Phosphorylation of the Prostacyclin Receptor during Homologous Desensitization

A CRITICAL ROLE FOR PROTEIN KINASE C*

E. M. Smyth, W. Hong Li, and G. A. FitzGerald‡

From the Center for Experimental Therapeutics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Agonist-induced phosphorylation of an epitope-tagged prostacyclin receptor (HAhIP) is mediated primarily by PKC (Smyth, E. M., Nestor, P. V., and FitzGerald G. A. (1996) J. Biol. Chem. 271, 33698–33704). Based on the two consensus sites for protein kinase C (PKC) phosphorylation in the C-terminal region mutant HA-hIPs were generated: S328A and S374A, in which an alanine replaced Ser-328 or Ser-374, respectively, S328A/S374A and C-DEL, in which the C-terminal portion was truncated at amino acid 313. Mutant receptors, stably expressed in HEK293 cells, coupled normally to cAMP production. Subsequently less coupling to inositol phosphate was apparent with S328A, S328A/S374A, and C-DEL compared with HAhIP or S374A. Point mutants resolved by SDS-polyacrylamide gel electrophoresis as a broad band with a molecular mass of 44–62, indicating that the receptors are glycosylated, and immunofluorescence staining demonstrated their membrane localization. C-DEL demonstrated a substantial reduction in glycosylation; bands with molecular masses of 38–54 (glycosylated), 30, and 27 kDa (unglycosylated) were apparent. Although membrane localization was evident, cellular localization was more diffuse. HAhIP and S374A underwent iloprost- and PMA-induced phosphorylation (1 and 5 μM, respectively, for 10 min). S328A and S328A/S374A showed a markedly less iloprost- and no PMA-induced phosphorylation. Phosphorylation of C-DEL was completely absent with either agonist. Electrospray mass spectrometry indicated that a peptide, including Ser-328, was phosphorylated in vitro by PKC, whereas one including Ser-374 was not.

Iloprost (1 μM, 10 min) desensitized HAhIP- and S374A-mediated adenylyl cyclase activation. A less impressive desensitization was evident with S328A and S328A/S374A, and no desensitization of C-DEL coupling was apparent. Exposure of transfected cells to iloprost (1 μM) for increasing times induced a rapid desensitization of subsequent iloprost-induced (1 μM) HAhIP and S374A adenylyl cyclase coupling. In contrast, no significant time-dependent desensitization of S328A, S328A/S374A, or C-DEL coupling was evident. These results indicate that PKC-dependent phosphorylation is of critical importance to homologous regulation of hIP, Ser-328 is a primary site for PKC phosphorylation of hIP.

The human prostacyclin receptor (hIP),† thought to mediate the potent anti-platelet, vasodilator, and anti-inflammatory actions of prostacyclin, is a member of the G protein-coupled receptor (GPCR) superfamily. Studies have demonstrated that the IP is coupled to stimulation of both adenylyl cyclase and phospholipase C (PLC) (1–3).

In general, the response to GPCRs tends to be tightly regulated by desensitization. This involves rapid receptor phosphorylation, which results in uncoupling of receptor-G protein interactions (4) and subsequent receptor internalization. These events result in a diminished response to agonist. Therefore, GPCRs may be degraded and/or recycled (5). Generally, at least two classes of kinases may participate in GPCR phosphorylation: second messenger-dependent kinases, such as PKC and protein kinase A, and the GPCR kinases (GRKs) (4). For example, β-adrenoreceptors may be phosphorylated and desensitized by the sequential action of protein kinase A and GRKs 2 and 3 (6, 7). Similarly, the thrombin (8) and angiotensin II-1A receptors (9) are phosphorylated in turn by PKC and GRKs.

The pathways involved in eicosanoid receptor regulation are not well understood, although stimulation-dependent phosphorylation and/or desensitization has been reported in the case of some prostaglandin E2 receptors (10, 11) and the thromboxane receptor (12, 13). We have demonstrated that this is also true of hIP (3). Phosphorylation of the hIP is primarily PKC-dependent, when it is overexpressed in vitro. In this study, we investigated further the pathways involved in hIP regulation and their implications for regulation of receptor function. Mutants of the hemagglutinin (HA)-tagged hIP were used to investigate the role of phosphorylation in desensitization of the hIP. We identified a single site of PKC-dependent phosphorylation as a critical determinant for desensitization of this biologically important (14) receptor.

