The prostanoid prostacyclin (prostaglandin I₂), mainly produced by the vascular endothelium, plays a key role in the local control of vascular hemostasis acting as a potent inhibitor of platelet aggregation and as a vasodilator (1, 2). The actions of prostacyclin generally counteract those of thromboxane A₂, and thus, the relative levels of these two prostanoids in the circulation are central to the maintenance of vascular hemostasis and vessel tone (3). Prostacyclin also exhibits proinflammatory and antiproliferative properties in vivo (4, 5) and may confer a cytoprotective effect against tissue injury during acute myocardial ischemia or in response to hypoxia (6).

Prostacyclin signals through interaction with its signature G protein-coupled receptor (GPCR) termed IP (7). The prostacyclin receptor (IP) is primarily coupled to activation of adenylyl cyclase via G(S) (3, 8, 9). Additional evidence indicates that the IP may couple to multiple G protein:effector systems including G(q)-dependent activation of PLC and to mobilization of intracellular calcium (10–13). Iloprost, a stable carbacyclin analogue of prostacyclin, can stimulate the opening of ATP-sensitive K⁺ channels in hyperpolarization and relaxation of the canine carotid artery (14). The IP agonists iloprost and cicaprost were shown to stimulate the GTPase activity of both G₄ and Gₛ, suggesting that the IP expressed in human erythroleukemia cells may potentially lead to activation and inhibition of adenyl cyclase, respectively (15). On the other hand, the IP expressed in the rat medullary thick ascending limb coupled exclusively to G₄, inhibiting adenyl cyclase as opposed to Gₛ (16).

We have recently established that the IP may be unique among GPCRs in that it is isoprenylated (12). Whereas isoprenylation of mouse (m) IP is not required for ligand binding, it is absolutely required for its activation of adenyl cyclase and for its efficient coupling to PLC (12, 13). In addition, a number of studies have confirmed that the IP undergoes rapid agonist-induced receptor phosphorylation, internalization/sequestration, and down-regulation in human platelets and other cell types (10, 17–20).

Whereas the primary manifestation of GPCR phosphorylation and sequestration was classically thought to be desensitization or dampening of the signal following agonist engagement, there is increasing evidence that this is not exclusively so (21). In fact, it is now widely held, for certain GPCRs at least, that “desensitization of the primary signaling event” may trig-

* This research was supported by grants (to B. T. K) from The Irish Heart Foundation, Enterprise Ireland, The Wellcome Trust, and The Health Research Board of Ireland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry, Merville House, University College Dublin, Belfield, Dublin 4, Ireland. Tel.: 353-1-7161507; Fax: 353-1-2837211; E-mail: Therese.Kinsella@UCD.ie.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Merville House, University College Dublin, Belfield, Dublin 4, Ireland. Tel.: 353-1-7161507; Fax: 353-1-2837211; E-mail: Therese.Kinsella@UCD.ie.

Received for publication, May 16, 2001, and in revised form, June 28, 2001 Published, JBC Papers in Press, July 6, 2001, DOI 10.1074/jbc.M104434200

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Published, JBC Papers in Press, July 6, 2001, DOI 10.1074/jbc.M104434200

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available on line at http://www.jbc.org

Orlaith A. Lawler, Sinead M. Miggin, and B. Therese Kinsella‡

From the Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, Merville House, University College Dublin, Belfield, Dublin 4, Ireland

The prostacyclin receptor (IP) is primarily coupled to Goₛ-dependent activation of adenylyl cyclase; however, a number of studies indicate that the IP may couple to other secondary effector systems perhaps in a species-specific manner. In the current study, we investigated the specificity of G protein:effector coupling by the mouse (m) IP overexpressed in human embryonic kidney 293 cells and endogenously expressed in murine erythroleukemia cells. The mIP exhibited efficient Goₛ coupling and concentration-dependent increases in cAMP generation in response to the IP agonist cicaprost; however, mIP also coupled to Goᵢ decreasing the levels of cAMP in forskolin-treated cells. mIP coupling to Goᵢ was pertussis toxin-sensitive and was dependent on protein kinase (PK) A activation status. In addition, the mIP coupled to phospholipase C (PLC) activation in a pertussis toxin-insensitive, Goᵢ, Gβγ, and PKC-independent but in a Goₛ and PKA-dependent manner. Whole cell phosphorylation assays demonstrated that the mIP undergoes cicaprost-induced PKA phosphorylation. mIP Ser³⁵⁷, a site-directed mutant of mIP, efficiently coupled to Goₛ but failed to couple to Goᵢ or to efficiently couple to Goₛ, PLC. Moreover, mIP Ser³⁷⁵ did not undergo cicaprost-induced phosphorylation confirming that Ser³⁵⁷ is the target residue for PKA-dependent phosphorylation. Finally, co-precipitation experiments permitted the detection of Goₛ, Goᵢ, and Goᵢ in the immunoprecipitates of mIP, whereas only Goᵢ was co-precipitated with mIP Ser³⁵⁷ indicating that Ser³⁵⁷ of mIP is essential for Goᵢ and Goᵢ interaction. Moreover, inhibition of PKA blocked co-precipitation of mIP with Goₛ or Goᵢ. Taken together our data indicate that the mIP, in addition to coupling to Goₛ, couples to Goᵢ and Goᵢ; however, Goᵢ and Goᵢ coupling is dependent on initial cicaprost-induced mIP:Goₛ coupling and phosphorylation of mIP by cAMP-dependent PKA where Ser³⁵⁷ was identified as the target residue for PKA phosphorylation.

Protein Kinase A-mediated Phosphorylation of Serine 357 of the Mouse Prostacyclin Receptor Regulates Its Coupling to Gₛ⁻, to Gᵢ⁻, and to Gᵢ⁻, and to Gᵢ⁻, and to Gᵢ-coupled Effector Signaling*

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 276, No. 36, Issue of September 7, pp. 33596–33607, 2001

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available on line at http://www.jbc.org

The prostacyclin receptor (IP) is primarily coupled to Goₛ-dependent activation of adenylyl cyclase; however, a number of studies indicate that the IP may couple to other secondary effector systems perhaps in a species-specific manner. In the current study, we investigated the specificity of G protein:effector coupling by the mouse (m) IP overexpressed in human embryonic kidney 293 cells and endogenously expressed in murine erythroleukemia cells. The mIP exhibited efficient Goₛ coupling and concentration-dependent increases in cAMP generation in response to the IP agonist cicaprost; however, mIP also coupled to Goᵢ decreasing the levels of cAMP in forskolin-treated cells. mIP coupling to Goᵢ was pertussis toxin-sensitive and was dependent on protein kinase (PK) A activation status. In addition, the mIP coupled to phospholipase C (PLC) activation in a pertussis toxin-insensitive, Goᵢ, Gβγ, and PKC-independent but in a Goₛ and PKA-dependent manner. Whole cell phosphorylation assays demonstrated that the mIP undergoes cicaprost-induced PKA phosphorylation. mIP Ser³⁷⁵, a site-directed mutant of mIP, efficiently coupled to Goₛ but failed to couple to Goᵢ or to efficiently couple to Goₛ, PLC. Moreover, mIP Ser³⁵⁷ did not undergo cicaprost-induced phosphorylation confirming that Ser³⁵⁷ is the target residue for PKA-dependent phosphorylation. Finally, co-precipitation experiments permitted the detection of Goₛ, Goᵢ, and Goᵢ in the immunoprecipitates of mIP, whereas only Goᵢ was co-precipitated with mIP Ser³⁵⁷ indicating that Ser³⁵⁷ of mIP is essential for Goᵢ and Goᵢ interaction. Moreover, inhibition of PKA blocked co-precipitation of mIP with Goₛ or Goᵢ. Taken together our data indicate that the mIP, in addition to coupling to Goₛ, couples to Goᵢ and Goᵢ; however, Goᵢ and Goᵢ coupling is dependent on initial cicaprost-induced mIP:Goₛ coupling and phosphorylation of mIP by cAMP-dependent PKA where Ser³⁵⁷ was identified as the target residue for PKA phosphorylation.
ger the “activation of (an)other secondary signaling cascade(s)” (21). For example, for many GPCRs, receptor phosphorylation and subsequent internalization has been shown to be essential for GPCR activation of the mitogen-activated protein kinase cascades and for transactivation of growth factor receptors (22). Moreover, in a limited number of cases, agonist-mediated receptor phosphorylation has been reported to trigger molecular switching whereby a receptor alters its coupling from one G protein:effector system to a different G protein:effector system (23, 24). Such molecular switching can facilitate GPCR coupling to more than one G protein:effector system (21).

