Investigation of the Mechanisms of G Protein: Effector Coupling by the Human and Mouse Prostacyclin Receptors

IDENTIFICATION OF CRITICAL SPECIES-DEPENDENT DIFFERENCES*

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We recently identified a novel mechanism explaining how the mouse (m) prostacyclin receptor (IP) couples to Goα, Goq, and Goi (Lawler, O. A., Miggin, S. M., and Kinsella, B. T. (2001) J. Biol. Chem. 276, 33596–33607) whereby mIP coupling to Goα and Goi is dependent on its initial coupling by Ser357. In the current study, the generality of that mechanism was expanded to Goq, whereby mIP coupling to Goq was inhibited by H-89. Abolition of the PKA site at Ser357, is replaced by a PKC site within the hIP, at Ser328. An analysis of their primary sequences revealed that the mIP to a PKC site generated mIP RP354,355QL that efficiently coupled to Gq, whereas the mIP to a PKA site generated mIP S325,326AL that failed to couple to Gq. Conversion of the PKC site of the hIP to a PKA site generated hIP S328A that efficiently coupled to Gq, but not to Gi, suggesting inhibition rather than coupling. Finally, conversion of the PKA site at Ser357 within the hIP to a PKC site generated hIP Q354,355LP that efficiently coupled to Gi.

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This article has been withdrawn by the authors. The authors of the paper have become aware that some features had been duplicated in Figs. 7, C and D, and 8G. As the original autoradiograms and scan images relating to the aforementioned figures are no longer available to investigate the matter, the authors wish to withdraw the article in the interests of maintaining their publication standards, while also respecting the highest standards of transparency and reliability of their research and of the JBC. Replica data sets for each of the figures in question that the authors state fully validate the findings and conclusions of the published article are available, and, accordingly, a revised version of the manuscript with the replica data sets can be obtained by contacting the corresponding author.
within the carboxy-terminal cytoplasmic (C) tail of the mIP, thereby switching mIP coupling from Gs to Gi and to Gq signaling (22).

Thus, it is evident that the IP is capable of coupling to multiple G protein effectors in a species- and/or tissue-specific manner. However, the molecular basis of this species-specific coupling has not been investigated in detail. Thus, in the present study, in view of the central roles of prostacyclin within the human vasculature, we sought to define the G protein coupling specificity of the hIP. Moreover, given the central role of PKA-mediated phosphorylation of the mIP in determining its G protein specificity and mechanisms of signaling, we sought to investigate whether the hIP undergoes a similar G protein switching mechanism accounting for its patterns of G protein coupling and intracellular signaling. Our data highlight critical differences in the signaling of the mIP and hIP that are regulated by their differential phosphorylation by the second messenger-regulated kinases PKA and PKC together with surrounding context sequence differences within those kinase recognition sites, thereby accounting for essential species-dependent differences in signaling by the mIP and hIP.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cicaprost was obtained from Schering AG (Berlin, Germany). Illoprost (1'Hiloprost, 15.5 Ci/mmol) was purchased from Amersham Biosciences. Fura2/AM, d-myoinositol 1,4,5-triphosphate, and its 3-deoxyhexosamidase acid (stable analogue of IP3) were purchased from Calbiochem. [3H]Orthophosphate (8000–9000 Ci/mmol) was obtained from PerkinElmer Life Sciences. [3H]AMP (15–30 Ci/mmol) was purchased from Amersham-Pharmacia Biotech. 

**Site-directed Mutagenesis**

Site-directed mutagenesis procedures were performed using the QuikChange site-directed mutagenesis kit (Stratagene). MIP coupling and intracellular signaling. Our data highlight critical differences in the signaling of the mIP and hIP that are regulated by their differential phosphorylation by the second messenger-regulated kinases PKA and PKC together with surrounding context sequence differences within those kinase recognition sites, thereby accounting for essential species-dependent differences in signaling by the mIP and hIP.

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**Measurement of cAMP—cAMP assays were carried out as previously described (14). Brieﬂy, cells were harvested into ice-cold PBS. Cells (1–2 × 106 cells/cm2) were homogenized in a modified Tyrode’s albumin buffer (26) containing 10 µM indomethacin.

**Measurement of IP_3 Levels—IP_3 binding assays were carried out as previously described (14). Brieﬂy, cells were harvested by scraping and washed twice with ice-cold PBS. Cells (1–2 × 106 cells/cm2) were homogenized in a modified Tyrode’s albumin buffer (26) containing 10 µM indomethacin.

