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The Critical Role of Transmembrane Prolines in Human Prostacyclin Receptor Activation

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ABSTRACT

The human prostacyclin receptor (hIP), a G protein-coupled receptor (GPCR), plays important roles in vascular smooth muscle relaxation as well as the prevention of platelet aggregation. It has been postulated that GPCR transmembrane (TM) prolines serve as molecular hinges or swivels and are necessary for proper binding and activation. By individually (as well as collectively) mutating these hIP prolines to alanine, the ability to form key structural and functional configurations was removed. Significant effects on both binding and activation were observed. Two highly conserved prolines across GPCRs, Pro-154, and Pro-254 (TMVI), showed the greatest effect on decreasing both binding and activation when changed to alanine. Along the extracellular boundary of the highly conserved transmembrane III domain, a proline-to-alanine mutation at position 89 (P89A) revealed normal binding affinity in comparison with the 1D4-epitope–tagged hIP (hIP1D4) wild-type control (K_i, iloprost = 3 ± 2 versus 7 ± 3 nM, respectively). In contrast, activation was markedly affected, with an EC_{50} of 12.0 ± 2.5 nM compared with that of 1.2 ± 0.3 nM (10-fold difference) for the hIP1D4. Movement within TMIII has been shown to be necessary for effective GPCR activation. Both the extracellular location (above the putative binding pocket) along with an exclusive effect upon activation suggest that this movement is facilitated by the presence of Pro-89 and independent from the actions of ligand binding. This finding strongly supports a model in which proline residues serve as molecular hinges or swivels, essential for coupling receptor binding to activation.

Prostacyclin plays an important role within the cardiovascular system, promoting vascular smooth muscle relaxation as well as preventing platelet aggregation. The actions of prostacyclin are mediated through a seven-transmembrane domain-spanning G protein-coupled receptor (GPCR), known as the IP or prostacyclin receptor. The gene for the human prostacyclin receptor (hIP) was cloned in 1994 (Boie et al., 1994; Katsuyama et al., 1994). As with other GPCRs, the hIP undergoes conformational changes within the seven transmembrane domains upon binding its native ligand, prostacyclin. This transformation is then conveyed to the cytoplasmic domain, where it ultimately promotes G protein activation. Recent biochemical and EPR studies have determined that TMIII and TMVI movements are essential for this activation (Farrens et al., 1996; Sheikh et al., 1996, 1999).

Limited structure-function studies on the IP have determined that TMVI and TMVII domains are involved in binding prostacyclin side groups, whereas TMI attaches to the cyclopentane ring (Kobayashi et al., 1997, 2000). Further studies have shown that glycosylation at Asn-78 and Asn-7 are important for membrane localization, ligand binding, and signal transduction (Zhang et al., 2001), and the C-terminal tail, which is isoprenylated (mice), affects the efficiency of G protein coupling (Hayes et al., 1999). Moreover, the cytoplasmic domain has been shown to contain a critical phosphorylation site (Ser-328) for homologous desensitization of the hIP receptor (Smyth et al., 1998). It remains unclear, both for the hIP as well as for other GPCRs, how the action of ligand binding is transmitted through the receptor protein (presumably via some conformational adjustment) to stimulate G protein Gs in the case of the hIP and trigger signal transduction cascades.

Experimental and simulation studies indicate that conformational modifications within transmembrane α-helices can be generated by proline-containing motifs (Sansom and Weinstein, 2000). Furthermore, work with the β_2-adrenergic receptor, by way of fluorescent labeling, has suggested that such conformational changes may be diminished upon activation by an agonist (Gether et al., 1997). The recent discovery of the crystal structure of rhodopsin (Palczewski et al., 2000) has revealed that transmembrane prolines, within α-helices I, IV, VI, and VII, induce kinks that are evident in

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ABBREVIATIONS: GPCR, G protein-coupled receptor; IP, prostacyclin receptor; hIP, human prostacyclin receptor; TM, transmembrane; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; PG, prostaglandin; HEM, HEPES/EGTA/$MgCl_2$.

the tertiary structure of the protein (Palczewski et al., 2000). Furthermore, each proline residue found within helices IV, V, VI, and VII is highly conserved (68, 85, 100, and 98%, respectively) across a variety of GPCRs (Baldwin et al., 1997) and serve as markers in the identification, parsing, and modeling of transmembrane domains (Baldwin et al., 1997; Sansom and Weinstein, 2000). In total, nine prolines are present within the transmembrane domains of the hIP receptor (Pro-17 TMI, Pro-69 TMII, Pro-89 TMIII, Pro-141 TMIV, Pro-154 TMIV, Pro-179 TMV, Pro-254 TMVI, Pro-285 TMVII, and Pro-289 TMVII) (Fig. 1). However, their functional roles in ligand binding and receptor activation are unknown. Of particular interest in our investigation are those prolines located within TMIII (Pro-89) and TMVI (Pro-254), the same TM domains that have been shown to move upon light activation within the rhodopsin receptor.

Our hypothesis was that transmembrane proline residues, in addition to forming critical helical bends, can act as molecular hinges or swivels, permitting the necessary transmembrane movement required for hIP and GPCR activation. Furthermore, we wished to ascertain the importance of transmembrane proline residues within the clinically important hIP receptor, particularly Pro-89 in TMIII, and determine which, if any, were essential for receptor activation and binding to prostacyclin. Each of the transmembrane proline residues was examined individually as well as in specific combinations. Our findings confirmed that effects of TM prolines on helical conformation and movements were variable and differential in regards to binding and activation. In particular, the TMIII proline (Pro-89) situated above the putative binding pocket had no effect on binding but was detrimental to activation when mutated to alanine (P89A). Conversely, the proline in TMV (P179A) located in a similar extracellular border position to Pro-89 had a significant negative effect on binding but allowed for normal, wild-type-like activation. Furthermore, Pro-254 in TMVI showed a significant decrease in both binding and activation when removed through mutation to alanine. The sum of these differential effects on hIP receptor binding and activation strongly supports the involvement of proline residues in transmembrane conformations and movements required for hIP activation.