EXPERIMENTAL PROCEDURES

Materials—Iloprost, the cAMP radioimmunoassay and the enhanced chemiluminescence kits, as well as all radiochemicals, were purchased from Amersham Pharmacia Biotech. Monoclonal anti-HA (16B12 clone) was obtained from the Berkley Antibody Company (Richmond, CA). A Transformerson site-directed mutagenesis kit was purchased from CLONTECH (Palo Alto, CA) and the TA cloning kit and pcDNA III were obtained from Invitrogen (San Diego, CA). Deoxycholic acid, isobutylmethylxanthine, ATP, cAMP, GTP, alumina, phosphatidylycholine, and phosphatidylserine were all purchased from Sigma. All cell culture reagents, G418, and Albumax were obtained from Life Technologies, Inc. Protein G-Sepharose was purchased from Amersham Pharmacia

† The abbreviations used are: hIP, human prostacyclin receptor; IP, prostacyclin receptor; PLC, phospholipase C; GPCR, G protein-coupled receptor; PKC, protein kinase C; GRK, GPCR kinase; HA, hemagglutinin; S328A, HAhIP/S328A; S374A, HAhIP/S374A; S328A/S374A, HAhIP/S328A/S374A; C-DEL, HAhIP/CDEL; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBS-T, TBS-Tween; PMA, phorbol 12-myristate 13-acetate.

‡ Robinette Foundation Professor of Cardiovascular Medicine. To whom correspondence should be addressed: Center for Experimental Therapeutics, University of Pennsylvania, 905 Stellar Chance Laboratories, 422 Curie Blvd., Philadelphia, PA 19104-6100. Tel.: 215-898-6446; Fax: 215-898-9004; E-mail: garret@spirit.gcrp.upenn.edu.

This paper is available on line at http://www.jbc.org
resulting pellet was resuspended in the same buffer and stored at -80 °C for further use. Cells grown in slide chamber assemblies (Nunc, Naperville, IL) were fixed and permeabilized with ice-cold 70% methanol, 30% acetone for 10 min at 4 °C. Membrane fractions collected at 115,000 × g (1 h at 4 °C). The resulting pellet was resuspended in the same buffer and stored at -80 °C for further use.

**Immunocytochemistry**—Cells grown in slide chamber assemblies (Nunc, Naperville, IL) were fixed and permeabilized with ice-cold 70% methanol, 30% acetone for 10 min at -20 °C and 5 min at room temperature. Fixed sections were treated with anti-HA (1:2000) in phosphate-buffered saline for 1 h at room temperature, and after being washed three times, each for 10 min, in phosphate-buffered saline, antigen-antibody complexes were visualized with a fluorescein isothiocyanate-labeled anti-mouse IgG antibody (1:500, Jackson Immunology, Westgrove, PA). Slides were mounted in Vectashield (Vector Labs, Burlingame, CA) and examined by fluorescence or confocal microscopy.

**cAMP Measurements**—Cells grown to confluence in 12-well plates were treated with isoproterenol (10 μM at 37 °C). Reactions were terminated by aspiration and cAMP extracted with ice-cold 75% ethanol for 30 min. Samples were dried under vacuum, reconstituted in assay buffer, and cAMP was quantified by radioimmunoassay (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. An exception was that half the recommended amounts of binding protein and ^{3}H-cAMP tracer were used in the samples and standards.

**Adenylyl Cyclase Assay—**Adenylyl cyclase activity was assayed by modification of the method of Solomon et al. (15). Assays were carried out in 50 μl Tris containing 3 mM MgCl₂, 0.15 mM EDTA, 0.05 mM GTP, 0.1 mM cAMP, 2.8 mM phosphoenolpyruvate, and 0.1 mM isobutylmethylxanthine. Each reaction contained 1 unit of myokinase, 1 unit of pyruvate kinase, and 2 μCi [γ-^{3}H]ATP (30 Ci/mmol) reactions were started by the addition of membranes (5 μg assay tube), prepared as outlined above, and after 30 min at 30 °C, were quenched by the addition of 1 ml of 5% trichloroacetic acid containing 30,000 CPM [^{3}H]cAMP (41 Ci/mmol). Samples were subjected to sequential chromatography through Dowex (AG W-X4, hydrogen form) and alumina (W-N, neutral) columns as described (15). Briefly, samples were eluted onto alumina with 3 ml of H₂O following application to Dowex columns and one wash with 2.5 ml of H₂O. CYClic AMP was eluted from the alumina columns with 4 ml of 0.1 M imidazole (pH 7.5). ^{32}P and ^{3}H in the eluates were estimated by scintillation counting. Recovery of cAMP from the columns was monitored by measuring the level of [^{3}H]cAMP in each sample.