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(101R, 1:300 dilution) at room temperature for 2 h. To investigate the effect of the protein kinase inhibitors, H-89 (10 μM) or GF 109203X (50 nM) were added and the cells were incubated for 2 min at 37 °C, 5% CO₂. Where appropriate, HA antibody (101R, 1:300 dilution) was used to immunoprecipitate the epitope-tagged IP receptors. The IP receptors were then resuspended at ~5 × 10⁶ cells/ml in HBS containing 10 mM LiCl. Cells (200 μl) were then preincubated at 37 °C for 10 min. To determine basal IP₃ levels, cells were incubated for 2 min at 37 °C, 5% CO₂, and IP₃ was measured. Where appropriate, the PKA inhibitor, H-89 (10 μM), was added prior to stimulation with cicaprost (1 μM). Furthermore, cells were stimulated for 2 min at 37 °C in the presence of cAMP (1 μM) or, for concentration response studies, were stimulated with cAMP 10⁻⁶ to 10⁻¹² M. To determine basal IP₃ levels, cells were incubated in the presence of an equivalent volume (50 μl) of the vehicle HBS. The IP₃ levels produced were determined using the IP₃ binding protein assay (28). Levels of IP₃ produced by ligand-stimulated cells over basal stimulation, in the presence of HBS, were expressed in picomoles of IP₃/mg of cell protein ± standard error (pmol/mg ± S.E.). The data presented are from representative experiments, each performed in duplicate.

**Measurement of Intracellular [Ca²⁺] Mobilization**—Measurements of [Ca²⁺], mobilization in Fura2/AM-preloaded cells was carried out essentially as previously described (24). Where appropriate, the PKA inhibitor, H-89 (10 μM) or PKC (10 μM), were added 2 min prior to stimulation with cAMP (1 μM). Drugs and inhibitors, with stock solutions dissolved in ethanol or Me₂SO, were diluted in modified Ca²⁺/Mg²⁺-free Hank’s buffered salt solution containing 20 mM HEPES, pH 7.67, 0.1% bovine serum albumin plus 1 mM CaCl₂ to the appropriate concentration such that addition of 20 μl of the diluted drug/inhibitor to 2 ml of cells resulted in the correct working concentration. In separate experiments, to examine the effect of the G protein on [Ca²⁺], mobilization, cells were transiently co-transfected with the plasmid pR5KβARK1 (495–869) encoding amino acid residues 459–689 from the C-tail of βARK1 (25 μg/10-cm dish) plus PADVA (10 μg/10-cm dish). To investigate the effect of PTx on [Ca²⁺], mobilization, cells were preincubated with PTx (50 ng/ml) for 16 h prior to stimulation with cAMP plus forskolin. For each [Ca²⁺], mobilization, cells were mobilized as a function of time (seconds) upon ligand stimulation or, alternatively, were calcium-loaded using Fura2/AM and the maximal fluorescence (Fₐ₈₅) was compared with that which occurred in HEK.mIP cells (14) stably overexpressing human (h)IP. The fluorescence signal was performed in 0.2% Triton X-100 to obtain a measure of [Ca²⁺], assuming a Kᵣ of 200 nM. The results presented in the figures are from representative experiments, each performed in four independent experiments and are shown as the mean ± S.E. Measurement of agonist-mediated IP₃ mobilization in Fura2/AM-preloaded cells was carried out essentially as previously described (22). Briefly, cells were washed a second time (Folbecco’s modified Eagle’s medium containing 5% FBS) and were metabolically labeled for 1 h in the same medium at 37 °C with 100 μCi/ml [³²P]orthophosphate (1000–9000 Ci/mmol) at 37 °C, 5% CO₂. Where appropriate, H-89 (10 μM), GF 109203X (50 nM), or the vehicle HEPES-buffered saline (HBS) was added for the duration of the labeling period. Thereafter, 1 μM cAMP or vehicle HBS was added for 10 min at 37 °C, 5% CO₂. Metabolic labeling of cells was terminated by transferring the dishes to ice and aspiration of the medium. Thereafter, cells were washed once in ice-cold PBS (2 ml/dish) and were lysed with 0.6 ml of radioimmuno precipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (v/v), 0.05% sodium deoxycholate (w/v), 0.1% SDS (w/v) containing 10 mM sodium fluoride, 25 mM sodium pyrophosphate, 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 15-min incubation on ice, cells were harvested and disrupted by sequentially passing through hypodermic needle (decreasing bore size: 20-, 21-, 23-, and 26-gauge), and soluble cell lysates were harvested by centrifugation for 15 min at 13,000 × g at room temperature. HEA 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 then washed twice by centrifugation at 13,000 × g at room temperature for 10 min and were washed three times in 0.3 ml of radioimmuno precipitation buffer and were finally 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 thereafter electroblotted onto PVDF membranes. Electrobots were then exposed to Eastman Kodak Co. X-Omat XR film to detect ³²P-labeled proteins. Thereafter, blots were subjected to Phosphorimage analysis, and the intensities of phosphorylation relative to basal phosphorylation were measured in arbitrary units of intensity relative to basal levels. In parallel experiments, cells were incubated under identical conditions in the absence of [³²P]orthophosphate; hIP, hIP(R325,326Q/5(R), hIP(R328A), mIP, and mIP(R354,355Q). Receptors were immunoprecipitated, and immunoblots were screened using the sheep anti-HA polyclonal antiserum conjugate (1:500) to check for recovery of each receptor type. Immunoreactive bands were quantified using the chemiluminescence detection system software (29), where sequences were aligned to show maximum homology. Amino acid sequence alignments were carried out using the ClustalW version 2.0 program. The statistical significance of differences was determined using the ClustalW program.
but not to Goi or to inhibition of adenylyl cyclase activity (Fig. 1, C and D, respectively). Whereas mIP coupling to Goi was inhibited by the PKA inhibitor H-89 (Fig. 1A) (22), the ability of the hIP expressed in HEL cells or in human platelets (data not shown) or in HEK.hIP cells to couple to Goi or to Goq was unaffected by H-89 (p = 0.94; Fig. 1E) or by the PKC inhibitor GF 109203X (p = 0.64, data not shown). These data indicate that, although the mIP couples to both Goq and Goi, the hIP couples to Goq but not to Goi.