Experimental Procedures

Materials. Ligand agonists were obtained from the following manufacturers: [^{3}H]iloprost (17.0 Ci/mmol), unlabeled iloprost, and the cAMP radioimmunoassay system were purchased from Amersham Biosciences (Piscataway, NJ); carbacyclin was obtained from Sigma-Aldrich (St. Louis, MO); and PGE_1 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). The hIP cDNA was a generous gift from Dr. Mark Abramovitz (Merck Frosst Canada Ltd., Kirkland, QC, Canada).

Construction of the hIP1D4-PMT4. The hIP cDNA flanked by 5′ *Eco*RI and 3′ *Not*I restriction sites was subcloned into the PMT4 vector. A new *Sal*I restriction site was created at residue 1023 by mutating a Thr to an Ala, using PCR mutagenesis (described below). A *Sal*I/*Not*I digestion released a 338-base pair fragment, which was replaced with an equivalently sized *Sal*I/*Not*I fragment from rhodopsin. This replaced the carboxyl terminal peptide SKAEASVACSLC from the hIP with SKTETSQVAPA (1D4 epitope) from rhodopsin. This allowed recognition by a high-affinity monoclonal antibody (1D4). Control experiments were performed to show that this had no significant effect on either binding or activation.

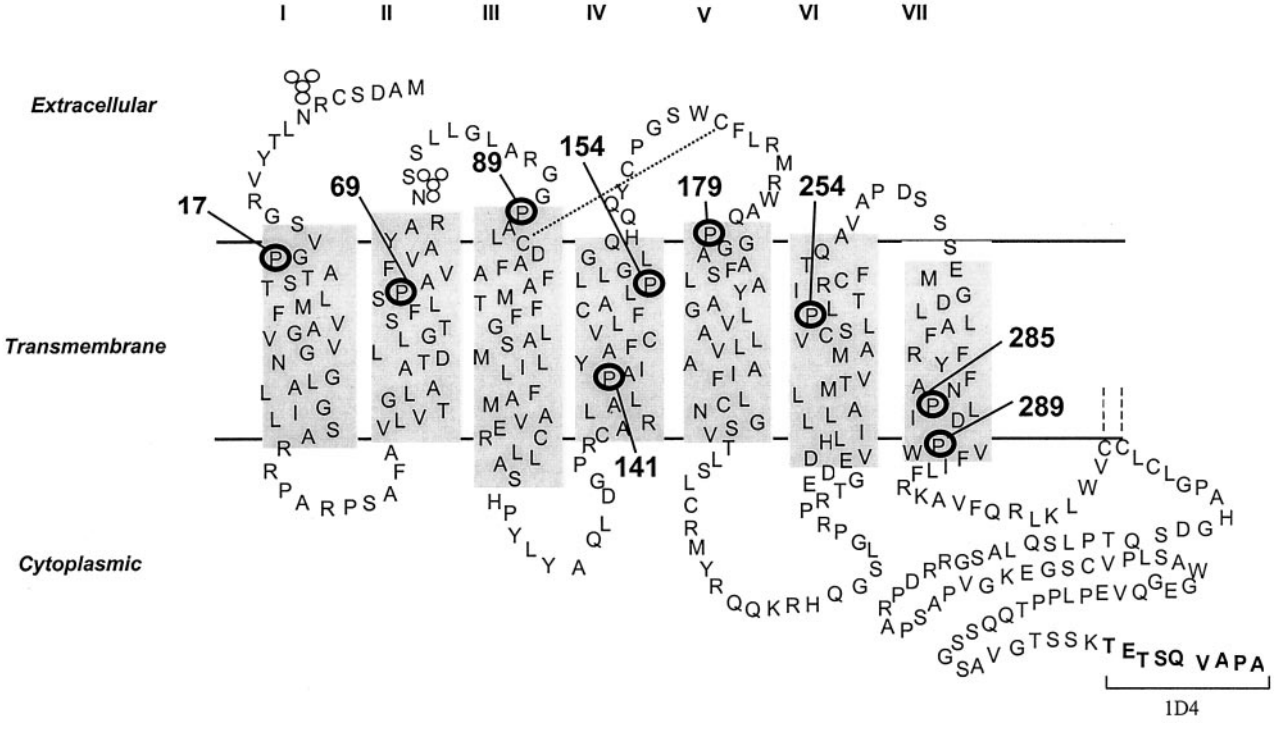

Fig. 1. Secondary structure model of the hIP receptor. Shown are the three domains (i.e., extracellular, transmembrane, and cytoplasmic). Within the extracellular region are consensus sequences for glycosylation (chain of circles at Asn-7 and Asn-78) and the conserved disulfide bond (indicated by the dotted line). The putative transmembrane helices are indicated by the shaded rectangles. The prolines studied, Pro-17 (TMI), Pro-69 (TMII), Pro-89 (TMIII), Pro-141 (TMIV), Pro-154 (TMIV), Pro-179 (TMV), Pro-254 (TMVI), Pro-285 (TMVII), and P289A (TMVII), are circled. The carboxyl tail in the cytoplasmic domain contains two palmitoylation sites (dashed lines) in addition to the 1D4 epitope tag (bold letters).