The molecular mechanisms whereby a single IP can couple to more than one G protein:effector system has not been explored in detail. Thus, in the present study we investigated the G protein-coupling characteristics of the mIP stably overexpressed in human embryonic kidney (HEK) 293 cells and in murine erythroleukemia (MEL) cells, which endogenously express mIP. Our studies confirmed that mIP, in addition to its coupling to Goq, may also couple Goq and Gs through a mechanism involving initial Goq signaling and cAMP-dependent protein kinase (PK) A phosphorylation of mIP at Ser567 within its C-tail region; thereafter the phosphorylated mIP exhibited activation of PLC and inhibition of adenyl cyclase and to Goq-mediated activation of PLC. These studies provide essential evidence demonstrating that agonist activation of the classical Gs-coupled mIP can trigger its phosphorylation and, in turn, favor its signaling to Gs and Goq-coupled signal transduction cascades.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cicaprost was obtained from Schering AG (Berlin, Germany). Iloprost, [3H]-Iloprost (15.3 Ci/mmol), and [3H]-CGP12177 (41.0 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Fura2/AM, t-tyrosequence 1,4,5-triphosphate, and its 3-deoxyhexosamid salt (stable analogue of IP3) were purchased from Calbiochem. [3H]-[25P]Orthophosphate (8,000–9,000 Ci/mmol) was obtained from PerkinElmer Life Sciences. Isoproterenol was purchased from Sigma. [3H]HIP (20–40 Ci/mmol) and [3H]-Camp (15–30 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. Polyvinylidene difluoride (PVDF) filters, TCA (trichloroacetic acid), and sodium metabisulfite were purchased from Roche Molecular Biochemicals. Oligonucleotides were synthesized by Genosys Biotechnologies. Anti-Goq (K-20), Goq/Go11 (C-19), and Go11 (I-20) antisera, horseradish peroxidase-conjugated goat anti-rabbit IgG, and anti-G protein-coupled receptor kinase 2 (C-15) antibody, against the carboxyl terminus of the β-adrenergic receptor kinase 1, were obtained from Santa Cruz Biotechnology. Mouse monoclonal 101R anti-HA antibody was obtained from BabCO.

**Materials and Methods**

Site-directed Mutagenesis of the mIP—Conversion of Ser567 of the mIP to Ala567, herein designated mIP567T, was performed using the Stratagene Quick Change site-directed mutagenesis kit using pHM: mIP (12) as template and oligonucleotides 5′-CTT GCC AGA CTA GCA GCC GGG AGA AGA GACC C-3′ (sense primer) and 5′-GTG TGT CCT TGG GCC CCG TGCC AGG TCG TCT GGA AAG-3′ (antisense primer, the sequence complimentary to mutator Ser (TCG) to Ala (GCC) codon is in boldface italics). The plasmid was verified by double-stranded DNA sequencing using Sequenase Version 2.0 (United States Biological Corp.). The plasmid pRK5βAR1K1 (495–689) encoding the carboxyl-terminal amino acid residues 495–689 of βAR1 (23) was kindly provided by Prof. Robert Lefkowitz, Howard Hughes Medical Institute, Duke University Medical Center. The plasmid pCMV-Goq was generated by subcloning the full-length cDNA for rat Goq into the EcoRI site of pCMV3. The plasmids pCMV-Goq and pCMV-Goa have been described previously (12, 26).

**Cell Culture and Transfections—** MEL clone 707 cells and HEK 293 cells were obtained from the American Type Culture Collection and were maintained at 37 °C in 5% CO2. MEL cells were routinely cultured in Dulbecco’s modified Eagle’s medium, 20% fetal bovine serum. HEK 293 cells were cultured in minimal essential medium with Earle’s salts and 10% fetal bovine serum. HEK 293 cells were transfected with 10 μg of pADVA and 25 μg of pcMV- or pHM-based vectors using the calcium phosphate/DNA co-precipitation procedure (25). For transient transfection, cells were treated with 10 μg of plasmid DNA for 48 h before harvesting. HEK.mIP, HEK.HAmIP, HEK.mIP567T, and HEK.bAR cell lines have been previously described (12). To create the HEK.HAmIP567T stable cell line, HEK 293 cells were transfected with 10 μg of ScAl linearized pADVA plus 25 μg of Peu1 linearized pMM.HAmIP567T. Forty-eight hours post-transfection, G418 (0.8 mg/ml) selection was applied. After ~2 days, G418-resistant colonies were selected, and individual HEK.HAmIP567T polyclonal stable cell lines or isolates were examined for IP expression by radioligand and binding. All isolates used throughout the study were derived from pure clonal stable cell lines.

**Radioligand Binding Studies—** Cells were harvested by centrifugation at 500 × g at 4 °C for 5 min and washed three times with phosphate-buffered saline. For membrane preparation, cells were resuspended in Homogenization Buffer (25 mM Tris-HCl, pH 7.5, 0.25 mM sucrose, 10 mM MgCl2, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride), and membrane fractions were prepared by homogenization followed by centrifugation (100,000 × g, 40 min at 4 °C). The pellet fraction (P100), representing crude membranes, was resuspended in Resuspension Buffer (10 mM MES-KOH, pH 6.0, 10 mM MnCl2, 1 mM MgCl2, 100 μg/ml antipain, and 100 μg/ml leupeptin). IP radioligand binding assays were carried out at 30 °C for 1 h using 100 μg of membrane protein (P100) in 100-μl reactions in the presence of 4 nM [3H]Iloprost (15.3 Ci/mmol) as described previously (12). β-Adrenergic receptor (βAR) radioligand binding assays were carried out on whole cells using 25 nM [3H]CGP-12177 (41.0 Ci/mmol) at 14 °C for 3 h using 100 μg of protein in a final volume of 100 μl essentially as described previously (27). Protein determinations were carried out using the Bradford assay (28).

**Measurement of cAMP—** cAMP assays were carried out as described previously (12). Briefly, cells were harvested by scraping and were washed three times in ice-cold phosphate-buffered saline. Cells (1–2 × 106 cells) were resuspended in 200 μl of HEPES-buffered saline (HBS; 140 mM NaCl, 4.7 mM KCl, 2.3 mM CaCl2, 1.2 mM KH2PO4, 11 mM glucose, 15 mM HEPES-NaOH, pH 7.4) containing 1 mM 3-isobutyl-1-methyloxanthine and were preincubated at 37 °C for 10 min. Afterwards, the agonists (50 μM) were added, and the cells were stimulated at 37 °C for 10 min in the presence of the ligand (1 μM ciprofloxacin, 10 μM forskolin, 1 μM ciprofloxacin plus 10 μM forskolin, 10 μM isoproterenol, or 10 μM isoproterenol plus 10 μM forskolin). For concentration-response studies, cells were stimulated with 1–10 μM ciprofloxacin. As controls, cells were stimulated in the absence of the ligand. The pregnancy of the response of HBS was determined in the absence of the ligand. To investigate the effect of PTX, cells were preincubated with PTX (50 ng/ml) for 16 h prior to stimulation with the respective ligand. To investigate the effect of kinase inhibitors on cAMP generation, cells were stimulated in the presence of 10 μM GF109203X (50 nM) was added, and the cells were incubated for 5 min at 37 °C. Intracellular IP3 levels were measured by guest on March 23, 2020http://www.jbc.org/Downloaded from
pADVA (10 μg/10-cm dish). The IP$_3$ levels produced were determined using the IP$_3$-binding protein assay (29, 30). Levels of IP$_3$ produced by ligand-stimulated cells over basal stimulation in the presence of HBS were expressed in pmol of IP$_3$/mg of cell protein ± S.E., and results are presented as -fold stimulation over basal (+fold increase ± S.E.). The data presented are representative of four independent experiments, each performed in duplicate.