To investigate the ability of the hIP to couple to Goq-mediated PLC activation, cicaprost-induced IP3 generation and concomitant increases in [Ca2+]i mobilization were investigated and were compared with that of the mIP. Stimulations of HEK.hIP, HEL, and HEK.mIP cells each resulted in efficient cicaprost-induced rises in [Ca2+]i, mobilization (Fig. 2, A–C, respectively), and increases in IP3 generation (Fig. 3A and data not shown), indicative of both hIP and mIP coupling to Goq-mediated PLC activation. Although cicaprost-induced [Ca2+]i mobilization (Fig. 2C, p = 0.24) and IP3 generation (22) by the control HEK.mIP cells were unaffected by preincubation with GF 109203X, H-89 significantly impaired mIP-mediated [Ca2+]i mobilization (Fig. 2A, p < 0.0005) and IP3 generation (22). In contrast, neither cicaprost-induced [Ca2+]i mobilization nor IP3 generation in HEK.hIP cells (Figs. 2A, 2D, and 3B) or in HEL cells (Fig. 2B and data not shown) were...
affected by H-89 or by GF 109203X. Thus, although the hIP and mIP both couple to Gq-mediated PLC activation, they display essential differences in their dependence on PKA and, hence, in their mechanism of Gq coupling whereby hIP independently couples to Gq, whereas mIP coupling is dependent on its PKA phosphorylation and consequent switching from Gs to Gq.

To fully exclude the possibility that cicaprost-induced [Ca\textsuperscript{2+}] mobilization might be mediated through Gi-derived Gi subunits, the effect of PTx and the Gi\beta\gamma sequestrant peptide, \(\beta\text{ARK1}_{459-689}\) (23) on [Ca\textsuperscript{2+}] mobilization was investigated. Neither preincubation of cells with PTx nor transient overexpression of \(\beta\text{ARK1}_{459-689}\) significantly affected cicaprost-induced [Ca\textsuperscript{2+}] mobilization in HEK.hIP cells (\(p > 0.35\) and \(p > 0.1\), respectively) or in HEK.mIP cells (22). Overexpression of the carboxyl-terminal residues of \(\beta\text{ARK1}\) was confirmed by Western blot analysis using anti-GRK 2 (Santa Cruz Biotechnology, As #C-15, data not shown).

Role of Second Messenger Kinases in hIP:G protein Coupling—We have previously established that mIP coupling to both Gs and Gq is dependent on its initial coupling to Go, and subsequent cAMP-dependent PKA phosphorylation, where Ser\textsuperscript{357} within the C-tail region of mIP was identified as the

FIG. 2. Effect of protein kinase inhibitors on cicaprost-induced [Ca\textsuperscript{2+}] mobilization. HEK.mIP (C) cells were preincubated for 2 min with 10 \(\mu\text{M}\) H-89 (Cic + H-89) or 50 \(\text{nM}\) GF 109203X (Cic + GF) prior to stimulation with 1 \(\mu\text{M}\) cicaprost. Data were calculated as changes in intracellular Ca\textsuperscript{2+} mobilization (\(\Delta\text{[Ca}^{2+}\text{]}\), \(\pm\) S.E., %). D–E, HEK.hIP (D) and HEK.mIP (E) cells were stimulated with 1 \(\mu\text{M}\) cicaprost. Data presented are representative profiles from at least three independent experiments and are plotted as changes in intracellular Ca\textsuperscript{2+} mobilization (\(\Delta\text{[Ca}^{2+}\text{]}\), nM) as a function of time (seconds) where cicaprost was added at the times indicated by the arrows. Actual mean changes in [Ca\textsuperscript{2+}], mobilized by HEK.hIP in the absence and presence of H-89, respectively; E, \(\Delta\text{[Ca}^{2+}\text{]}\) = 152 \(\pm\) 192 nM in the absence and 192 \(\pm\) 7.9 nM in the presence of H-89, respectively. **, \(p < 0.01\).