**Inositol Phosphate Production**—Cells grown to 70–80% confluence in 12-well plates coated with 0.2% gelatin were labeled overnight with 2 μCi/ml myo[^3]H]inositol in DMEM (without inositol) containing 0.5% albumax, 50 units/ml penicillin, and 50 μg/ml streptomycin. 30 min before stimulation, cells were treated with 20 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 for 30 min at room temperature. Samples were neutralized (final pH 8–9) with 3 ml 10 mM ammonia. Total inositol phosphates were recovered by anion exchange using Dowex 1-X8 AG anion exchange resin (formate form). Samples were applied to the resin, washed with 40 μl formic acid/ammonium formate (pH 5) (16). The inositol phosphates were eluted with 2 μl formic acid/ammonium formate (pH 5) (16).
mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 1 tablet/50 ml complete protease inhibitor mixture, 10 mM sodium fluoride, and 10 mM NaH$_2$P$_2$O$_7$), drawn though a 23-gauge needle 6 times and centrifuged at 14,000 rpm. The resulting supernatants were precleared by adding 100 μl of 10% (w/v) protein G-Sepharose to each tube and rotating for 60 min. Anti-HA-protein G-Sepharose was prepared by adding 1 μl anti-HA ascites per lysate to 10% protein G-Sepharose and rotating it for 60 min. The HAhIP was immunoprecipitated from pre-cleared lysates by adding 100 μl of the anti-HA-protein G-Sepharose to each lysate and rotating for 2 h. Protein G was precipitated at 14,000 rpm for 1 min, washed three times with RIPA buffer, and finally resuspended in 60 μl of SDS-PAGE sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.002% bromphenol blue, 100 mM dithiothreitol). Samples were then boiled for 10 min and subjected to electrophoresis as outlined above. Gels were dried for phosphorimaging.

In Vivo IP Phosphorylation—Cells were plated in 60-mm dishes and grown to 70–80% confluence. 32P-labeling was carried out using 150–200 μCi/ml [32P]orthophosphate in phosphate-free DMEM containing 0.5% bovine serum albumin (Fisher, Malvern, PA) for 60 min at 37 °C. Labeled cells were treated with the stimulant. Dishes were placed on slides were permeabilized and stained with anti-HA antibody. A, all HAhIP mutants were examined using standard immunofluorescent microscopy. B, confocal micrographs were taken from the top and through the center of C-Del-transfected cells. Arrows highlight several cells where membrane staining was evident in both planes of view.

**FIG. 3.** Immunofluorescence staining of HEK 293 cells expressing HA-hIP or mutant receptors. Cells plated on slides were permeabilized and stained with anti-HA antibody. A, all HAhIP mutants were examined using standard immunofluorescent microscopy. B, confocal micrographs were taken from the top and through the center of C-Del-transfected cells. Arrows highlight several cells where membrane staining was evident in both planes of view.
ice, and the overlying medium was removed at the end of the reaction. Cells were lysed with RIPA, following one wash with ice-cold phosphate-buffered saline, and the HAhIP was immunoprecipitated as outlined above. Following phosphorimaging of dried gels, rectangles of equal size were drawn around each of the bands observed, and the intensity quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

In Vitro IP Phosphorylation—Based on the PKC consensus site sequences found in hIP, peptides containing either serine 328 (hIP328: LASGRDRDPRAP) or serine 374 (hIP374: AGVTTSSKAE) were synthesized. A third peptide, hIP230 (LGPRPRTGEDEV), which does not contain a consensus site for PKC phosphorylation was also generated.