*Measurement of Intracellular [Ca$^{2+}$] Mobilization—*Measurements of [Ca$^{2+}$], in Fura2/AM-preloaded cells were carried out essentially as described previously (26). To investigate the effect of PTXs, cells were preincubated with PTXs (50 ng/ml) at 37 °C in 5% CO$_2$ for 16 h prior to stimulation with cicaprost (1 μM). Prior to the appropriate treatment (4 min), cells were subjected to PhosphorImager analysis, and the intensities of phoshorylation relative to basal phosphorylation were determined and were expressed in arbitrary units of intensity relative to basal levels. In parallel experiments, cells were incubated under identical conditions in the absence of [32P].

**RESULTS**

**Statistical analysis** was carried out using the unpaired Student’s t test using GraphPad Prism Version 2.0 (GraphPad Software Inc., San Diego, CA). p values of less than or equal to 0.05 were considered to indicate a statistically significant difference.

**Mouse Prostacyclin Receptor G Protein Coupling in Response to Adenyl Cyclase Activation—**In the current study we sought to investigate the specificity of G protein:effector coupling by the mIP and to define the mechanism(s) whereby the mIP may couple to more than one G protein:effector system. In addition, as a comparative control, we investigated signaling by the β$_2$AR, which has been reported to switch coupling from G$_a_1$ to G$_a_1$ in a PKA-dependent mechanism (23). Thus, our studies were performed in HEK 293 cells stably overexpressing the mIP (HEK.mIP cells), in MEL cells that endogenously express mIP, and in HEK β$_2$AR cells stably overexpressing the human β$_2$AR (23).

**Data Analyses**

**Animal care** and experimental procedures were performed in accordance with the recommendations of the manufacturer’s recommendations.

**Measurement of Agonist-mediated IP Phosphorylation in Whole Cells—**Agonist-mediated IP phosphorylation in whole HEK.HAmIP (isolate 2 cells) and HEK.HAmIP$^{S357A}$ cells were carried out essentially as described previously (29). Briefly, cells were washed once in ice-cold phosphate-buffered saline (2 ml/dish) and were lysed with 0.6 ml of Radioimmune Precipitation Buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (v/v), 0.1% sodium deoxycholate (w/v), 0.1% SDS) containing 10 mM sodium fluoride, 25 mM sodium molybdate, 10 mM ATP, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM benzamidine hydrochloride, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Following a 15-min incubation on ice, cells were harvested and disrupted by sequentially passing through hypodermal needles of decreasing bore size (2G, 2G1, 2G2, and 2G3), and soluble cell lysates were harvested by centrifugation for 15 min at 13,000 × g at room temperature. HA epitope-tagged IP receptors were immunoprecipitated using the anti-HA antibody (101R, 1,300 dilution) at room temperature for 2 h followed by the addition of 10 μl of protein G-Sepharose 4B (Sigma) and further incubation at room temperature for 1 h. Immune complexes were collected by centrifugation at 13,000 × g at room temperature for 5 min and were washed three times in 0.3 ml of Radioimmune Precipitation Buffer and were then resuspended in 1× Solubilization Buffer (10% β-mercaptoethanol (v/v), 2% SDS (w/v), 30% glycerol (v/v), 0.025% bromphenol blue (w/v), 50 mM Tris-HCl, pH 6.8; 60 μl). Samples were boiled for 5 min and then loaded onto 10% polyacrylamide gels, analyzed by SDS-PAGE, and then loaded onto 10% polyacrylamide gels, analyzed by SDS-PAGE, and then subjected to PhosphorImager analysis, and the intensities of phoshorylation relative to basal phosphorylation were determined and were expressed in arbitrary units of intensity relative to basal levels. In parallel experiments, cells were incubated under identical conditions in the absence of [32P].

**Results**

**Prostacyclin Receptor Signaling to G$_a_3$, G$_a_1$, and G$_q$**

**Co-immunoprecipitation of the mIP and its Associated G Proteins—**HEK.mIP$^{S357A}$ and HEK.HAmIP$^{S357A}$ cells were transiently transfected with the respective pCMV-Ga plasmids encoding Ga$_a_3$, Ga$_a_1$, and Ga$_q$ (25 μg/10-cm dish) and pADVA (10 μg/10-cm dish). Approximately 48 h post-transfection, cells were either preincubated for 10 min with 10 μM H-89 or, as a control, with an equivalent volume of the vehicle HBS at 37 °C in 5% CO$_2$ prior to stimulation with 1 μM cicaprost for 10 min at 37 °C in 5% CO$_2$. Reactions were terminated, and HA-tagged IP receptors were immunoprecipitated using the anti-HA 101R antibody (1:300, blotted, and analyzed essentially as described previously (12)). Samples were resuspended in 1× Solubilization Buffer, boiled for 5 min, then analyzed by SDS-PAGE on 10% polyacrylamide gels, and thereafter electropho$^{b}$ted onto PVDF membranes. Membranes were then screened by immunoblot analysis using the appropriate Ga$_a_3$ (920-500, 1:500), Ga$_a_1$ (1:3000), or Ga$_q$ (1:500) antisera where horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) was the secondary antibody used in each case. Immuno$^{b}$roteins were visualized using the chemiluminescence detection system according to the manufacturer’s recommendations.

**Data Analyses**

**Agonist-mediated IP Phosphorylation in Whole Cells—**Agonist-mediated IP phosphorylation in whole HEK.HAmIP (isolate 2 cells) and HEK.HAmIP$^{S357A}$ cells were carried out essentially as described previously (29). Briefly, cells were washed once in ice-cold phosphate-buffered saline (2 ml/dish) and were lysed with 0.6 ml of Radioimmune Precipitation Buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (v/v), 0.1% sodium deoxycholate (w/v), 0.1% SDS) containing 10 mM sodium fluoride, 25 mM sodium molybdate, 10 mM ATP, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM benzamidine hydrochloride, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Following a 15-min incubation on ice, cells were harvested and disrupted by sequentially passing through hypodermal needles of decreasing bore size (2G, 2G1, 2G2, and 2G3), and soluble cell lysates were harvested by centrifugation for 15 min at 13,000 × g at room temperature. HA epitope-tagged IP receptors were immunoprecipitated using the anti-HA antibody (101R, 1,300 dilution) at room temperature for 2 h followed by the addition of 10 μl of protein G-Sepharose 4B (Sigma) and further incubation at room temperature for 1 h. Immune complexes were collected by centrifugation at 13,000 × g at room temperature for 5 min and were washed three times in 0.3 ml of Radioimmune Precipitation Buffer and were then resuspended in 1× Solubilization Buffer (10% β-mercaptoethanol (v/v), 2% SDS (w/v), 30% glycerol (v/v), 0.025% bromphenol blue (w/v), 50 mM Tris-HCl, pH 6.8; 60 μl). Samples were boiled for 5 min and then loaded onto 10% polyacrylamide gels, analyzed by SDS-PAGE, and then subjected to PhosphorImager analysis, and the intensities of phoshorylation relative to basal phosphorylation were determined and were expressed in arbitrary units of intensity relative to basal levels. In parallel experiments, cells were incubated under identical conditions in the absence of [32P].