FIG. 3. Analysis of IP\textsubscript{3} generation by HEK.hIP cells. HEK.hIP cells transiently co-transfected with Gq were stimulated with cicaprost (10\textsuperscript{-12} to 10\textsuperscript{-6} M), and levels of IP\textsubscript{3} generation were measured (A). Alternatively, HEK.hIP cells transiently co-transfected with Go\textsubscript{13} (B) were stimulated with 1 \(\mu\text{M}\) cicaprost (Cic) or were preincubated for 5 min with H-89 (10 \(\mu\text{M}\), Cic + H-89) or with GF 109203X (50 \(\text{nM}\), Cic + GF) prior to stimulation with 1 \(\mu\text{M}\) cicaprost. In each case, basal IP\textsubscript{3} levels were determined by exposing the cells to the vehicle HBS under identical incubation conditions. Levels of IP\textsubscript{3} produced in ligand-stimulated cells relative to vehicle-treated cells (basal IP\textsubscript{3}) were expressed as \(-\)fold stimulation of basal (\(-\)fold increase in IP\textsubscript{3} \(\pm\) S.E., n = 4).
target residue for PKA phosphorylation (22). Optimal alignment of the primary sequences of the C-terminal cytoplasmic (C) tail regions of the hIP (residues 299–388) and mIP (residues 328–417), aligned to show maximum homology using the ClustalW software (29), are shown. The consensus PKC phosphorylation site within the hIP is underlined, and the putative phospho-target residue Ser328 is highlighted in boldface (32). Throughout the alignment, gaps, indicated by the dash symbol (-), were inserted to optimize the alignment; identical amino acids are indicated by an asterisk; conservative substitutions are represented by a colon, and semi-conservative substitutions are indicated by a period. Sequences for the hIP and mIP are based on published sequences (21, 30, 31).

![Alignment of human and mouse prostacyclin receptor amino acid sequences.](Image)

**Fig. 4.** Alignment of human and mouse prostacyclin receptor amino acid sequences. The deduced amino acid sequences of the carboxy-terminal cytoplasmic (C) tail regions of the hIP (residues 299–388) and mIP (residues 328–417), aligned to show maximum homology using the ClustalW software (29), are shown. The consensus PKC phosphorylation site within the hIP is underlined, and the putative phospho-target residue Ser328 is highlighted in boldface (32). Throughout the alignment, gaps, indicated by the dash symbol (-), were inserted to optimize the alignment; identical amino acids are indicated by an asterisk; conservative substitutions are represented by a colon, and semi-conservative substitutions are indicated by a period. Sequences for the hIP and mIP are based on published sequences (21, 30, 31).
On the other hand, the hIP S328A did not undergo significant cicaprost-induced phosphorylation (Fig. 7C). The identities of the broad radiolabeled band between 46 and 66 kDa were confirmed to be those of the immunoprecipitated HA-tagged hIP and its variant hIPQL325,326RP in parallel immunoprecipitations whereby efficient, quantitative recovery of each receptor type was detected by Western blot analysis using the anti-HA 3F10 peroxidase antibody (Fig. 7D, lanes 1 and 2, respectively). Moreover, quantitative recovery of hIP S328A from HEK.hIP S328A cells confirmed that the absence of significant phosphorylation of hIP S328A was not attributed to reduced receptor expression or due to reduced immunoprecipitation (Fig. 7D, lane 3).

Role of Second Messenger Kinases in mIP-G protein Coupling—As previously stated, coupling of the mIP to both Gaq and Goq is dependent on its initial coupling to Goq and consequent PKA phosphorylation of mIP at Ser357 thereby switching mIP coupling from Goq coupling to Gaq and Goq coupling (22). In the present study, we sought to fully explore the role of PKA in mediating mIP switching by investigating whether alteration of the consensus PKA target site surrounding Ser357 to a PKC target site would alter the G protein coupling specificity of the mIP. Thus, mIP RP354,355QL, a variant of mIP was generated whereby Arg354-Pro355, within the PKA consensus sequence RPAS357GRR, were converted to Gln354-Leu355, to generate a putative PKC consensus sequence QLAS357GRR (32).