**TABLE I**

|                | cAMP production | Inositol phosphate production |
|----------------|-----------------|-------------------------------|
| HAhIP          | 0.10 ± 0.03 (n = 3) | 78.4 ± 19.4 (n = 4)         |
| S374A          | 0.12 ± 0.07 (n = 3) | 78.8 ± 10.8 (n = 5)       |
| S328A          | 0.22 ± 0.09 (n = 4) | 142.98 ± 36.8 (n = 4)     |
| S328A/S374A    | 0.11 ± 0.04 (n = 3) | 192.3 ± 11.9 (n = 3)      |
| C-DEL          | 0.27 ± 0.14 (n = 3) | 169.0 ± 35.1 (n = 4)      |

**RESULTS**

Expression of HAhIP Mutants—Western blot analysis (Fig. 2) of membranes prepared from HEK 293 cells transfected with HAhIP or its mutants demonstrated that, similar to the non-mutated receptor (HAhIP), each of the point mutants resolved as a broad band with a molecular mass from 44 to 66 kDa. The mass of C-DEL was shifted to a 38–54-kDa band, indicating the loss of 74 amino acids from the C-terminal tail. Two lower molecular weight species (30 and 27), not seen with any of the

**Fig. 4. Intracellular cAMP production in HEK 293 cells expressing HAhIP or mutant receptors.** Cells were treated with increasing concentrations of iloprost for 10 min, and cellular cAMP was extracted and quantified as outlined under “Experimental Procedures.” Basal (B) levels were measured in the absence of agonist stimulation. Data are from one experiment in duplicate, which was repeated 3–4 times with similar results.

**Fig. 5. Total inositol phosphate production in HEK 293 cells expressing HAhIP or mutant receptors.** Cells were treated with increasing concentrations of iloprost for 10 min, and inositol phosphates extracted and quantified as outlined under “Experimental Procedures.” Basal (B) levels were measured in the absence of agonist stimulation. Data are from one experiment in duplicate, which was repeated 3–5 times with similar results.
other receptors, were evident in membranes prepared from C-DEL-transfected cells. The level of expression of the mature receptor was similar in HAhIP-, S328A-, S374A-, and S328A/S374A- and apparently lower in C-DEL- transfected cells. Immunofluorescence staining of transfected cells showed the expected pattern of membrane localization of all mutant receptors (Fig. 3A). This was similar to HAhIP-transfected cells for all point mutant receptors (S328A, S374A, and S328A/S374A), whereas somewhat more diffuse staining was apparent in cells transfected with C-DEL. Analysis of HAhIP and C-DEL localization by confocal microscopy confirmed the membrane localization of both receptors, although cytosolic localization of the latter was also apparent (Fig. 3B).

Second Messenger Coupling of HAhIP Mutants—We have previously shown that activation of HAhIP in this system leads to an increase in both intracellular cAMP and inositol phosphates (3). As demonstrated in Fig. 4A, treatment of transfected cells with increasing concentrations of iloprost caused an increase in cAMP levels. Coupling of HAhIP and mutant receptors to cAMP production was comparable (Fig. 4, B–E). Basal levels (0.7–2.2 pmol cAMP/10⁶ cells) and EC₅₀ values for iloprost (Table I) were comparable between each of the cell lines. Thus, all receptors were functional, and the mutations did not substantially affect receptor coupling to the Gₛ-cAMP pathway.

Iloprost stimulated inositol phosphate production in HAhIP- and S374A-transfected cells with similar potency and efficacy (Fig. 5, A and B and Table I). However, stimulation of inositol phosphate production reached only 2–3-fold over basal in cells transfected with S328A, S328A/S374A, and C-DEL (Fig. 5, C–E) and EC₅₀ values for iloprost (Table I) were slightly higher. Similar changes in coupling to inositol phosphate production were seen with a second prostacyclin analog, cicaprost (data not shown).

Phosphorylation of HAhIP Mutants—Previously, we reported a rapid iloprost-stimulated phosphorylation of HAhIP (3). A similar pattern of rapid phosphorylation was evident only with cells transfected with the S374A mutant (Fig. 6A). Both HAhIP- and S374A-transfected cells showed a 4–6-fold increase in phosphorylated receptor following treatment with iloprost for increasing time intervals. The minor dip evident in the S374A time course is probably a result of loading errors because this was not a consistent observation. Furthermore, PMA-induced phosphorylation also occurred in these cells (Fig. 6B), indicating that PKC-mediated phosphorylation occurs despite the substitution of serine 374. In contrast, both S328A- and S328A/S374A-transfected cells showed a marked reduction of iloprost-induced phosphorylation. Although iloprost-induced phosphorylation occurred over the same rapid time course, maximum increases of only 2–3-fold were achieved (Fig. 6A). PMA-induced S328A and S328A/S374A phosphorylation was completely absent (Fig. 6B). Cells transfected with C-DEL
showed no phosphorylation with either agonist. These data indicate that serine 328 is the locus of iloprost-induced PKC-mediated rapid HAhIP phosphorylation.