Construction of Mutations. A PCR protocol was used for site-directed mutagenesis. Two complementary primers were designed extending 10 to 12 nucleotides 3′ and 5′ from the desired mutation site. The reaction mixture contained 1× *Pfu* reaction buffer, 200 ng of DNA construct, 150 ng each of two primers (sense and antisense), 0.2 mM dNTPs, and 2.5 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The cycling protocol involved heating and cooling at 95°C for 30 s, 55°C for 1 min, and 68°C for 10 min, for 16 cycles. The products were then digested with the restriction enzyme *Dpn*I (Promega, Madison, WI) for 3 h to remove native wild-type strands, leaving only the synthesized strands. Ten microliters of PCR product were used to transform competent DH5α cells ($\sim 2 \times 10^9$ cells), followed by DNA extraction from the selected clones. Large plasmid preparations were performed using Wizard *Plus* Maxiprep kits (Promega). All mutant constructs were confirmed through DNA sequencing at the Dartmouth Medical School Molecular Biology Core Facility.

Transfection of COS-1 Cells. COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Cellgro; Mediatech, Herndon, VA) supplemented with penicillin, streptomycin, glutamine, and 10% fetal bovine serum. At 80% confluence, transient transfections were performed. In brief, the 150-mm plates were initially washed with DMEM followed by the addition of the DNA mixture (20 μg per plate) in DEAE-dextran (0.2 mg/ml DMEM). After a 6-h incubation, 0.1 mM chloroquine was added for 1 h and then removed with DMEM washes. Cells were harvested 72 h after transfection.

Membrane Preparations. COS-1 cell plasma membrane preparations have been described previously (Perez et al., 1991); this technique is based on differential centrifugation using sucrose gradients (Ozols, 1990). Membranes were prepared as follows: the cells were washed in phosphate-buffered saline and harvested using a cell scraper. The cells were then washed twice in 0.25 M sucrose followed by vigorous vortexing (providing shear forces) for 3 min. A low-speed spin was performed at 1260*g* for 5 min, and the supernatant centrifuged at 30,000*g* for 15 min. The pellet was then washed twice in HEM (20 mM HEPES, pH 7.4, 1.5 mM EGTA, and 12.5 mM $MgCl_2$), followed by resuspension in HEM containing 10% glycerol and stored at −70°C. A Bradford protein assay was performed to quantitate membrane proteins.

Ligand Binding. The ligand-binding characteristics of the expressed receptors were determined in a series of radioligand binding studies using [^{3}H]iloprost, an IP receptor-specific agonist. The competition study involved duplicate wells containing 50 μg of total membrane protein, HEM buffer, and 15 nM [^{3}H]iloprost, and nonradiolabeled iloprost at 11 concentrations extending from 10 μM to 0.01 nM. Nonspecific binding was determined with the addition of a 500-fold excess of nonradiolabeled iloprost. For saturation studies, 1 to 100 nM [^{3}H]iloprost was used. After 1.5 h incubation at 4°C, reactions were stopped by the addition of ice-cold 10 mM Tris/HCl buffer, pH 7.4, and were filtered through Whatman GF/C glass filters (Whatman, Clifton, NJ) using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). The filters were washed five times with ice-cold Tris buffer. The filters were counted in the presence of 5 ml of Liquiscint (National Diagnostics, Atlanta, GA). Data were analyzed using Prism software (GraphPad Software, San Diego, CA). IC_{50} values were converted to K_i values using the Cheng-Prusoff equation. K_i values were expressed as mean ± S.E. Analysis of variance (post-test Newman-Keuls) and unpaired Student's *t* test were used to determine significant differences ($p < 0.05$).

Western Analysis. The presence of mutant protein with low affinity to iloprost was determined through Western analysis, using monoclonal antibodies targeting the 1D4 epitope tag. Thirty microliters of membrane preparation, containing 30 μg of membrane protein, were subjected to 10% SDS-polyacrylamide gel electrophoresis. This was transferred to nitrocellulose membrane, immunoblotted using a 1D4 monoclonal antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody, and detected with enhanced chemiluminescence reagents.

cAMP Determination. The wild-type and mutant constructs were analyzed for signal transduction capabilities. Transfections were performed on 25-mm plates using 2 μg of DNA as described. After 72 h, the cells were washed twice with phosphate-buffered saline plus 4 mM EDTA and 2 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and incubated at 20°C for 10 min. This was followed by addition of defined concentrations of iloprost to selected plates. Basal and maximal stimulation were performed in triplicate in the presence or absence of 1 μM iloprost. Dose-response curves were determined by the addition of six different concentrations (1 μM to 10 pM) in duplicate. After 20 min, the cells were harvested and boiled for 3 min followed by centrifugation. Fifty microliters of the resultant supernatant (total of 300 μl) were used to determine cAMP production in the competition assay. cAMP levels were measured using the radio-receptor competition assay (Amersham Biosciences). In brief, [^{3}H]cAMP was used in competition for a cAMP-binding protein against known concentrations of nonradiolabeled cAMP followed by determination of the unknowns. The reaction was allowed to proceed for 2 h at 4°C. Charcoal was used to remove excess unbound cAMP. The sample was then subjected to counting with 5 ml of Liquiscint. Results were analyzed with Prism software. Mean ± S.E.M. was calculated for basal and maximal cAMP production. For the dose response, a best-fit dose-response curve was calculated, and the EC_{50} was determined for wild-type and mutant constructs. Analysis of variance (post-test Newman-Keuls) and unpaired Student's *t* tests were used to determine significant differences ($p < 0.05$).

Results

For optimal expression and detection by Western analysis, the hIP receptor sequence was subcloned into the PMT4 expression vector, and the 1D4 epitope tag was added to the C terminus. Initial control experiments were performed to assess the effect of the 1D4 epitope tag and the PMT4 vector upon binding and activation. After determining that the 1D4 tag and the PMT4 vector confer robust expression, binding, and activation (similar to wild-type hIP) and that no background IP (i.e., no native IP receptors) were present on our COS-1 cell expression system, individual proline residues within each of the seven transmembrane helices were mutated to alanine within the hIP1D4PMT4.