Characterization of HEK.mIP RP354,355QL cells, recombinant HEK 293 cells stably overexpressing mIP RP354,355QL, by saturation radioligand binding confirmed high level receptor expression (Table I). Consistent with their ability to couple to Goq, both the mIP RP354,355QL (Fig. 8A) and mIP (Fig. 8C) exhibited efficient, cicaprost-induced increases in cAMP generation and transient co-transfection of HEK.mIP RP354,355QL cells with pCMV-Gaq significantly augmented that cAMP generation (2.29-fold augmentation, p < 0.01). In contrast to that of HEK.mIP cells (Fig. 8C), stimulation of HEK.mIP cells with cicaprost failed to inhibit, but rather augmented, forskolin-induced cAMP generation by mIP RP354,355QL. (Fig. 8A, p < 0.05). Moreover, although PTx (Fig. 8C) and H-89 (Fig. 8D) abolished mIP coupling to Goq, cAMP generation by HEK.mIP RP354,355QL cells was not affected by PTx (Fig. 8A) or by H-89 (Fig. 8B, p > 0.05) or by the PKC inhibitor GF 109203X (data not shown). Next, the ability of the mIP RP354,355QL to couple to Goq and PLC activation was investigated and was compared with
Fig. 6. Analysis of signaling by the hIP<sup>328A</sup>. A-C, HEK.hIP (<i>A</i>, lane 1), HEK.hIP<sup>328A</sup> (<i>C</i>, lane 1), HEK.hIP<sup>328A</sup> (<i>C</i>, lane 1), or control HEK 293 (<i>A</i>, lane 5) cells, metabolically labeled with [32P]orthophosphate, were incubated for 10 min with the vehicle HBS (<i>A</i>–<i>C</i>, lane 1) or were stimulated for 10 min with 1 μM cicaoprost (<i>A</i>, lanes 2 and 5; <i>B</i>–<i>D</i>, lane 2). Additionally, cells were preincubated with 10 μM H-89 (A–C, lane 3) or 50 nM GF 109203X (<i>A</i>–<i>C</i>, lane 4) for 10 min prior to stimulation with 1 μM cicaoprost for 10 min. Thereafter, HA epitope-tagged IP receptors were immunoprecipitated using the anti-HA antibody 101R. Immunoprecipitates were resolved by SDS-PAGE and electrophorosed onto PVDF membranes and were then exposed to X-Omat XAR-5 film (Kodak) for 5–10 days. Thereafter, blots were subject to PhosphorImager analysis, and the intensities of cicaoprost-mediated hIP, hIP<sup>325.326RP</sup>, and hIP<sup>328A</sup> phosphorylation relative to basal phosphorylation, in the presence of HBS, were determined and expressed in arbitrary units as follows: hIP: 1 μM cicaoprost, 9.48-fold; 1 μM cicaoprost plus 50 nM GF 109203X, 1.17-fold; 1 μM cicaoprost plus 10 μM H-89, 9.77-fold; hIP<sup>325.326RP</sup>: 1 μM cicaoprost, 8.46-fold; 1 μM cicaoprost plus 10 μM H-89, 9.48-fold; 1 μM cicaoprost plus 10 μM H-89, 1.20-fold; HEK.hIP: 1 μM cicaoprost, 1.20-fold; HEK.hIQ: 1 μM cicaoprost, 1.36-fold; HEK.hIPS328A: 1 μM cicaoprost plus 50 nM GF 109203X, 1.19-fold; 1 μM cicaoprost plus 10 μM H-89, 1.09-fold; HEK 293 cells: 1 μM cicaoprost, 1.20-fold. D, HEK.hIP, HEK.hIP<sup>325.326RP</sup>, HEK.hIPS328A, or control HEK 293 cells (lanes <i>1</i>–<i>4</i>, respectively) were subject to immunoprecipitation using the anti-HA antibody 101R, and immunoprecipitates were resolved by SDS-PAGE and electrophorosed onto PVDF membranes. Membranes were screened using the anti-HA 3F10 peroxidase-conjugated antibody, and immunoreactive bands were visualized by chemiluminescence detection. The positions of the molecular mass markers (kDa) are indicated to the left and right of A and D, respectively. Data presented are representative of three independent experiments.