In vitro phosphorylation of hIP peptides corroborated this finding. Three hIP peptides were used in these experiments: hIPS328 and hIPS374, containing the consensus sites for PKC phosphorylation, and hIPT230, containing a threonine residue that is not in a PKC consensus sequence. hIPS328 was phosphorylated by PKC, but hIPS374 and hIPT230 were not (Fig. 7). A known PKC substrate, α-pseudosubstrate peptide (18), was used as a positive control. Analysis of the peptides by mass spectrometry confirmed these findings (Fig. 8). In the case of the α-pseudosubstrate peptide, roughly 50% of the total peptide was phosphorylated. Less than 1% of the hIPS328 peptide, by contrast, was phosphorylated. However, no phosphorylation of the hIPS374 or hIPT230 peptides was detected.

Desensitization of HAhIP mutants—Pretreatment with iloprost induced rapid desensitization of receptor coupling to adenylyl cyclase in membranes prepared from HAhIP-transfected cells (Fig. 9). Treatment of cells for 10 min with 1 μM iloprost, conditions which maximally phosphorylate the receptor (Fig. 6 and Ref. 3), induced a striking reduction in concentration-dependent iloprost-stimulated adenylyl cyclase activation (Fig. 9A). This effect was rapid; it was evident after only a 15 s treatment (Fig. 9B). Substitution of serine 374 with alanine had minimal impact on either desensitization of the concentration-dependent response of membrane adenylyl cyclase to iloprost (Fig. 10A), or the time course of desensitization to 1 μM iloprost (Fig. 11A). In contrast, truncation of the C-terminal tail completely prevented iloprost-induced desensitization (Figs. 10D and 11D). Substitution of serine 328 with alanine either alone or in combination with serine 374, resulted in a markedly reduced level of desensitization. Although pretreatment with 1 μM iloprost produced some abrogation of iloprost-stimulation of adenylyl cyclase in S328A- and S328A/S374A-transfected cells, this was only evident at lower agonist concentrations (Fig. 10, B and C). In addition, pretreatment of S328A- or S328A/S374A-transfected cells with 1 μM iloprost for increasing times did not produce a significant level of desensitization, in contrast to HAhIP and S374A-transfected cells (Fig. 11, B and C).

Similar to adenylyl cyclase coupling, pretreatment of cells with iloprost also desensitized coupling of HAhIP and S374A to inositol phosphate production (data not shown). Accurate determination of the desensitization of this response in cells expressing S328A, S328A/S374A, or C-DEL was not possible, because of the already low level of iloprost-stimulated inositol phosphate generation achieved with these mutants.
expressed as the mean ± S.E., and are from 3–7 experiments each carried out in duplicate. Where error bars are not evident, they are obscured by the plot symbol.

**Fig. 10. Desensitization of mutant receptors.** Adenylyl cyclase activity was stimulated with increasing concentrations of iloprost as indicated, in membrane preparations from cells transfected with (A) S374A, (B) S328A, (C) S328A/S374A or (D) C-DEL and pretreated with (open circles) or without (closed circles) 1 μM iloprost for 10 min. Data are expressed as the mean ± S.E., and are from 3–7 experiments each carried out in duplicate. Where error bars are not evident, they are obscured by the plot symbol.

**DISCUSSION**

The hIP is rapidly desensitized in response to agonist stimulation. Furthermore, PKC-mediated phosphorylation is of primary importance in hIP regulation and the site(s) of phosphorylation are located in the C-terminal region of the receptor. These observations offer initial insight into the molecular basis of IP desensitization. Given the recently highlighted importance of this eicosanoid response and in cardiovascular biology (14) and the prospect of tachyphylaxis complicating chronic therapy with novel IP agonists, regulation of the response to IP stimulation is an issue of biological significance.