Characterization of High-Efficiency hIP Expression System. The terminal epitope tag had no significant effect on agonist binding (Fig. 2A). The hIP in PMT4 without the 1D4 epitope tag (hIPPMT4) showed an iloprost (high-affinity stable analog of prostacyclin)-binding affinity (K_i ± S.E.M.) of 16 ± 5 nM ($n = 3$) in comparison with the hIP1D4PMT4 of 7 ± 3 nM ($n = 7$). Expression of hIP in the vector pcDNA (in the absence of the 1D4 epitope tag) also indicated no significant difference in affinity, $K_i = 12 \pm 4$ nM ($n = 3$). These results for iloprost binding to the hIP receptor, in the presence of the 1D4 epitope in two different expression vectors, are comparable with those described previously for hIP (K_i = 11 nM) (Narumiya et al., 1999). A single binding site produced the best curve fit ($r^2 > 0.98$). This has also been observed in other studies on the human IP (Namba et al., 1994). However, studies (in mouse IP) have shown two binding sites (i.e., high and low affinity) (Hayes et al., 1999). The differences may be due to the different expression systems or interspecies variation.

The C-terminal epitope tag had no effect on activation in the prostacyclin receptor as assessed by cAMP production.

Iloprost dose responses were performed in parallel with mock transfection, hIP in the PMT4 vector (hIPPMT4), and in the presence of the 1D4 epitope tag (hIP1D4PMT4) to determine EC_{50}. The mock transfection showed no significant increase in cAMP over the dose range 0.01 nM to 1 μM (Fig. 2B). In the presence and absence of the 1D4 epitope tag in the expression vector PMT4, the EC_{50} values were 1.2 $\pm$ 0.3 nM (n = 12) and 3.5 $\pm$ 1.3 nM (n = 3), respectively. This difference was not statistically significant as determined by an unpaired Student's t test. One such experiment of at least three separate experiments (performed in duplicate) is shown in Fig. 2B. The 1D4 epitope tag replaces the hIP sequence containing Cys-414, thought to be important for isoprenylation in mouse IP, upon mutation to a serine (C414S) (Hayes et al., 1999). Our results indicate no loss of receptor function in the absence of this cysteine. This result may be due to differences in species or cell type, the ability of the inserted rhodopsin sequence to compensate for the changes in IP, or the possibility that the serine substitution was phosphorylated (leading to desensitization). When performed in parallel, our studies indicate that the presence or absence of the 1D4 epitope tag showed no significant difference in either binding or activation and is comparable with previously published results for the human IP.

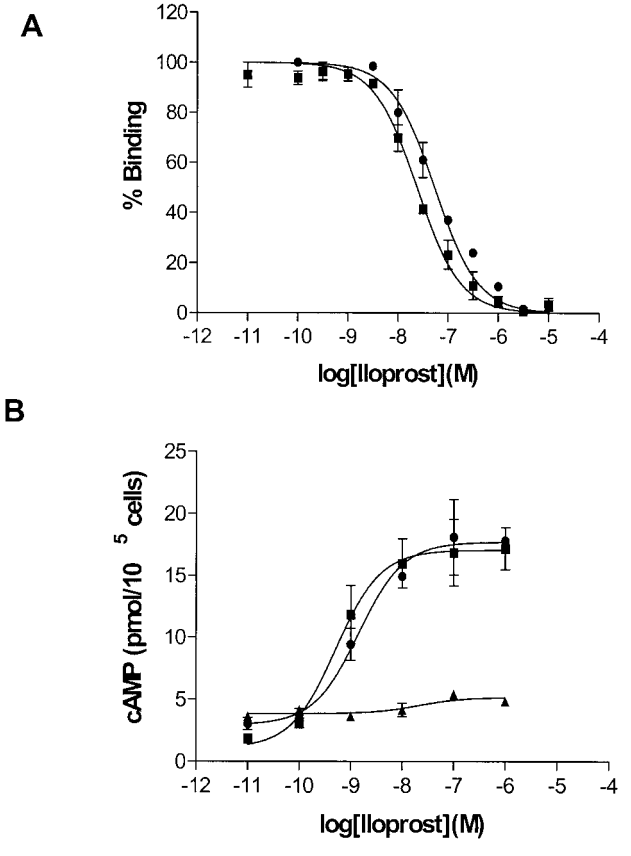

Fig. 2. A, competition binding studies for constructs hIP1D4PMT4 (■) and hIPPMT4 (●), which had no 1D4 epitope tag. Concentrations of iloprost from 10 μM to 0.01 nM were used as the nonradiolabeled competitor. Shown are the mean and S.E. from at least three separate experiments performed in parallel. IC_{50} values were determined from the best-fit single slope curve (GraphPad Prism). B, cAMP activation of the control constructs as described in A. A mock transfection control (▲) was also determined for cAMP stimulation. Iloprost concentrations ranging from 1 μM to 0.01 nM were used. Results are the mean and S.E. (picomoles of cAMP per 10^5 cells transfected) from one experiment, with all constructs performed in parallel. EC_{50} was determined from best-fit single slope curve (GraphPad Prism). At least three repetitions were performed, all showing similar results.