Fig. 7. Cicaoprost induced-phosphorylation of hIP, hIP<sup>325.326RP</sup>, and hIP<sup>328A</sup>. A–C, HEK.hIP (<i>A</i>, lanes 1–4), HEK.hIP<sup>325.326RP</sup> (<i>B</i>), HEK.hIP<sup>328A</sup> (<i>C</i>), or control HEK 293 (<i>A</i>, lane 5) cells, metabolically labeled with [32P]orthophosphate, were incubated for 10 min with the vehicle HBS (<i>A</i>–<i>C</i>, lane 1) or were stimulated for 10 min with 1 μM cicaoprost (<i>A</i>, lanes 2 and 5; <i>B</i>–<i>D</i>, lane 2). Additionally, cells were preincubated with 10 μM H-89 (<i>A</i>–<i>C</i>, lane 3) or 50 nM GF 109203X (<i>A</i>–<i>C</i>, lane 4) for 10 min prior to stimulation with 1 μM cicaoprost for 10 min. Thereafter, HA epitope-tagged IP receptors were immunoprecipitated using the anti-HA antibody 101R. Immunoprecipitates were resolved by SDS-PAGE and electrophorosed onto PVDF membranes and were then exposed to X-Omat XAR-5 film (Kodak) for 5–10 days. Thereafter, blots were subject to PhosphorImager analysis, and the intensities of cicaoprost-mediated hIP, hIP<sup>325.326RP</sup>, and hIP<sup>328A</sup> phosphorylation relative to basal phosphorylation, in the presence of HBS, were determined and expressed in arbitrary units as follows: hIP: 1 μM cicaoprost, 9.48-fold; 1 μM cicaoprost plus 50 nM GF 109203X, 1.17-fold; 1 μM cicaoprost plus 10 μM H-89, 9.77-fold; hIP<sup>325.326RP</sup>: 1 μM cicaoprost, 8.46-fold; 1 μM cicaoprost plus 10 μM H-89, 9.48-fold; 1 μM cicaoprost plus 10 μM H-89, 1.20-fold; HEK.hIP: 1 μM cicaoprost, 1.20-fold; HEK.hIP<sup>325.326RP</sup>, HEK.hIPS328A, or control HEK 293 cells (lanes <i>1</i>–<i>4</i>, respectively) were subject to immunoprecipitation using the anti-HA antibody 101R, and immunoprecipitates were resolved by SDS-PAGE and electrophorosed onto PVDF membranes. Membranes were screened using the anti-HA 3F10 peroxidase-conjugated antibody, and immunoreactive bands were visualized by chemiluminescence detection. The positions of the molecular mass markers (kDa) are indicated to the left and right of A and D, respectively. Data presented are representative of three independent experiments.
Unlike that of the mIP, stimulation of HEK.mIPRP354,355QL cells with cicaprost did not result in significant increases in cicaprost-induced IP₃ generation (Fig. 8E, p > 0.32) or in [Ca²⁺]ᵢ mobilization (Fig. 8F, p > 0.50). Moreover, neither H-89 nor GF 109203X affected mIPRP354,355QL-mediated IP₃ generation (Fig. 8E) or [Ca²⁺]ᵢ mobilization (data not shown) but inhibited mIP-mediated Gq coupling and PLC activation (22). Whole cell phosphorylation studies established that, unlike the mIP (22), the mIPRP354,355QL did not undergo PKA- or PKC-induced phosphorylation in response to cicaprost.
stimulation; despite this, high levels of expression of mIPRP354,355QL were confirmed by radioligand binding studies (Table I) and by the efficient, quantitative recovery of the HA-tagged mIPRP354,355QL in the immunoprecipitates from HEK.mIPRP354,355QL cells but not from control HEK 293 cells (Fig. 8G).

Thus, taken together, these data highlight the essential role of PKA phosphorylation of Ser357 of the mIP in independently mediating its coupling to both Goi and to Gq, whereby conversion of critical determinants of the PKA recognition site to that of a defined PKC phosphorylation site inhibits cicaprost-induced mIP phosphorylation and switching from Goi and, thereby, inhibits its coupling to Goi and to Gq.

**DISCUSSION**

Prostacyclin plays a central role in the local control of vascular hemostasis acting as an endothelium-derived inhibitor of platelet aggregation and as a vasodilator (5). Mice deficient in the IP show increased susceptibility to thrombotic stimuli, exhibit diminished pain perception and inflammatory responses (3, 33), and develop more pronounced hypertension and vascular remodeling following hypoxic exposure relative to wild type mice (34). Although IPs are thought to primarily couple to adenylyl cyclase (5, 35), in certain species/cell types and a number of independent mechanisms have been proposed (40, 41). We have recently established that the mIP couples to both activation and to inhibition of adenylyl cyclase, via Gq (22). Moreover, Ser 328 of the hIP has been confirmed to be a direct target for cicaprost-induced PKC, but not PKA, phosphorylation (22).