Four mutant HAhIP were generated (Fig. 1) and transfected into HEK 293 cells. Point mutants (S328A, S374A, and S328A/S374A) were expressed at levels similar to the nonmutated receptor, localized to the cell membrane, and coupled normally to the Gαs-adenylyl cyclase pathway. In contrast, immunofluorescence studies and Western blotting suggest that truncation of the C-terminal tail has some effect on receptor processing and localization. We have previously shown that the HAhIP is glycosylated in its mature form and that deglycosylation shifts the molecular weight from 44–62 kDa to 39 kDa (3). Although a reasonable level of mature, and presumably glycosylated, C-DEL receptor was expressed in HEK 293 cells transfected with the C-DEL mutant (Fig. 2), a substantial amount of the protein was at lower molecular weights, most likely because of a failure of normal glycosylation. The lack of complete receptor processing appeared to have some effect on membrane localization of the C-DEL mutant. Although some of the receptor was inserted into the membrane, as expected, immunofluorescent staining indicated a more diffuse pattern of expression, suggesting incomplete membrane localization. Studies with GPCRs have not examined the role or mechanism of receptor glycosylation. Analysis of the hIP amino acid sequence has indicated that glycosylation would occur on the extracellular N-terminal of the protein (1). It is unclear why deletion of the intracellular tail of the receptor should affect a post-translational modification on the extracellular N-terminal region of the protein, and why or how this can affect subcellular localization. These questions are currently under investigation. Whatever the reason for reduced expression of the mature processed receptor, coupling to adenylyl cyclase appears to be unaffected. Iloprost-stimulated cAMP production in C-DEL-transfected cells is comparable with that in cells transfected with HAhIP.

Although having no affect on iloprost-stimulated cAMP production, loss of serine 328, either as a point mutation to an alanine or as part of a C-terminal tail deletion, dramatically reduces agonist-stimulated inositol phosphate production. The reasons for this are not clear. It may be that this amino acid is crucial for coupling of HAhIP to this pathway. Alternatively, removal or substitution of serine 328 with alanine may alter a portion of the receptor in such a way that it can no longer couple to the particular G protein(s) involved in this pathway. For example, point mutations of single residues in the C-terminal tail largely abolish angiotensin II-1A receptor coupling to Gαs (19). Preservation of Gαs coupling to the Ser-328 mutants raises the possibility that different regions of the receptor may mediate coupling to different G proteins. A further and intriguing possibility is that PKC-mediated phosphorylation, absent in the serine 328 and C-DEL mutants, is required to switch HAhIP coupling from one G protein (Gαs) to another, as was shown recently for protein kinase A-mediated phosphorylation of the β2-adrenoreceptor (20). It is not clear how this switch of receptor coupling to PLC activation could be dependent on PKC phosphorylation of the receptor, because this kinase is activated downstream of the PLC-inositol phosphate-Ca2+ cascade in the first place. However, studies indicate that PKC can be
Adenylyl cyclase activity was stimulated with 1 μM iloprost in membrane preparations from cells transfected with S374A (A), S328A (B), S328A/S374A (C), or C-DEL (D) and pretreated with or without (zero time) 1 μM iloprost for increasing times as indicated. Data are expressed as the mean ± S.E. from three to four experiments each carried out in duplicate. Where error bars are not evident, they are obscured by the plot symbol.

The S328A and S328A/S374A mutants, both of which demonstrated phosphorylation of HAhIP phosphorylation, but it is possible that it is PLC-independent. Abrogation of receptor phosphorylation, such as that seen with the serine 328 and C-DEL mutant receptors, may subsequently interfere with coupling to more than one G protein.