**Results**

**Prostacyclin Receptor Signaling to G$_a_3$, G$_a_1$, and G$_q$**

**Co-immunoprecipitation of the mIP and its Associated G Proteins—**HEK.mIP$^{S357A}$ and HEK.HAmIP$^{S357A}$ cells were transiently transfected with the respective pCMV-Ga plasmids encoding Ga$_a_3$, Ga$_a_1$, and Ga$_q$ (25 μg/10-cm dish) and pADVA (10 μg/10-cm dish). Approximately 48 h post-transfection, cells were either preincubated for 10 min with 10 μM H-89 or, as a control, with an equivalent volume of the vehicle HBS at 37 °C in 5% CO$_2$ prior to stimulation with 1 μM cicaprost for 10 min at 37 °C in 5% CO$_2$. Reactions were terminated, and HA-tagged IP receptors were immunoprecipitated using the anti-HA 101R antibody (1:300, blotted, and analyzed essentially as described previously (12)). Samples were resuspended in 1× Solubilization Buffer, boiled for 5 min, then analyzed by SDS-PAGE on 10% polyacrylamide gels, and thereafter electropho$^{b}$ted onto PVDF membranes. Membranes were then screened by immunoblot analysis using the appropriate Ga$_a_3$ (920-500, 1:500), Ga$_a_1$ (1:3000), or Ga$_q$ (1:500) antisera where horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) was the secondary antibody used in each case. Immuno$^{b}$roteins were visualized using the chemiluminescence detection system according to the manufacturer’s recommendations.
increases in cAMP generation, co-incubation with cicaprost and forskolin caused significant decreases in cAMP in both HEK.mIP (p < 0.0001) and MEL (p < 0.0001) cells. These results suggest that the mIP can couple to both Go and Ga. Similar results were obtained upon examining cAMP generated by the βAR (HEK.βAR cells) in the presence of 25 nM [3H]CGP-12177. Data are presented as the mean ± S.E. (n = 4).

| Cell type | [3H]Iloprost bound | pmol mg⁻¹ protein |
|-----------|---------------------|-------------------|
| HEK.mIP   | 3.28 ± 0.22         |                   |
| MEL       | 0.55 ± 0.02         |                   |
| HEK.mIP[357A] | 3.43 ± 0.47   |                   |
| HEK.HAmIP isolate 1 | 2.37 ± 0.12 |                   |
| HEK.HAmIP isolate 2 | 1.56 ± 0.21 |                   |
| HEK.293   | 1.57 ± 0.24         |                   |
| HEK.293   | 0.01 ± 0.001        |                   |

TABLE I

Radioligand binding assays were carried out on membrane fractions of MEL cells, HEK 293 cells, or HEK 293 cells stably overexpressing either mIP (HEK.mIP cells), mIP[357A] (HEK.mIP[357A] cells), HA-tagged mIP (HEK.HAmIP isolates 1 and 2), or HA-tagged mIP[357A] (HEK.HAmIP[357A]) in the presence of 4 nM [3H]Iloprost.

Radioactive binding assays were carried out on whole HEK 293 cells or on HEK 293 cells stably overexpressing the βAR (HEK.βAR cells) in the presence of 25 nM [3H]CGP-12177. Data are presented as the mean ± S.E. (n = 4).

To establish whether the mIP-Gα coupling is dependent on the concentration of ligand used for stimulation, HEK.mIP cells were stimulated with cicaprost (1 μM) in the absence and presence of forskolin (Fig. 3A). mIP generated significant concentration-dependent increases in cAMP consistent with its coupling to Go. However, in cells co-incubated with forskolin, 1 μM cicaprost led to concentration-dependent augmentation of cAMP generation consistent with mIP coupling to Go. However, at the higher cicaprost concentrations (≥1 μM), cicaprost led to a concentration-dependent reduction in cAMP generation indicative of mIP coupling to Go. In the presence of PTX, cicaprost led to concentration-dependent increases in cAMP generation in the presence (Fig. 3B) and absence of forskolin (data not shown) over the complete range of cicaprost concentrations (1 μM–1 μM). Moreover, in the presence of H-89, cicaprost resulted in concentration-dependent increases in cAMP generation in the presence (Fig. 3B) and absence of forskolin (data not shown) over the complete range of cicaprost concentrations providing further evidence that Go coupling by mIP is dependent on PKA activation status.

Mouse Prostacyclin Receptor Coupling to PLC—We extended these studies to investigate the mechanism whereby the Gα, coupled mIP may also couple to the Go, PLC effector system (12). Stimulation of cells with cicaprost resulted in a significant increase in IP3 generation in HEK.mIP cells compared with control, nontransfected HEK 293 cells (Figs. 4A and 10A, p < 0.0009). Preincubation of HEK.mIP cells with PTX or GF 109203X had no significant effect on IP3 generation (PTX, p > 0.911; GF 109203X, p > 0.668); however, preincubation with H-89 significantly decreased cicaprost-induced IP3 generation (p < 0.005). Because the levels of cicaprost-stimulated IP3 were quite low (1.56 ± 0.13-fold increase in IP3 in response to 1 μM cicaprost), HEK.mIP cells were transiently co-transfected with Go, to increase the levels of measurable IP3 (Fig. 4B). Overexpression of Go, was confirmed by Western blot analysis using anti-Gα, specific antisera (Fig. 4C). The presence of Go, augmented (p < 0.002) and led to a significant increase in cica-

To extend these studies, we also investigated cicaprost-induced PLC activation by monitoring mobilization of [Ca2+]i. Consistent with previous studies (12), stimulation of HEK.mIP cells with cicaprost resulted in efficient transient rises in [Ca2+]i mobilization (Fig. 5A). Preincubation of HEK.mIP cells with PTX (Fig. 5B) prior to stimulation with cicaprost had no significant effect on [Ca2+]i mobilization (p > 0.627). Consistent with this, transient overexpression of the carboxyl-terminal residues (amino acid residues 459–689) of βARK1 (25) to sequester Gβγ subunits had no significant effect on cicaprost-induced [Ca2+]i mobilization (Fig. 5C, p > 0.32). Overexpression of the carboxyl-terminal residues of βARK1 was confirmed by Western blot analysis (Fig. 5D). Preincubation of HEK.mIP cells with the PKC inhibitor GF 109203X had no significant effect on [Ca2+]i mobilization by the mIP (Fig. 5E, p > 0.24). In contrast, preincubation of HEK.mIP cells with H-89 resulted in near complete inhibition of cicaprost-induced [Ca2+]i mobilization (Fig. 5F, p < 0.001).

Thereafter to confirm that the source of cicaprost-induced [Ca2+]i mobilization is derived from PLC/IP3-mediated intracellular Ca2+ stores, we examined the effect of the PI-PLC inhibitor U73122 and, as a control, the phosphatidylinositol-specific phospholipase C inhibitor D-609 (31, 32) on cicaprost-induced [Ca2+]i mobilization. Preincubation of HEK.mIP cells with U73122 abolished [Ca2+]i mobilization by the mIP (Fig. 6B, p < 0.0001), whereas preincubation of the cells with D-609 had no significant effect on cicaprost-induced [Ca2+]i mobilization (Fig. 6C, p > 0.07). As an additional control, preincubation of HEK.mIP cells with U73122 did not affect ionomycin-induced [Ca2+]i mobilization ensuring that U73122 did not affect the general measurement of [Ca2+]i mobilization in Fura2/AM-preloaded cells per se (p < 0.001, data not shown).

Whole Cell Phosphorylations of the mIP—To establish whether the mIP may be subject to cicaprost-induced PKA phosphorylation, whole cell phosphorylations of the mIP were investigated in HEK 293 cells stably overexpressing an amino-
terminal extracellular HA epitope-tagged mIP (HEK.HAmIP cells). Consistent with previous reports (12, 33) and Table I, the presence of the HA epitope tag did not affect the ligand binding characteristics or cell signaling properties of mIP (data not shown). Initially the specificity of the anti-HA 101R antiserum was investigated in HEK.HAmIP cells and not shown).
HEK.HAmIP cells with the PKA inhibitor H-89 (Fig. 7B). Alternatively, HEK.β2AR cells (panel C) were stimulated with either 10 μM isoproterenol (Iso), 10 μM forskolin (Fsk), or 10 μM isoproterenol plus 10 μM forskolin (Iso + Fsk). In each case, basal cAMP levels were determined by exposing the cells to the vehicle under identical reaction conditions. Levels of cAMP produced in ligand-stimulated cells relative to basal cAMP levels were expressed as fold-stimulation of basal (fold increase in cAMP ± S.E., n = 4). Basal levels of cAMP generation in HEK.mIP cells and in MEL cells were 0.71 ± 0.04 pmol/mg of cell protein (n = 4) and 0.98 ± 0.06 pmol/mg of cell protein (n = 4), respectively.