Ligand Binding. Iloprost (high-affinity analog of prostacyclin), carbacyclin (low-affinity analog of prostacyclin), and PGE_1 (low-affinity nonselective agonist for the hIP receptor) were used to assess whether or not proline-to-alanine mutations disrupted agonist binding. Binding of agonist in regards to the individual proline-to-alanine mutations revealed a spectrum of affinities, ranging from no significant effect ($p >$ 0.05) to mild defects in binding, to severe binding deficits ($p <$ 0.001) (Table 1). Wild-type binding affinities for iloprost were observed after mutations within TMI (P17A = 52 nM), TMII (P69A = 22 nM), TMIII (P89A = 3 nM), TMIV (P141A = 14 nM), and TMVII (P285A = 31 nM) (Table 1). The affinities were not significantly different from the wild-type hIP receptor, as determined by analysis of variance and Student's t test. P89A (TMIII) showed no significant difference in affinity for iloprost, carbacyclin, or PGE_1 (Fig. 3 and Table 1). A significant decrease in binding affinity ($p < 0.05$) was seen with the P179A mutation (TMV) (K_i = 87 nM) for iloprost and carbacyclin (K_i = 660 nM) (Table 1). The proline-to-alanine mutation at the highly conserved NPXXY motif in TMVII had no effect on binding (P289A = 4 nM). Mutations at P154A (TMIV) and P254A (TMVI) were the most severe in their effects on binding, with little specific binding shown, even at 100 nM [^{3}H]iloprost (from saturation binding). This was calculated to be 7 and 3%, respectively, compared with the wild-type protein at equivalent expression.

cAMP Production. The results of the cAMP assays reflect the activity exhibited by the hIP1D4PMT4 (wild-type) and mutant receptors upon activation by iloprost. Basal and maximal activities, in addition to EC_{50}, were determined. On assessing the basal activities, the proline-to-alanine mutations exhibited no significant variations compared with hIP1D4PMT4 (data not shown). Although ligand binding was normal, dose response (EC_{50}) for cAMP production showed a significant variation (by at least 5-fold) with P89A in TMIII (Fig. 4), in addition to P17A (TMI), P69A (TMII), and P289A (TMVII), compared with that of wild-type protein (Table 2). In contrast, proline mutations within TMV (P179A), although exhibiting abnormal binding, showed normal EC_{50} values similar to wild-type hIP (Table 2). Only the P154 and P254 mutations profoundly affected both activation and binding (Tables 1 and 2; Fig. 4). The severe defect in activation may be partially due to the severe defect in binding. Both TMIV (P141A, P154A) and TMVII (P285A, P289A), each of which contains two prolines, showed decreased activation for one residue (TMIV - P154A and TMVII - P289A) and wild-type activation for the other (TMIV - P141A and TMVII - P285A) (Table 2). These results show a discernible differential effect of proline mutations on binding and activation.

Protein Quantitation with Saturation Binding and Western Analysis. Receptor expression was quantitated using saturation binding with iloprost, as outlined under *Experimental Procedures*. The single mutants, when transfected in parallel (20 μg of DNA construct per 150-mm plate), expressed between 0.4 and 4.8 pmol of receptor/mg of COS-1 cell membrane protein (Table 1). P141A and P285A expressed 2- to 3-fold better than wild-type protein (Table 1). Western analysis on membrane preparations expressing hIP in the presence of the 1D4 monoclonal antibody tag showed

at least three bands ranging from approximately 39 to 45 kDa (Fig. 5). The hIP1D4 bands of higher molecular mass reflect the different states of glycosylation, as is typically seen with GPCR expression in eukaryotic cells. In some instances, such as with rhodopsin in COS-1 cells, these different levels of glycosylation are represented as a smear (Hwa et al., 1997); but more often than not, they can be seen as distinctly higher molecular mass bands, as is the case with 1D4-tagged β_2- and AT_1 receptors (Noda et al., 1994, 1996). All 10 constructs were transfected in parallel with equivalent DNA concentrations (20 μg per 150-mm plate). The membranes were also prepared in parallel using 30 μg of membrane protein as determined by Bradford assay. Protein presence was determined by Western analysis, and the results can be seen in Fig. 5. Furthermore, a correlation between these results and those of the saturation binding assays is evident as well. Differences observed (e.g., P285A and P141A versus hIP1D4PMT4) are probably due to protein aggregation or degradation. The 1D4 epitope tag (2 μg per 10 ml of Tris-buffered saline/Tween 20) is effective in detecting the hIP1D4PMT4; however, its ability to accurately quantitate receptor numbers requires further investigation. Nevertheless, the two receptors that exhibited very poor binding affinities (P154A and P254A) are being expressed at approximately one-fifth the levels of the hIP1D4PMT4.

Combination Proline Mutants. With the individual binding and activation characteristics of each proline mutation established, our next objective was to combine mutations to verify whether the effects of each mutation were independent and localized, thus becoming additive upon combination. A series of combination mutations were constructed, first composed of double mutations followed by triple mutations (Tables 3 and 4). As a result, hIP receptors with progressively fewer and fewer proline residues within the TM domains were created. Two constructs had three prolines replaced by alanines, P17A/P69A/P89A (TMI, TMII, and TMIII) and P141A/P179A/P285A (TMIV, TMV, and TMVII). These combinations were chosen because of their adjacent helical positions and the fact that each individual mutation within TMI, TMII, and TMIII had significant negative effects on activation, as opposed to binding. Mutations with severe effects on both binding and activation (e.g., P154A and P254A) were excluded from such combinations. The results for each double mutation revealed ample expression, along with wild-type-like binding (Table 3). However, activation reflected that of the most severe mutation (Table 4). Upon combining three proline-to-alanine mutations within a single construct, both binding and activation were severely affected (Tables 3 and 4), as was protein expression, with both of the triple proline mutants expressing four to five times less receptor protein compared with the wild-type hIP1D4PMT4.