The hIPQ₃₂₅,₃₂₆RP exhibited both Gq and Go coupling to Gqₐₙ and adenylyl cyclase (Fig. 9A), consistent with both mIP (14, 21, 22) and hIP (12, 15, 32) coupling to Gq. Although the sequence RPAS₃₅₇GRR within the mIP was predicted to act as a PKA site (32), we have confirmed that this site acts as a PKA, but not a PKC, site, and cicaprost-induced PKA phosphorylation of Ser328 of the hIP was inhibited by PTx and H-89. Hence, conversion of critical determinants of the PKA recognition site to that of a defined PKC phosphorylation site inhibits cicaprost-induced mIP phosphorylation and switching from Goi and, thereby, inhibits its coupling to Goi and to Gq.

In contrast, the hIPQ₂₃₂₅,₃₂₆RP exhibited both Gq and Go coupling to Gqₐₙ and PLC activation. Although stimulation of HEK.mIP and HEK.hIP cells with cicaprost also yielded significant increases in IP3 generation and [Ca²⁺]ᵢ, hIP in independently couples to Goi and to Gq, but does not undergo PKA phosphorylation and switching and does not couple to Goi (Fig. 9B).

While the mIP and rIP exhibit extensive amino acid sequence identity (94% identity), the hIP and mIP only share 73% overall identity, exhibiting greater divergence within their C-terminal regions (66% identity). Alignment of the C-tail regions of the mIP (21) and the hIP (30–32) revealed that the previously identified PKA site at Ser357 within the mIP (RPAS₃₅₇GRR), is replaced by a consensus target site for PKC within the hIP (QLAS₃₂₈GRR) whereby the Arg-Pro (RP) versus Gln-Leu (QL) represent the only divergent residues and therefore act as the determinants of PKA versus PKC phosphorylation of the targeted Ser. Although the sequence RPAS₃₅₇GRR within the mIP may actually be predicted to couple both a PKA or PKC site (32, 21), we have confirmed that the site acts as a PKA, but not a PKC, site, and cicaprost-induced PKA phosphorylation of Ser328 of the hIPQ₃₂₅,₃₂₆RP was inhibited by PTx and H-89. Hence, conversion of critical determinants of the PKA recognition site to that of a defined PKC phosphorylation site inhibits cicaprost-induced mIP phosphorylation and switching from Goi and, thereby, inhibits its coupling to Goi and to Gq.

Although Stimulation of HEK.mIP, HEK.hIP and HEL cells and human platelets with cicaprost led to concentration-dependent increases in cAMP generation, consistent with mIP and hIP coupling to Goi and hIP coupling to Gq. Whereas stimulation of the mIP with cicaprost inhibited forskolin (Fsk)-induced cAMP generation, consistent with mIP coupling to Goi, stimulation of the hIP augmented Fsk-induced cAMP generation indicative of the hIP coupling to Goi, but not to Gq. Moreover, although PTx and H-89 abolished mIP coupling to Goi, cAMP generation by the hIP was not affected by these agents.

The hIPQ₃₂₅,₃₂₆RP exhibited both Goi coupling with Goi, and Gq coupling inhibited by PTx and H-89. This receptor also exhibited Goi coupling that was unaffected by H-89 or by GF 109203X or PTx (data not shown), consistent with the independent coupling of hIPQ₃₂₅,₃₂₆RP to Goi and PLC activation. Although the hIP underwent cicaprost-induced phosphorylation, consistent with previous reports (12, 13), this phosphorylation was unaffected by H-89 but was inhibited by GF 109203X. Although the hIPQ₃₂₅,₃₂₆RP also underwent cicaprost-induced phosphorylation, it was unaffected by GF 109203X but was inhibited by H-89. Hence, conversion of the PKC site within the hIP to that of a PKA site generated hIPQ₃₂₅,₃₂₆RP, which can switch from Goi to Goi through a PKA-dependent mechanism (Fig. 9C), and implies that the contextual sequence surrounding Ser328 in the hIPQ₃₂₅,₃₂₆RP is essential for Goi coupling following its phosphorylation by PKA.

To further explore the essential requirement of the latter PKA phosphorylation at Ser328 in mediating hIPQ₃₂₅,₃₂₆RP switching and coupling to Goi, hIPS₂₃₂₈A was generated. Although the hIPS₂₃₂₈A coupled to Goi, it failed to exhibit coupling to Goi. The hIPS₂₃₂₈A also mediated increases in IP3 generation and [Ca²⁺]ᵢ, mobilization consistent with its independent coupling to Goi/PLC activation. Whole cell phosphorylations established that, unlike the hIP or the hIPQ₃₂₅,₃₂₆RP, the hIPS₂₃₂₈A did not undergo significant cicaprost-induced phosphorylation. Hence, the hIPS₂₃₂₈A independently couples to both Goi and Gq, similar to the hIP, but does not couple to Goi.