The Role of the C-tail of the mIP in Receptor:G Protein Coupling—It has previously been established that the isoprenylation-defective mIPSSLc, in which the critical Cys<sup>414</sup> of mIP was mutated to Ser<sup>414</sup>, cannot couple to G<sub>aq</sub>, to mediate cAMP increases and cannot efficiently couple to G<sub>aq</sub>PLC activation (12) implying that the C-tail of the mIP may be important in receptor:G protein coupling. Thus, we sought to investigate whether the isoprenylation-defective mIPSSLc can couple to G<sub>aq</sub>. Consistent with previous studies (12), HEK.mIPSSLc cells exhibited a significantly impaired ability to generate cicaprost-mediated cAMP generation compared with HEK.mIP cells (Fig. 8, compare panel A to panel C). In fact the levels of cicaprost-induced cAMP generation in HEK.mIPSSLc cells were not significantly different from that produced by the control, nontransfected HEK 293 cells over the range of 1 pM–100 nM cicaprost (data not shown), but at 1 μM cicaprost a small, although significant, increase in cAMP generation was observed in HEK.mIPSSLc cells (Fig. 8A, p < 0.05). Moreover, in the presence of forskolin, cicaprost (1 μM) did not result in a diminution of cAMP levels, indicative of G<sub>i</sub> coupling by mIPSSLc (Fig. 8A), but consistent with previous data (Fig. 2B) it signifi-
by exposing the cells to the vehicle HBS under identical reaction conditions. Levels of IP3 produced in ligand-stimulated cells relative to which Ser357 was converted to Ala357, thereby destroying the HAmIPS357A cells (Table I). Both the HEK.HAmIPS and of the HEK.HAmIP cells (HEK.HAmIP isolate 2) that exhibited studies we chose to use a second pure clonal cell line or isolate relative levels of the respective receptor expression, for further pared with the mIP may not be due to simple differences in the sequence where Ser357 represents the predicted target residue for Ser357 did not affect the ligand binding characteristics of the mIP, radioligand binding assays were carried out on HEK.mIPSSLC membranes (Table I). No significant differences were observed between the levels of mIP expression in HEK.mIP and HEK.mIPSSLC cells (p > 0.0985). Computational analysis of the C-tail region of the IP revealed a putative PKA phosphorylation site in the mIP sequence where Ser357 represents the predicted target residue for PKA phosphorylation (34). Thus, to determine the importance of this putative PKA phosphorylation motif, site-directed mutagenesis was used to generate mIP S357A, a variant of mIP in which Ser357 was converted to Ala357, thereby destroying the putative PKA phosphorylation site within the C-tail of mIP. Stable cell lines overexpressing the epitope-tagged (HA) mIP S357A (HEK.HAmIP S357A) were established and characterized by radioligand binding (Table I). Whereas through transient expression studies it was established that mutation of Ser357 did not affect the ligand binding characteristics of the mIP S357A compared with the wild type mIP, the level of mIP S357A expression in HEK.HAmIP S357A cells was appreciably lower than that of mIP expressed in HEK.mIP or in HEK.HAmIP cells (Table I). Thus, to ensure that any potential difference in the signaling behavior of the mIP S357A when compared with the mIP may not be due to simple differences in the relative levels of the respective receptor expression, for further studies we chose to use a second pure clonal cell line or isolate of the HEK.HAmIP cells (HEK.HAmIP isolate 2) that exhibited similar levels of mIP expression to that of the HEK.HAmIP S357A cells (Table I). Both the HEK.HAmIP and HEK.HAmIP S357A cells showed significant cicaprost-mediated, concentration-dependent increases in cAMP generation (Fig. 9A). Moreover, co-transfection of cells with Goα (Fig. 9B) significantly augmented cAMP generation in HEK.HAmIP cells (p < 0.003) and in HEK.HAmIP S357A cells (p < 0.0001) where Goα overexpression was confirmed by Western blot analysis (Fig. 9B, inset). However, whereas mIP S357A exhibited efficient Goα coupling (p < 0.001) unlike that of the mIP isolates, it did not exhibit Goα coupling as evidenced by its failure to reduce, but rather augmented, cAMP levels in cells co-stimulated with cicaprost plus forskolin when compared with cells incubated with forskolin alone (Fig. 9C). cAMP generation by the mIP S357A was not affected by the presence of H-89 (Fig. 9D) or GF 109203X (data not shown).

Next the ability of mIP S357A to couple to Goα and PLC activation was examined. HEK.HAmIP cells produced a concentration-dependent increase in IP3 generation upon stimulation with 1 nm–10 μM cicaprost (Fig. 10A, p < 0.001 at 10 μM cicaprost). In contrast, neither the HEK.HAmIP S357A nor the nontransfected HEK 293 cells showed any significant increases in IP3 even at the highest concentration used (p > 0.249 at 10 μM and p > 0.314 at 10 μM, respectively). Moreover, co-transfection of cells with Goα did not augment cicaprost-mediated IP3 generation in HEK.HAmIP S357A cells (data not shown). Stimulation of both the mIP and the mIP S357A did lead to significant increases in [Ca2+2], mobilization (Fig. 10B); however, the level of [Ca2+2], mobilization by the mIP S357A was significantly less than that of the mIP (p < 0.003) and was only marginally greater than that mobilized in nontransfected HEK 293 cells in response to cicaprost (Ref. 12 and data not shown).

Whole Cell Phosphorylations of the mIP S357A—Whole cell phosphorylations in HEK.HAmIP S357A cells (Fig. 7B) con-
firmed that HAmIP<sub>357A</sub> did undergo increased phosphorylation, represented as a broad phosphoprotein band of 36–66 kDa, in response to stimulation with cicaprost (Fig. 7B, compare lanes 1 and 2). However, the level of HAmIP<sub>357A</sub> phosphorylation was not significantly different from that observed in HEK 293 cells (Fig. 7A, lane 5; p > 0.05) but was substantially less when compared with that of the HAmIP (Fig. 7, compare panel A, lane 2 to panel B, lane 2; p < 0.05) despite the fact that HAmIP<sub>357A</sub> was expressed at levels similar to that of the HAmIP (Fig. 7C, lanes 1 and 2; Table I). Moreover, preincubation of HEK.HAmIP<sub>357A</sub> cells with GF 109203X or H-89 (Fig. 7B, lanes 3 and 4, respectively) had no effect on the level of mIP<sub>357A</sub> phosphorylation in cicaprost-stimulated cells.

mIP and mIP<sub>357A</sub> Receptor:G Protein Interactions—To investigate the G protein interactions of the mIP and the mIP<sub>357A</sub> more closely, HEK.HAmIP (Fig. 11A) and HEK.HAmIP<sub>357A</sub> (Fig. 11B) cells were transiently transfected with the cDNA encoding the α subunit of G<sub>q</sub>, (Fig. 11, panels A and B, lanes 1 and 2), G<sub>q</sub> (Fig. 11, panels A and B, lanes 3 and 4), or G<sub>q</sub> (Fig. 11, panels A and B, lanes 5 and 6). Receptor:G protein interactions were examined by immunoprecipitating the HAmIP or HAmIP<sub>357A</sub> receptor using
the anti-HA 101R antisera followed by screening the immunoprecipitates for co-precipitation of the respective Go subunit using anti-Go-specific antisera. Whereas nonstimulated, vehicle (HBS)-treated cells showed no receptor:G protein interaction (data not shown), stimulation of both HEK.HAmIP and HEK.HAmIP<sub>S357A</sub> cells with cicaprost (1 μM) for 10 min permitted co-precipitation of Go<sub>j</sub>. However, whereas stimulation of HEK.HAmIP cells with cicaprost resulted in co-precipitation of HAmIP with Go<sub>j</sub> and Go<sub>i</sub>, precipitation of neither Go<sub>j</sub> nor Go<sub>i</sub>, along with HAmIP<sub>S357A</sub> was observed. The presence of HAmIP and HAmIP<sub>S357A</sub> in the immunoprecipitates was confirmed by screening the blots with the anti-HA 3F10 horseradish peroxidase-conjugated antibody (data not shown). Moreover, pretreatment of HEK.HAmIP cells with H-89 blocked co-precipitation of Go<sub>j</sub> (Fig. 11C, lanes 3 and 4) and Go<sub>i</sub> (Fig. 11C, lanes 5 and 6) but had no effect on co-precipitation of Go<sub>j</sub> along with HAmIP (Fig. 11C, lanes 1 and 2).