Discussion

The hIP is an important receptor critical for cardiovascular function, and our current knowledge of its structure and function is limited. The exact locations and configurations of the binding pocket and activation domains are currently being investigated. However, the way in which receptor-ligand binding is coupled to activation of signal transduction remains undetermined. It is our suggestion that transmembrane proline residues facilitate this process by forming molecular hinges or swivels, conferring the necessary transmembrane movements required for GPCR activation. Support for this hypothesis can be seen with Pro-89 in TMIII, of which location is above both the putative binding pocket as well as with the highly conserved Cys-92 (involved in critical disulfide bond) and Asp/Glu-Arg (D/E, R) sequence (critical for G protein activation). The aim of this study was to ascertain the importance of transmembrane proline residues (particularly Pro-89 in TMIII) within the hIP receptor and determine which, if any, were essential for receptor activation and binding to prostacyclin. In addition, each transmembrane proline residue was examined individually, as well as in specific combinations, to determine whether any of the effects being observed were localized or global occurrences.

Our initial focus was geared toward determining the roles of individual proline residues, particularly Pro-89 (TMIII) as well as Pro-254 (TMVI), in ligand binding and signal transduction activation. The recent discovery of the crystal structure of rhodopsin, as well as EPR and biochemical data on transmembrane movements during activation, have provided us with a solid GPCR model by which to compare and interpret the results from our investigations into the structure and function of the hIP receptor. Our findings suggest that Pro-89 in TMIII plays a critical role in receptor function, coupling the processes of ligand binding and activation of G protein. Other TM prolines may exhibit similar or differen-

TABLE 1

Ligand binding studies for the single proline-to-alanine mutations

Shown are K_i values (mean ± S.E.) from at least three separate experiments (duplicates of 12 different concentrations). Receptor expression was determined by saturation binding from membranes prepared from COS-1 cells transfected with 20 μg of DNA per plate.

	Iloprost	Carbacyclin	PGE_1	Expression
	nM	*nM*	*nM*	*pmol/mg protein*
hIP1D4PMT4 (WT)	7 ± 3	121 ± 51	301 ± 90	2.1 ± 0.5
P17A (I)	52 ± 10	270 ± 52	896 ± 173	1.7 ± 0.3
P69A (II)	22 ± 6	128 ± 31	139 ± 28	0.9 ± 0.2
P89A (III)	3 ± 2	56 ± 22	149 ± 109	0.7 ± 0.4
P141A (IV)	14 ± 3	233 ± 56	697 ± 207	4.8 ± 2.7
P154A (IV)	>500**	N.D.	N.D.	0.4 ± 0.1
P179A (V)	87 ± 9*	660 ± 81*	880 ± 83	1.0 ± 0.3
P254A (VI)	>500**	N.D.	N.D.	0.4 ± 0.2
P285A (VII)	31 ± 5	243 ± 44	794 ± 161	4.2 ± 0.6
P289A (VII)	4 ± 1	354 ± 154	545 ± 307	0.8 ± 0.2

N.D., not determined.
* $p < 0.05$.
** $p < 0.001$.

tial roles, such as Pro-179 (TMV), which seems crucial for proper binding, yet holds little effect on activation, and Pro-254 (TMVI), which seems uniformly necessary for both processes.

Role of TM Prolines in Activation. In converting each proline residue to alanine, our aim was to replace those residues that are most incompatible with α-helix formation (prolines) with those residues that are most compatible, namely alanine (Lyu et al., 1990; O'Neil and DeGrado 1990). Thus, the net effect of this exchange straightened the bends that arise from transmembrane prolines (Palczewski et al., 2000). Because of the centralized and extracellular surface location of many of these residues (Fig. 1), it was expected that each proline-to-alanine mutation would significantly disrupt the ligand-binding pocket of the receptor, resulting in an alteration of normal binding affinity. However, this was not the case, with most of the mutations having normal binding affinities (Fig. 6A). Although the mutations in TMI, TMII, TMIII, and TMVII had little effect on binding, they had a significant effect on activation (Fig. 6B). Conversely, the P179A mutation had abnormal binding but a normal EC_{50} for cAMP production. These findings support the notion that structural requirements for ligand binding and activation in the hIP are different (Fig. 6).

Studies on rhodopsin (Farrens et al., 1996; Sheikh et al., 1996; Cai et al., 1999) and other GPCRs (Sheikh et al., 1999) have shown that movements of TMIII and TMVI are the most critical for GPCR activation. Furthermore, structural changes upon activation have also been noted around TMI and TMII (Yang et al., 1996; Altenbach et al., 1999; Klein-Seetharaman et al., 1999). As seen in Fig. 1, the three prolines in TMI, TMII, and TMIII are located on the extracellu-

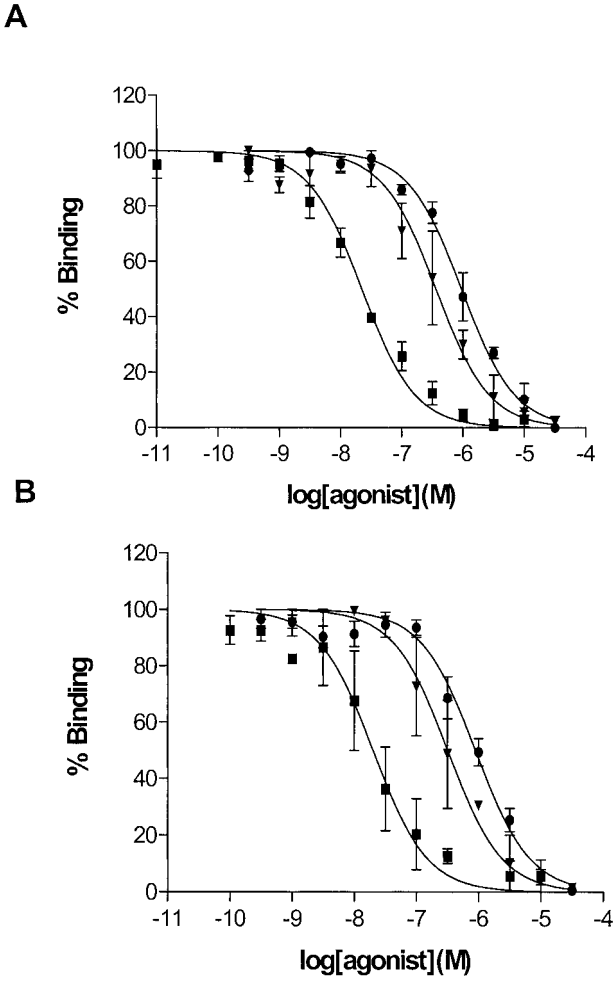

Fig. 3. Competition binding curves comparing hIP1D4PMT4 (A) and P89A (B) were performed as described in Fig. 2A. The agonists used were iloprost (■), carbacyclin (▲), and PGE_1 (●). Mean and S.E. were determined for each concentration of agonist from at least three separate experiments. IC_{50} values were converted to K_i values using the Cheng-Prusoff equation.

