it is involved principally in the regulation of protein transport. Indeed, many of PDE6δ’s protein partners are themselves involved in vesicular transport. Thus, Arl family proteins have been implicated in transport to and from the trans-Golgi network (31), while Rab13 mediates the continuous recycling of occludin to the cell surface (32). The isoprenylation-deficient IPSSLΔ mutant, which we have determined is incapable of interacting with PDE6δ (Fig. 2D), is also defective in terms of agonist-induced sequestration (24). As such we examined whether IP-PDE6δ might modulate the agonist-induced internalization of IP, a process that remains ill-defined.

Ectopic expression of PDE6δ in HEPG2 cells facilitated the rapid, and at least partially reversible, internalization of IP in the presence of agonist (Fig. 3A). These observations were not related to extraneous changes in the expression level of functional IP due to co-expression of PDE6δ, since the maximal CAMP generation in both settings was identical. It may be argued that, since PDE6δ interacts with a number of proteins involved in vesicular transport, modification of IP internalization occurred secondary to a general PDE6δ trafficking effect. However, interference with the IP-PDE6δ interaction, by mutation of Cys383, would not prevent these additional PDE6δ interactions, yet trafficking of the IPSSLΔ mutant was unaltered by co-expression of PDE6δ (Fig. 3B). This finding argues against a generalized vesicular transport effect of PDE6δ and for a shift in IP trafficking directed specifically by its interaction with the isoprenylated COOH terminus of IP. Moreover, the similarity with which the wild type and mutant IP internalized in PDE6δ-null HEPG2 cells (open bars; Fig. 3, A and B), together with the inability of PDE6δ to modify sequestration of the mutant (Fig. 3B), suggests strongly that the inability of the mutant to interact with PDE6δ in HEK293 cells was responsible for the trafficking impairment reported in that model (24).

While rapid ligand-dependent IP sequestration has been demonstrated previously in platelets (33), neither the endocytotic pathways utilized during, nor the regulatory elements involved in IP internalization, have been identified in cells, which like platelets, endogenously express the receptor. We examined the contribution of PDE6δ in facilitating IP internalization in hASMC, a cell type relevant to IP-mediated cardiovascular protection (1, 34), using siRNA-mediated knockdown. Strikingly, while significant cicaprost-induced IP internalization was observed in hASMC transfected with control siRNA, the response to cicaprost was abolished in hASMC depleted of PDE6δ (Fig. 4B), supporting a specialized role for PDE6δ in IP trafficking in endogenously expressing cells.

In addition to modulation of receptor sequestration, recycling of IP upon removal of agonist, a phenomenon that restores responsiveness in both transfected (5) and native cells (7, 8), was enhanced by PDE6δ (Fig. 5), secondary to its specific interaction with the isoprenylated IP. We did not extend this time course beyond 2 h and thus have not considered the potential impact of PDE6δ on restoration of surface IP expression by longer term mechanisms including de novo protein synthesis (7, 35). However, it appears that the rapid return of IP to the cell surface was directed, at least in part, by PDE6δ.
The apparent reversibility of IP internalization in PDE6δ-overexpressing HEPG2 cells may similarly be explained by enhanced IP recycling. Receptor trafficking is a dynamic process, with ligand-induced internalization and constitutive recycling occurring in parallel (36). The absolute density of IP present at the plasma membrane at any given point following its activation is determined by a combination of these processes. In HEK293 cells, IP utilizes multiple internalization pathways (5), contributing to post-endocytic receptor sorting and the rapidity with which IP recycles to the surface. Indeed, PDE6δ appears to direct the IP along a pathway characterized by rapid internalization (Fig. 3A) and recycling (Fig. 5). Overexpression of PDE6δ may force this pathway to predominate committing the IP to rapidly recycle to the cell surface following its sequestration, rendering ligand-dependent internalization reversible.

We have not examined directly the consequence of this altered trafficking pathway for IP signaling; such investigations are not generally informative because the absolute cellular level of a second messenger, in this case cAMP, measured so long after initial activating event, reflects multiple cellular mechanisms, such as hydrolysis by cAMP-specific phosphodiesterases, and indirect kinase-driven effects on receptor and/or effector activity and is therefore not a faithful readout of receptor function. Instead we have focused on events that modulate cell surface expression of the receptor, a critical determinant of both termination and restoration of receptor activation. The marked shift in trafficking of IP evoked by co-expression, native or ectopic, of PDE6δ and restoration of receptor activation. The marked shift in trafficking of IP evoked by co-expression, native or ectopic, of PDE6δ and restoration of receptor activation. The relative predictability of IP evoked by co-expression, native or ectopic, of PDE6δ and restoration of receptor activation. The marked shift in trafficking of IP evoked by co-expression, native or ectopic, of PDE6δ and restoration of receptor activation.

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