**DISCUSSION**

Prostacyclin is central to the regulation of vascular hemostasis and may also mediate other somewhat diverse cellular actions under both physiologic and pathophysiologic settings (3, 8, 35). Mice deficient in the IP exhibit altered pain perception and inflammatory reactions as well as exhibiting an impaired response to thrombotic stimuli (4). Whereas IP is primarily coupled to G<sub>j</sub>-dependent adenylyl cyclase activation, ligand activation of multiple signaling pathways by IP agonists has been identified, such as in mouse BNu2cl3 mast cells (36), in mouse preadipocyte Ob1771 cells (37), in human erythrocyt-
mIP expressed in both cell types also coupled to inhibition of adenylyl cyclase in a PTx-sensitive, G_{i}-dependent manner (data relating to PTx studies in MEL cells are not shown). Consistent with mIP coupling to G_{i}, stimulation of HEK.HAmIP cells but had no effect on mIP:G_{i} coupling. On the other hand, molecules cloning has confirmed the existence of sin-

FIG. 10. Cicaprost-induced IP_{3} generation and [Ca^{2+}], mobilization by the mIP^{357A}. Panel A, HEK.HAmIP isolate 2 (IP), HEK.HAmIP^{357A} (IP^{357A}), and HEK 293 (HEK) cells were stimulated with cicaprost (1 nM–10 μM), and the levels of IP_{3} generation were measured (panel A). In each case, basal IP_{3} levels were determined by exposing the cells to the vehicle HBS under identical reaction conditions. Levels of IP_{3} produced in ligand-stimulated cells relative to basal levels were expressed as -fold stimulation of basal (-fold increase in IP_{3} levels of IP_{3} generation in HEK.HAmIP isolate 2 (IP) and HEK.HAmIP^{357A} (IP^{357A}) cells (data not shown). HEK.HAmIP^{357A} cells (panel C) and HEK.HAmIP^{357A} cells preincubated for 10 min with 10 μM H-89 (panel D) were stimulated with either 1 μM cicaprost, 10 μM forskolin (Fsk), or 1 μM cicaprost plus 10 μM forskolin (Cic + Fsk). In each case, basal IP_{3} levels were determined by exposing the cells to the vehicle HBS under identical reaction conditions. Levels of cAMP produced in ligand-stimulated cells relative to basal cAMP levels were expressed as -fold stimulation of basal (n = 4 respectively).

FIG. 9. The effect of kinase inhibitors on cicaprost-induced cAMP generation by the mIP^{357A}, HEK.HAmIP isolate 2 (IP) and HEK.HAmIP^{357A} (IP^{357A}), cells were stimulated with 1 μM–1 μM cicaprost (panel A). Alternatively HEK.HAmIP isolate 2 (IP) and HEK.HAmIP^{357A} (IP^{357A}) cells that had been transiently co-transfected with either the vector pCMV5 (-) or with the cDNA for G_{i} (+) were stimulated with 1 μM cicaprost (panel B). The inset to panel B represents a typical Western blot confirming overexpression of G_{i} in HEK.HAmIP isolate 2 cells (75 μg of total cellular protein analyzed) and in HEK.HAmIP^{357A} cells (data not shown). HEK.HAmIP^{357A} cells (panel C) and HEK.HAmIP^{357A} cells preincubated for 10 min with 10 μM H-89 (panel D) were stimulated with either 1 μM cicaprost, 10 μM forskolin (Fsk), or 1 μM cicaprost plus 10 μM forskolin (Cic + Fsk). In each case, basal IP_{3} levels were determined by exposing the cells to the vehicle HBS under identical reaction conditions. Levels of cAMP produced in ligand-stimulated cells relative to basal cAMP levels were expressed as -fold stimulation of basal (-fold increase in cAMP levels of cAMP generation in HEK.HAmIP isolate 2 (IP) and HEK.HAmIP^{357A} (IP^{357A}) cells were 0.53 ± 0.05 pmol/mg of cell protein (n = 4) and 0.79 ± 0.06 pmol/mg of cell protein (n = 4), respectively.

kemia (MEG01) cells (39). Coupling of IP to changes in intracellular Ca^{2+}, rather than to activation of adenylyl cyclase, has been reported in rabbit cortical collecting ducts (40), whereas the IP expressed in the rat medullary thick ascending limb coupled exclusively to G_{i}-mediated inhibition of adenylyl cyclases rather than to activation of adenylyl cyclase (16). Whereas molecular cloning has confirmed the existence of single IPs in human, mouse, rat, and bovine tissues (11, 41–43), the cloned human and mouse IP are reported to couple to activation of both adenylyl cyclase and PLC{beta} effectors (10–13, 41). To date, IP isoforms are not known to exist unlike other prostanoid receptors, and thus it appears that differential mRNA splicing mechanisms per se cannot account for the mul-
tiple patterns of IP signaling within a given species (3, 44). Thus, the key objective of the present study was to investigate the molecular mechanisms whereby a single IP may couple to more than one G protein:effector system by examining in detail the signaling properties of the mIP either stably overexpressed in HEK 293 (HEK.mIP) cells or in native MEL cells.

Consistent with mIP coupling to G_{i}, stimulation of HEK.
mIP and MEL cells resulted in significant cicaprost-induced increases in cAMP generation. However, contrary to previous reports, mIP expressed in both cell types also coupled to inhibition of adenylyl cyclase in a PTx-sensitive, G_{i}-dependent manner (data relating to PTx studies in MEL cells are not shown). The PKA inhibitor H-89 abolished mIP:G_{i} coupling in HEK.mIP cells but had no effect on mIP:G_{s} coupling. On the
other hand, the PKC inhibitor GF 109203X had no effect on mIP coupling to either Goα or Goq. To establish whether the specificity of mIP coupling to Goα or Goq was concentration-dependent, HEK.mIP cells were stimulated with a range of cicaprost concentrations in the absence and presence of forskolin. In the absence of forskolin, mIP generated significant concentration-dependent increases in measurable cAMP consistent with its coupling to Goq. However, in the presence of forskolin, stimulation of mIP with low concentrations of cicaprost (<1 nM) generated significant concentration-dependent increases in measurable cAMP consistent with its coupling to Goq, whereas at higher concentrations (>1 nM) cicaprost yielded a concentration-dependent reduction in cAMP generation indicative of mIP coupling to Goα. Preincubation with PTX or H-89, but not GF 109203X (data not shown), blocked mIP-mediated Goα coupling but had no effect on mIP coupling to Goq. Preincubation with the range of cicaprost concentrations used. Taken together these data indicate that mIP can couple to both Goα and to PTX-sensitive Goq to mediate activation and inhibition of adenylyl cyclase, respectively. Coupling of mIP to Goq, but not Goα, appears to require a critical concentration of cAMP and is dependent on PKA activation.

We extended our studies to investigate the mechanism whereby the Gα-coupled mIP may also couple to PLC activation. Cicaprost stimulation of HEK.mIP cells led to significant increases in IP3 generation and mobilization of [Ca2+], H-89, but not PTX or GF 109203X, almost completely abolished cicaprost-mediated signaling indicating that mIP-mediated IP3 generation and [Ca2+]i mobilization occurred in a PTX-insensitive, PKC-independent, PKA-dependent mechanism(s). Moreover, overexpression of the carboxyl-terminal residues of βARK1, to sequester Gβγ subunits, confirmed that cicaprost-mediated IP3 generation (data not shown) or [Ca2+]i mobilization were not through Gβγ-stimulated mechanisms such as through Gβγ regulation of PLCβ isozymes (45). Consistent with these data, transient overexpression of Goq led to significant augmentations of cicaprost-mediated IP3 generation and mobilization of [Ca2+]i (Ref. 12 and data not shown) in a PTX-insensitive, PKC-independent, PKA-dependent mechanism(s). Furthermore, the PI-PLC inhibitor U73122 (32) abolished cicaprost-induced [Ca2+]i mobilization confirming that the primary source of mIP-mediated [Ca2+]i mobilization is from IP3-regulated intracellular stores. Thus, taken together these data indicate that mIP, in addition to coupling to Goq and Goi, can couple to PLC and that PLC coupling occurs in a Goq-mediated, PTX-insensitive, PKC-independent, but PKA-dependent, manner. Additionally, coupling of mIP to both Goi and Goq is dependent on its prior coupling to Goq to stimulate cAMP-dependent PKA activation. Moreover, subsequent whole cell phosphorylation assays established that the mIP is subject to cicaprost-mediated phosphorylation in a GF 109203X-independent, H-89-dependent manner indicating that mIP coupling to Goq and Goi may involve direct PKA-mediated phosphorylation of the mIP itself.