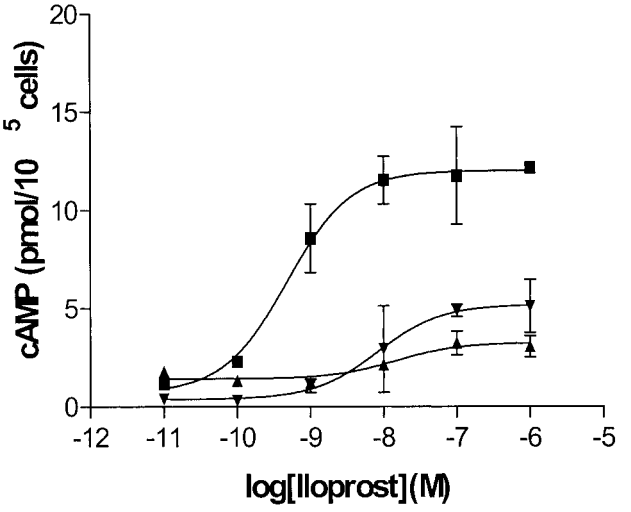

Fig. 4. cAMP activation for hIP1D4PMT4 (■), P89A (▼), and P254A (▲) as described in Fig. 2B. This graph represents one of at least three separate repetitions performed in duplicate for each construct. Shown are the mean and S.E. (picomoles of cAMP per 10^5 cells). EC_{50} was determined using GraphPad Prism.

TABLE 2

cAMP response for the single proline-to-alanine mutations

EC_{50} values (mean ± S.E.) for single proline-to-alanine mutations from at least three separate experiments (duplicates of six different concentrations). Bold numbering indicates significant decrease in EC_{50} compared with wild-type (hIP1D4PMT4).

	Iloprost	EC_{50} MUT / WT
	nM	
hIP1D4PMT4 (WT)	1.2 ± 0.3	1
P17A (I)	6.3 ± 1.3	**5**
P69A (II)	25.7 ± 5.4	**21**
P89A (III)	12.0 ± 2.5	**10**
P141A (IV)	1.1 ± 0.3	1
P154A (IV)	28.0 ± 18.0	**23**
P179A (V)	0.9 ± 0.1	1
P254A (VI)	8.2 ± 3.0	**7**
P285A (VII)	0.9 ± 0.3	1
P289A (VII)	6.3 ± 2.2	**5**

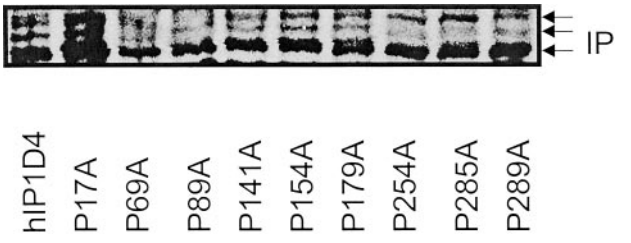

Fig. 5. Western analysis of the proline mutations using the high-affinity monoclonal antibody 1D4 toward the carboxyl terminal tail epitope tag. Comparisons were made with all the proline mutations, P17A, P69A, P89A, P141A, P154A, P179A, P254A, P285A, and P289A, all transfected in parallel using 20 μg of DNA per 150-mm plate. Thirty micrograms of membrane protein were loaded into each well.

lar face of the hIP receptor, where ligand is believed to bind in most GPCRs. Therefore, it is difficult to imagine why binding was not significantly affected (in contrast to activation) in proline mutations located above both the putative binding pocket as well as the site for activation, as was the case with P89A in TMIII. However, our hypothetical model for TMIII accounts for such observations, based on the principle that activation (in contrast to binding) requires TM movements to be transmitted to the cytoplasmic face. The absence of a binding defect (K_i = 3 nM) with P89A (TMIII) shows that the proline-to-alanine mutation has no effect on binding pocket structure. Furthermore, the normal basal activity suggests that the cytoplasmic domain also remains unperturbed. Thus, the basal receptor is minimally affected by P89A. It is known that GPCR activation requires TM movements (Farrens et al., 1996), and TMIII within the hIP receptor is probably no exception, having many conserved residues (e.g., ER at the base of TMIII) important for receptor activation (Acharya and Karnik, 1996; Kim et al., 1997). Thus, leaving these conserved residues intact and changing only one particular amino acid (Pro-89) along the extracellular face, TM movement must be inhibited to significantly affect activation. Accordingly, because activation is the only process being inhibited by this lack of movement, and not binding, a proline-generated hinge mechanism as outlined in our model seems likely to account for such observations. It is pertinent to note that a proline is located at this identical position in rhodopsin (Pro-107), the third residue extracellular to the conserved cysteine. Interestingly, invoking this model would suggest that the disulfide bond also plays an active role in receptor activation, possibly being broken upon TMIII movement. By inhibiting this movement, we have seemingly uncoupled binding from activation. However, in addition to a detailed study on prolines within rhodopsin, direct visualization with either NMR, crystallography, or EPR in the unbound and agonist-bound state would be required to definitively support this model.