Iloprost-induced HAhIP phosphorylation was significantly reduced in serine 328, and completely prevented in C-DEL mutant cell lines. Studies have demonstrated that the C-terminal tail and 3rd intracellular loop are the primary regions where phosphorylation of GPCRs tends to occur (4). It appears from the present work that the site(s) of HAhIP phosphorylation in response to agonist stimulation are located in the C-terminal tail and that serine 328 may be the locus of PKC-mediated phosphorylation. In agreement with this suggestion, phosphorylation, albeit at low levels, of the hIP3S74 peptide was established by mass spectrometry. Although it may be argued that the reduction in inositol phosphate coupling, and thus by inference PKC activation, could account for the reduction in iloprost-induced serine 328 and C-DEL mutant phosphorylation, this does not appear to be an adequate explanation for several reasons. First, the concentration of iloprost used in the phosphorylation studies (1 μM), increased inositol phosphate production by roughly 2–3-fold (Fig. 5). Thus, it would be expected that under the conditions employed in the phosphorylation and desensitization experiments, some PLC and therefore PKC activation, should have occurred. Second, we have shown phosphorylation of HAhIP at concentrations of iloprost that produced an increase in inositol phosphate in this range (3). Thus, it is likely that there would be sufficient PKC activation to phosphorylate the mutant receptors, despite a reduced coupling efficiency to inositol phosphate production. Finally, in contrast to HAhIP and S374A, serine 328 and C-DEL mutants did not undergo PKC-mediated phosphorylation when the kinase was activated directly with PMA or indirectly with thrombin (data not shown). This indicates that regardless of PLC coupling, mutation of serine 328 led to a loss of PKC-mediated phosphorylation of the HAhIP. It is also possible that the complete lack of phosphorylation of the C-DEL mutant may be related to its altered processing and localization. However, as outlined above, a substantial level of functional, membrane-associated C-DEL receptor was expressed, and because second messenger coupling was intact to a level that would induce phosphorylation, it would be expected to undergo phosphorylation in the same way at as the other receptors. Given the presence of several serine and threonine residues in the C-terminal region of the receptor and the previously established association of this region with GPCR phosphorylation and desensitization, it is more likely that the lack of phosphorylation was a result of the deletion of the sites where this modification occurs.

Although a marked reduction was observed, phosphorylation was not entirely abrogated in serine 328 mutants. Instead, an approximate 2-fold phosphorylation could be induced with iloprost in both the S328A and S328A/S374A cell lines. Consistent with these observations, pharmacological inhibition of PKC with GF109203X does not completely inhibit iloprost-stimulated HAhIP phosphorylation (3). The mechanism and significance of this residual component of hIP phosphorylation is unknown. Many GPCRs are phosphorylated both by second messenger-activated kinases (PKC or protein kinase A) and GRKs (6–9). It is presently unknown whether GRK-mediated phosphorylation of hIP occurs.

The pattern of phosphorylation closely correlated with desensitization of mutant receptors in response to pretreatment with iloprost. HAhIP and S374A, both of which are phosphorylated in response to iloprost, undergo significant desensitization when pretreated with agonist and both responses were observed as quickly as 15 s after addition of iloprost, whereas the nonphosphorylatable C-DEL does not undergo agonist-induced desensitization under the conditions of our experiments. The S328A and S328A/S374A mutants, both of which demonstrate a minor component of iloprost-induced phosphorylation but are not PKC-phosphorylated, do not desensitize to the same degree as the native hIP. However, a modest degree of desensitization does occur. It is unclear whether this is related to the GRK-mediated phosphorylation, which we speculate occurs with these mutants. A higher variability in the desensitization experiments was seen with the serine 328 mutants. This may reflect the low level of non-PKC-mediated phosphorylation in these receptors.

Although PKC appears to be the major phosphorylating and desensitizing kinase in this particular overexpression system, this may not be the case in all cells that normally express the hIP. For example, GRK-mediated phosphorylation may play a greater role in desensitization of endogenous receptors in vivo. Indeed, GRK-mediated regulation of GPCRs has been shown to be of major importance in the case of the β2-adrenoreceptors, where GRKs have an important role in modulating myocardial contractility in mice (23, 24). Furthermore, levels of GRK2 are increased in human heart failure (25) and hypertension (26). Whether or not the findings presented here reflect the regulation of endogenous IP must await studies in cells that normally express this receptor. However, the finding that hIP is a substrate for PKC raises the possibility of a role for this kinase in homologous and/or heterologous regulation of this receptor.

In summary, we provide the first evidence for the functional importance of phosphorylation in hIP desensitization. Recently, Murata et al. (14) have provided compelling data in IP-deficient mice for the importance of this eicosanoid in hemostatic and inflammatory responses in vivo (14). Given these
and other observations (27, 28), the response to prostacyclin is likely to be tightly regulated. Insight into the molecular basis of hIP desensitization may afford therapeutic opportunities to extend and/or to amplify the response to this homeostatic mediator in vivo.

Acknowledgment—We acknowledge Ginger Griffis for assistance with the cell culture.