We have previously established that mIP may be unique among GPCRs in that it is isoprenylated within its C-tail domain (12, 13). Disruption of isoprenylation through site-directed mutagenesis to generate mIPSSLC established that isoprenylation of mIP is required for its efficient Goα- and Goq-mediated activation of adenylyl cyclase and PLC, respectively, suggesting that the C-tail of the mIP may be involved in receptor::G protein interaction (12). Consistent with previous studies (12), the isoprenylation-defective mIPSSLC exhibited significantly diminished coupling to both adenylyl cyclase and PLC activation compared with the wild type mIP. Moreover, mIPSSLC failed to couple to Goq to mediate inhibition of forskolin-induced cAMP generation indicating that (a) the C-tail domain of mIP may be involved in receptor::Goα interaction and/or that (b) failure of mIPSSLC to couple to Goq may be due to its initial inability to couple to Goq to stimulate PKA-mediated mIP phosphorylation and subsequent coupling to Goq, and indeed to Goi.

Analysis of the primary sequence of the mIP revealed the presence of a putative PKA phosphorylation site within the C-tail region of the mIP where Ser357 represents the predicted target residue for phosphorylation (34). Thus, through the use of site-directed mutagenesis, the role of Ser357 was investigated. Whereas mIP357A exhibited Goq activation of adenylyl
Fig. 12. Model of mIP coupling to Go, mediated activation of adenylyl cyclase (AC) (a), Go, coupled inhibition of adenylyl cyclase (b), and Go, coupled activation of PI-PLC (c). a, ligand activation of the mIP stimulates Go, mediated activation of adenylyl cyclase leading to increases in cAMP generation and, in turn, activation of PKA. b, activated PKA phosphorylates the mIP at Ser357 favoring mIP:Go, coupling leading to decreases in adenylyl cyclase activity and, in turn, decreases in cAMP generation. PKA-mediated mIP phosphorylation and coupling to PTx-sensitive Go, may be inhibited by H-89, c, alternatively PKA-mediated phosphorylation of mIP at Ser357 may promote mIP:Go, coupling leading to activation of PI-PLC and, in turn, leading to increases in IP3 generation and mobilization of [Ca2+]i. 

PKA-mediated mIP phosphorylation and coupling to Go, may be inhibited by H-89, and the PI-PLC inhibitor U73122 blocks mIP-mediated IP3 generation and mobilization of [Ca2+]i.

cyclase similar to that of the wild type mIP, it failed to couple to Go, or to Go, to mediate inhibition of adenylyl cyclase and activation of PLC, respectively. The fact that mIP, but not mIP357A, exhibited cicaprost-mediated Go, and Go, coupling confirmed that cicaprost is acting through the mIP itself and not through another, perhaps related prostanoid, receptor to mediate these effects. Unlike that which occurred for the wild type mIP, whole cell phosphorylation assays established that mIP357A did not undergo significant agonist-induced PKA phosphorylation. Finally, cicaprost stimulation of cells overexpressing HA epitope-tagged forms of the mIP and mIP357A permitted co-immunoprecipitation of Go, confirming direct, agonist-induced interaction of both mIP and mIP357A with Go, On the other hand, whereas stimulation of HEK.HAmIP cells with cicaprost resulted in co-precipitation of HAmIP with both Go, and Go, co-precipitation of neither Go, nor Go, along with HAmIP357A was observed. Furthermore, in support of this, inhibition of PKA blocked co-precipitation of Go, and Go, with HAmIP but had no effect on co-precipitation of Go, with HAmIP suggesting that for mIP interaction with Go, and/or Go, to occur mIP must be phosphorylated on Ser357 by PKA. Thus, taken together the data presented in this study provide a mechanism whereby the classical Go,-coupled mIP may couple both to Go,-mediated inhibition of adenylyl cyclase and to Go,-mediated activation of PLC. This mechanism, as outlined in the model presented in Fig. 12, requires initial mIP coupling to Go, leading to elevation of cAMP and PKA activation. Thereafter PKA-mediated phosphorylation of mIP at Ser357 within the C-tail domain of mIP in turn favors its coupling to Go, leading to inhibition of adenylyl cyclase, and to Go, leading to activation of PLC.

The proposed mechanism of mIP coupling to both Go, and to Go, is not unlike that which has been previously reported for the β2AR-mediated switching from Go, to Go, (21, 23). In their model, the β2AR can switch coupling from Go, to Go, in a mechanism involving initial Go, activation of adenylyl cyclase and subsequent PKA phosphorylation within the third intracellular loop of the β2AR; the phosphorylated β2AR, in turn, exhibited diminished coupling to Go, and increased coupling to Go, thereby switching its coupling from Go, to Go, (21, 23, 46). In our studies, we identified Ser357 within the C-tail of the mIP itself as being the critical residue for PKA phosphorylation and, hence, regulating the Go, to Go, coupling mechanism. Our studies do not rule out the possibility that other components of the signaling pathway may also be targets for the PKA-mediated phosphorylation and contribute to the differential signaling event; however, the fact that mIP357A can couple efficiently to Go, to generate cAMP and activate PKA but not to Go, makes it unlikely that other signaling elements are involved. One of the manifestations of the β2AR switching its coupling from Go, is that the Go,-coupled receptor can stimulate activation of the mitogen-activated protein kinase cascades in a PTx-sensitive, Gβγ- and Src-mediated cascade (23). Whether a similar mechanism applies to the Go,-coupled mIP will require further investigation.

Many Go,-coupled GPCRs can activate both cAMP and IP3/ Ca2+ signaling pathways, and three mechanisms have been proposed to explain this dual G protein coupling. One mechanism is independent GPCR coupling to Go, and to a Go, member, such as G15 or Go13, to initiate independent activation of adenylyl cyclase and PLCβ (24). The other two mechanisms involve cAMP-regulated PKA activation of PLCβ or PKA-induced switching of receptor coupling from Go, to Go, (24). In the latter mechanism, such as that reported to occur for the vasoactive intestinal peptide receptor, both PLC-mediated IP3 generation and mobilization of [Ca2+]i, are regulated through PTx-sensitive, Gδ-derived Gβγ subunits rather than through Go-coupled mechanisms (24). In our studies investigating mIP, as stated, we established that PKA phosphorylation of Ser357 within the C-tail of the mIP itself as the critical residue to regulate coupling to both Go, and Go,; thereafter the phosphorylated mIP can independently regulate Go, and Go-mediated inhibition of adenylyl cyclase and activation of PLC, respectively. Thus, our mechanism differs from that proposed for the vasoactive intestinal peptide receptor in that mIP-mediated PLC activation is PTx-insensitive, Go-, and Gβγ-independent. Moreover, mIP-mediated PLC activation occurs in a PKA-dependent, Go,-coupled mechanism suggesting that mIP must undergo PKA-mediated switching from Go, to Go, to activate PLC activation as opposed to independently coupling to Go, and Go, members as proposed in the first mechanism (24). Similar to that previously stated for mIP-Go, coupling, our studies do not fully exclude the possibility that other components of the signaling pathway, such as PLCβ, may also be targets for the PKA-mediated phosphorylation as proposed in the second mechanism (24); however, the fact that mIP357A can couple efficiently to Go, but does not couple to Go,-PLC activation makes it unlikely.