As previously stated, coupling of the mIP to both Goi and to Gq is dependent on its initial coupling to Goi and consequent
PKA phosphorylation of mIP at Ser\textsuperscript{357} thereby switching mIP from Go\textsubscript{a} coupling to Go\textsubscript{i} and Go\textsubscript{q} coupling (22). Herein, we also investigated whether alteration of the critical divergent residues within the consensus PKA target site surrounding Ser\textsuperscript{357}, which converts it to a PKC target site identical to that found within the hIP, would alter the G protein coupling specificity of the mIP. Although the mIP\textsuperscript{S,354,355QL} and Go\textsubscript{a} as not undergo measurable cia-prost-induced phosphorylation. Hence, conversion of the PKA site to a PKC site within the mIP yielded mIP\textsuperscript{S,354,355QL} that can independently couple to Go\textsubscript{a} but that does not undergo agonist-induced phosphorylation on Ser\textsuperscript{357} and, hence, cannot switch coupling from Go\textsubscript{a} to Go\textsubscript{i} or to Go\textsubscript{q} (Fig. 9D).

Taken together, these data with the hIP and the mIP and their mutants have indicated the critical requirement for the dipeptide RP, as opposed to a QL, in the -3 and -2 positions in addition to a phosphorylated Ser (representing +1) in determining mIP coupling to Go\textsubscript{i} and Go\textsubscript{q} and in determining hIP coupling to Go\textsubscript{a}, but not to Go\textsubscript{q}. Further experiments are required to dissect the importance of the contextual nature of the individual residues within the dipeptide RP sequence and/or any knock-on or consequent structural changes in determining that Go\textsubscript{i}/Go\textsubscript{q} interaction and coupling. A similar type of PKA-dependent mechanism is involved in regulating the human \(\beta_2\) adrenergic receptor switching from Go\textsubscript{i} to Go\textsubscript{q} (40). In this case, a 14-amino acid peptide containing a PKA recognition sequence RRSS within the third intracellular loop of the \(\beta_2\) adrenergic receptor could stimulate GTP\textsuperscript{S} binding by Go\textsubscript{a} but not by Go\textsubscript{q}; on the other hand, the PKA-phosphorylated peptide showed weak Go\textsubscript{i} activation and strong Go\textsubscript{q} activation (44). Similar to our findings with the mIP sequence (22), although the RRSS could be predicted to act as both a PKA and PKC recognition site, it was preferentially phosphorylated by PKA and not by PKC (44).

In essence, these studies highlight critical differences in mIP and hIP:G protein coupling and intracellular signaling. Although the mIP can couple to Go\textsubscript{a} and Go\textsubscript{i} and Go\textsubscript{q}, coupling to the latter G protein:effector systems is dependent on PKA-mediated phosphorylation and switching (Fig. 9A). The hIP, on the other hand, can independently couple to Go\textsubscript{a} and to Go\textsubscript{q},
but cannot couple to Goα (Fig. 9B). mIP coupling to Goα, versus that of hIP, is primarily regulated by a targeted carboxylate-induced PKA consensus sequence at Ser357 of the hIP, which is replaced by a PKC consensus sequence in the nIP, at Ser328. Conversion of the PKC recognition site within the nIP to a PKA site within hIPQL325,326RP facilitates its coupling to Goα (Fig. 9C). Therefore, emphasizing the importance of the contextual nature of the Arg225-Pro326 residues in addition to phosphorylation of Ser328 in regulating Goα coupling. mIP coupling to Goα is also regulated by a targeted carboxylate-induced PKA phosphorylation site at Ser357 and conversion of that PKA recognition sequence from a PKA to a PKA site (in mIPRL354,355QLL) abolished mIP coupling to Goα and Goαq (Fig. 9D). The hIP independently couples to Goαq and is not regulated by PKA or PKC phosphorylation of hIP at Ser328 as neither (a) conversion of the PKC site to the PKA site by hIPQL325,326RP nor (b) abolition of the PKC site by site-directed mutagenesis within hIP322AA impaired the ability of the hIP to couple to Goαq/PLC activation. Thus, it appears that other sequence differences within the hIP, as opposed to the mIP, act as determinants in facilitating its independent coupling to Goαq. Given the extent of sequence variation that exists between the hIP and the mIP, particularly within their C-tail sequences and third intracellular loop regions, it is likely that these domains may also contain the sequence determinants of Goαq interactions with the hIP.

These critical differences in mechanisms of intracellular signaling by the hIP and the mIP may confer important species-dependent differences to the cellular responses to prostacyclin stimulation under both physiologic/pathophysiologic settings. Precise appreciation of these mechanisms may offer a potential for differential targeting of prostacyclin-signaling within species and settings. Finally, data presented here confirm the existing knowledge of the generality of the coupling, particularly in relation to the Go protein Gαi signaling.

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