REFERENCES

1. Boie, Y., Rushmore, T. H., Darmon-Goodwin, A., Grygorczyk, R., Sliptez, D. M., Metters, K. M., and Abramovitz, M. (1994) J. Biol. Chem. 269, 12173–12178
2. Namba, T., Oida, H., Sugimoto, Y., Kakizuka, A., Negishi, M., Ichikawa, A., and Narumiya, S. (1994) J. Biol. Chem. 269, 9986–9992
3. Smyth, E. M., Nestor, P. V., and FitzGerald, G. A. (1996) J. Biol. Chem. 271, 33696–33704
4. Freedman, N. J., and Lefkowitz, R. J. (1996) Recent Prog. Horm. Res. 51, 319–353
5. Koenig, J. A., and Edwards, J. M. (1997) Trends Pharmacol. Sci. 18, 276–278
6. Freedman, N. J., Liggett, S. B., Drachman, D. E., Pei, G., Caron, M. G., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 17953–17961
7. Hausdorff, W. P., Lohse, M. J., Bouvier, M., Liggett, S. B., Caron, M. G., and Coughlin, S. R. (1994) J. Biol. Chem. 269, 1125–1130
8. Ishii, K., Chen, J., Koch, W. J., Freedman, N. J., Lefkowitz, R. J., and Couglin, S. R. (1999) J. Biol. Chem. 274, 1125–1130
9. Opperman, M., Freedman, N. J., Alexander, R. W., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 15266–15272
10. Katoh, H., Watabe, A., Sugimoto, Y., Ichikawa, I., and Megishi, M. (1995) Biochim. Biophys. Acta 1244, 41–48
11. Bastepe, M., and Ashby, B. (1997) Mol. Pharmacol. 51, 343–349
12. Kinsella, B. T., O’Mahony, D. J., and FitzGerald, G. A. (1997) J. Biol. Chem. 269, 29914–29919
13. Habib, A., Vezza, R., Creminson, C., Maclouf, J., and FitzGerald, G. A. (1997) J. Biol. Chem. 272, 7191–7200
14. Murata, T., Usukuki, P., Matsuzaka, T., Hirata, M., Tamaska, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N., Uono, N., Oh-Ishi, S., and Narumiya, S. (1997) Nature (Lond.) 388, 678–682
15. Solomon, T., Londos, C., and Rodbell, M. (1994) Anal. Biochem. 58, 541–548
16. Seuwen, K., Lagarde, A., and Pouyssegur, J. (1998) EMBO J. 7, 161–168
17. Vezza, R., Habib, A., Li, H., Lawson, J. A., FitzGerald, G. A. (1996) J. Biol. Chem. 271, 30028–30033
18. Nakadate, T., Jeng, A. Y., and Blumberg, P. M. (1987) J. Biol. Chem. 262, 11507–11513
19. Sano, T., Ohyama, K., Yamano, Y., Nakagomi, Y., Nakazawa, S., Kikyo, M., Shirai, H., Blank, J. S., Exton, J. H., and Inagami, T. (1997) J. Biol. Chem. 272, 23631–23636
20. Daaka, Y., Lutrell, L. M., and Lefkowitz, R. J. (1997) Nature (Lond.) 390, 88–91
21. Nishizuka, Y. (1992) Neurosci. Res. 15, 3–5
22. Newton, A. C. (1995) J. Biol. Chem. 270, 11049–11058
23. Milano, C. A., Allen, L. F., Rockman, H. A., Dobler, P. C. McMinn, T. R., Chien, K. R., Johnson, T. D., Bond, R. A., and Lefkowitz, R. J. (1994) Science 264, 582–586
24. Koch, W. J., Rockman, H. A., Samama, P., Hamilton, R.-A., Bond, R. A., Milano, C. A., and Lefkowitz, R. J. (1995) Science 268, 1350–1353
25. Ungerer, M., Bohm, M., Elce, J. S., Erdmann, E., and Lohse, M. J. (1993) Circulation 87, 454–463
26. Gros, B., Benovic, J. L., Tan, C. M., and Feldman, R. D. (1997) J. Clin. Invest. 99, 2687–2693
27. Fisch, A., Tobusch, K., Veit, K., Meyer, J., and Darius, H. (1997) Circulation 96, 765–766
28. Giovannozzi, S., Acomazzzo, M. R., Letari, O., Oliva, D., and Nicosia, S. (1997) Biochem. J. 325, 71–77

PKC Critical Role in Prostacyclin Receptor Desensitization