Several GPCRs are known to activate dual or multiple signaling systems, such as the thrombin receptor, the histamine H2 receptor, or the parathyroid hormone receptor to name but a few (47). In many instances, this can occur in a species- or cell type-specific manner. However, the molecular mechanisms whereby a given GPCR may couple to multiple G protein-coupled effectors are, on the whole, poorly understood. Our studies extend those previously reported for the β2AR (23) and for the vasoactive peptide receptor (24) and establish that second messenger kinase- such as PKA-mediated switching is not restricted to G/G, mechanisms but may also be extended to dual switching from Go, to both Go, and G, mechanisms independently. Moreover, our studies add further evidence to the growing awareness that mechanisms such as GPCR phosphorylation and internalization, previously thought to be exclusively asso-
associated with the desensitization of GPCR signaling, may trigger activation of other signaling events, such as switching receptor coupling from one G protein-coupled cascade to other G protein-coupled cascade(s), or to initiation of other signaling events, such as activation of mitogen-activated protein kinase cascades and/or transactivation of growth factor signaling (21, 22).

REFERENCES

1. Campbell, W. B. (1990) in Goodman and Gilman’s The Pharmacological Basis of Therapeutics (Gilman, A. G., Rall, T. W., Nies, A. S., and Taylor, P., eds) 8th Ed., pp. 600–617, Pergamon Press, New York
2. Vane, J. R., and Botting, R. M. (1995) Am. J. Cardiol. 73, 3A–10A
3. Narumiya, S., Sugimoto, Y., and Fumita, U. (1989) Physiol. Rev. 79, 1193–1236
4. Murata, T., Ushikubi, F., Matsuoka, T., Hirata, M., Yamasaki, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N., Ueno, A., Oh-ishi, S., and Narumiya, S. (1997) Nature 388, 678–682
5. Zucker, T. P., Bonisch, D., Hasse, A., Grosser, T., Weber, A. A., and Schror, K. (1998) Eur. J. Pharmacol. 345, 213–220
6. Sakai, A., Yajima, M., and Nishio, S. (1996) Life Sci. 47, 711–719
7. Coleman, R. A., Smith, W. L., and Narumiya, S. (1994) Pharmacol. Rev. 46, 205–229
8. Wise, H., and Jones, R. L. (1996) Trends Pharmacol. Sci. 17, 17–21
9. Armstrong, R. A. (1996) Pharmacol. Ther. 72, 171–191
10. Smyth, E. M., Li, W. H., and Fitzgerald, G. A. (1998) J. Biol. Chem. 273, 23258–23266
11. Namba, T., Oida, H., Sugimoto, Y., Kakinaka, A., Negishi, M., Ichikawa, A., and Narumiya, S. (1994) J. Biol. Chem. 269, 9966–9972
12. Hayes, J. S., Lawler, O. A., Walsh, M.-T., and Kinsella, B. T. (1999) J. Biol. Chem. 274, 23707–23718
13. Lawler, O. A., Miggin, S. M., and Kinsella, B. T. (2001) Br. J. Pharmacol. 132, 1639–1649
14. Siegel, G., Carl, A., Adler, A., and Stock, G. (1989) Ecosamnoids 2, 213–222
15. Schwanner, I., Offermanns, S., Spicher, K., Seifert, R., and Schultz, G. (1995) Biochim. Biophys. Acta 1265, 8–14
16. Hebert, R. L., O’Connor, T., Neville, C., Burns, K. D., Laneville, O., and Peterson, L. (1998) Am. J. Physiol. 275, F904–F914
17. Leigh, P. J., and MacDermot, J. (1985) Br. J. Pharmacol. 85, 237–247
18. Krane, A., MacDermot, J., and Keen, M. (1994) Biochim. Biophys. Acta 47, 953–982
19. Giovanazzi, S., Accomazzo, M. R., Letari, O., Oliva, D., and Nicosia, S. (1997) Biochim. J. 325, 71–77
20. Smyth, E. M., Austin, S. C., Reilly, M. P., and Fitzgerald, G. A. (2000) J. Biol. Chem. 275, 30447–30454
21. Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 18677–18680
22. Luttrell, L. M., Daaka, Y., and Lefkowitz, R. J. (1999) Curr. Opin. Cell Biol. 11, 177–183
23. Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) Nature 390, 88–91
24. Luo, X., Zeng, W., Xu, X., Popov, S., Davignon, I., Willie, T. M., Munday, S. M., and Muallim, S. (1999) J. Biol. Chem. 274, 17684–17699
25. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197
26. Kinsella, B. T., O’Mahony, D. J., and Fitzgerald, G. A. (1997) J. Pharmacol. Exp. Ther. 281, 957–964
27. Gagnon, A. W., Kallal, L., and Benovic, J. L. (1998) J. Biol. Chem. 273, 6976–6981
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
29. Walsh, M.-T., Foley, J. F., and Kinsella, B. T. (2000) J. Biol. Chem. 275, 20412–20423
30. Godfrey, P. P. (1992) in Signal Transduction: A Practical Approach (Milligan, G., ed) pp. 105–120, IRL Press, Oxford
31. Thompson, A. K., Mostafapour, S. P., Denlinger, L. C., Bleasdale, J. E., and Fisher, S. K. (1991) J. Biol. Chem. 266, 23586–23602
32. Kobayashi, H., Horima, S., Nakahata, N., and Ohizumi, Y. (2000) J. Neurochem. 74, 2167–2173
33. Smyth, E. M., Nestor, P. V., and Fitzgerald, G. A. (1996) J. Biol. Chem. 271, 35986–35970
34. Blom, N., Kreegipuu, A., and Brunak, S. (1998) Nucleic Acids Res. 26, 382–386
35. Dogne, J. M., de Leval, X., Delarge, J., David, J. L, Masereel, B. (2000) Curr. Med. Chem. 7, 609–628
36. Oka, M., Negishi, M., Nishigaki, N., and Ichikawa, A. (1993) Cell. Signal. 5, 643–650
37. Vassaux, G., Gaillard, D., Ailhaud, G., and Negrel, R. (1992) J. Biol. Chem. 267, 11092–11097
38. Schwanner, I., Seifert, R., and Schultz, G. (1992) Biochem. J. 281, 301–307
39. Watanabe, T., Yatomi, Y., Sunaga, S., Miki, I., Ishii, A., Nakao, A., Higashihara, M., Seyama, Y., Ogura, M., and Saito, H. (1991) Blood 78, 2328–2336
40. Hebert, R. L., Regnier, L., and Peterson, L. N. (1995) Am. J. Physiol. 268, F145–F154
41. Boie, Y., Rushmore, T. H., Darmon-Goodwin, A., Grygorczyk, R., Slipetz, D. M., Metters, K. M., and Abramovitz, M. (1994) J. Biol. Chem. 269, 12173–12178
42. Nakagawa, O., Tanaka, I., Uusi, T., Harada, M., Sasaki, Y., Itoh, H., Yoshimasa, T., Namba, T., Narumiya, S., and Nakao, K. (1994) Circulation 90, 1643–1647
43. Sasaki, Y., Uusi, T., Tanaka, I., Nakagawa, O., Sando, T., Takahashi, T., Namba, T., Narumiya, S., and Nakao, K. (1994) Biochim. Biophys. Acta 1224, 601–605
44. Kilpatrick, G. J., Dautzenberg, F. M., Martin, G. R., and Eglen, R. M. (1999) Trends Pharmacol. Sci. 20, 294–301
45. Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997) Annu. Rev. Biochem. 66, 475–509
46. Okamoto, T., Murayama, Y., Hayashi, Y., Inagaki, M., Ogata, E., and Nishimoto, I. (1991) Cell 67, 723–730
47. Watson, S., and Arkinstall, S. (eds) (1994) The G-protein Linked Receptor Factsbook, Academic Press, London
Protein Kinase A-mediated Phosphorylation of Serine 357 of the Mouse Prostacyclin Receptor Regulates Its Coupling to Gs-, to Gβγ, and to Gq-coupled Effector Signaling

Orlaith A. Lawler, Sinead M. Miggin and B. Therese Kinsella

J. Biol. Chem. 2001, 276:33596-33607.
doi: 10.1074/jbc.M104434200 originally published online July 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104434200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 18 of which can be accessed free at http://www.jbc.org/content/276/36/33596.full.html#ref-list-1
During a recent review of this article, the authors realized that there may have been unspecified reordering of lanes in Fig. 11B and possible duplication of lanes 2 between Fig. 11, B and C. As the original data were no longer available, replicate data are provided. This correction does not affect the results or conclusions of this work. The authors wish to apologize for any inconvenience this error may have caused.