Localized versus Global Effect. To address the question regarding localized versus global effect with each individual proline mutation, IP receptors were constructed with a different combination of missing prolines. Individual proline mutations were progressively added to the constructs (excluding the highly conserved Pro-154, Pro-254, and Pro-289), and characterizations were made based in regards to binding and activation. The double mutations were tolerated relatively well, with the final outcome reflecting the worst individual mutation. The triple proline mutations (P17A/P69A/P89A and P141A/P179A/P285A) were not tolerated, although individually each had a moderate effect on either binding or activation, suggesting that minor changes in other helices could accommodate such alterations. In combination, these mutations could no longer be withstood by the receptor, revealing a dramatic decrease in binding, activation, and expression. This further supports the necessity of the structural and functional contributions provided by proline residues for coupling, binding, and activation. Furthermore, the fact that the double mutations reflect the worst single mutations suggests that each mutation had produced a localized effect as opposed to a global disruption.

Critical Role of Conserved TM Prolines in Binding and Activation. Considerable disruption of both binding and activation was seen with mutation of the conserved TMIV proline Pro-154 (68% conservation among all GPCRs) and TMVI at Pro-254 (100% conservation). Retinitis pigmentosa mutations within rhodopsin are known to occur at equivalent sites (P171L, Q, S, and P267L and R), suggesting the importance of this position in both binding and activation. Our findings within the hIP receptor also support this theory of conserved residue importance and resultant binding and activation deficits upon mutation. Pro-289 is a highly conserved residue (98% conservation) found within TMVII and part of an important NPXXY (in the case of the hIP DPXXF) motif. Mutation of this residue to alanine showed abnormal activation, consistent with other studies performed on the rat m3 muscarinic receptor (Wess et al., 1993), the C5A receptor (Kolakowski et al., 1995), and the LH/CG (Fernandez and Puett 1996; Hong et al., 1997). Interestingly, the P285, a second proline found within TMVII, is conserved in approximately 10% of GPCRs. As would be expected, the activation and binding characteristics of the P285A mutant resembled that of the wild-type receptor.

Intracellular coupling efficiency is dependent upon stoichiometry of protein levels (receptor/G protein/adenylyl cyclase), uniform accessibility, and equivalent function of individual components within a cell (Ostrom et al., 2000, 2001). The use of overexpression systems (transient transfected cells and polyclonal stable cell lines) for wild-type and mutant protein can disrupt this balance, with heterogeneous expression between cells and within cell compartments (endoplasmic reticulum retention or incorrect localization of mutant receptors). Changes in receptor density can have profound effects on signal transduction (Niswender et al., 1999). We have tried to address these issues by performing binding assays on cell surface plasma membrane preparations (Ozols 1990; Perez et al., 1991) thus only measuring cell surface receptors that are directly exposed to an agonist. In addition, we ob-

TABLE 3

Ligand binding studies for the combination proline-to-alanine mutations.

Shown are K_i values (mean ± S.E.) from at least three separate experiments (duplicates of 12 different concentrations). Receptor expressions were determined by saturation binding from membranes prepared from COS-1 cells transfected with 20 μg of DNA per plate.

	Iloprost	Carbacyclin	PGE_1	Expression
	nM	nM	nM	pmol/mg protein
hIP1D4PMT4 (WT)	7 ± 3	121 ± 51	301 ± 90	2.1 ± 0.5
P17A/P179A	38 ± 5	267 ± 71	890 ± 121	1.2 ± 0.3
P69A/P89A	33 ± 7	265 ± 56	548 ± 144	1.2 ± 0.3
P141A/P285A	16 ± 2	177 ± 34	842 ± 143	3.9 ± 1.5
P17A/P69A/P89A	>500*	N.D.	N.D.	0.4 ± 0.1
P141A/P179A/P285A	>500*	N.D.	N.D.	0.6 ± 0.2

N.D., not determined.
* $p < 0.001$.

served that our binding and activation results, performed in parallel with wild-type control, remained consistent and reproducible from multiple separate COS-1 cell transfections. Despite inherent limitations in interpreting such results, data from overexpression systems remain valuable. The only definitive means to resolve these important issues is to study endogenous receptors on cells or native tissues in the presence of such mutations (naturally occurring mutations or knock-in) where heterogeneity between and within cells is less pronounced.

Our findings highlight the importance of transmembrane prolines in the processes of ligand binding and signal transduction activation. In addition to providing necessary conformational bends within the transmembrane helices, they can serve as molecular hinges or swivels, facilitating transmembrane movements. When the local structural properties of these proline residues are altered (i.e., they no longer confer movement or proper configuration), significant differential effects on binding and activation can result. Of great importance is the effect of the P89A mutation within TMIII, which highlights the involvement of this residue in the critical movement required for hIP activation. The propensity for creating molecular hinges or swivels is a fundamental necessity for coupling the processes of binding to activation. In the absence of select prolines, ligand binding and signal transduction activation, although closely related actions, can be uncoupled.

TABLE 4

cAMP response for the combination proline-to-alanine mutations

EC_{50} values (mean ± S.E.) for combination proline-to-alanine mutations. Bold numbering indicates significant decrease in EC_{50} compared with wild-type (hIP1D4PMT4).

	Iloprost	EC_{50} MUT / WT
	nM	
hIP1D4PMT4 (WT)	1.2 ± 0.3	1
P17A/P179A	9.4 ± 0.7	**8**
P69A/P89A	10.5 ± 1.5	**9**
P141A/P285A	0.6 ± 0.3	1
P17A/P69A/P89A	2824 ± 2167	**2353**
P141A/P179A/P285A	57 ± 46	**48**

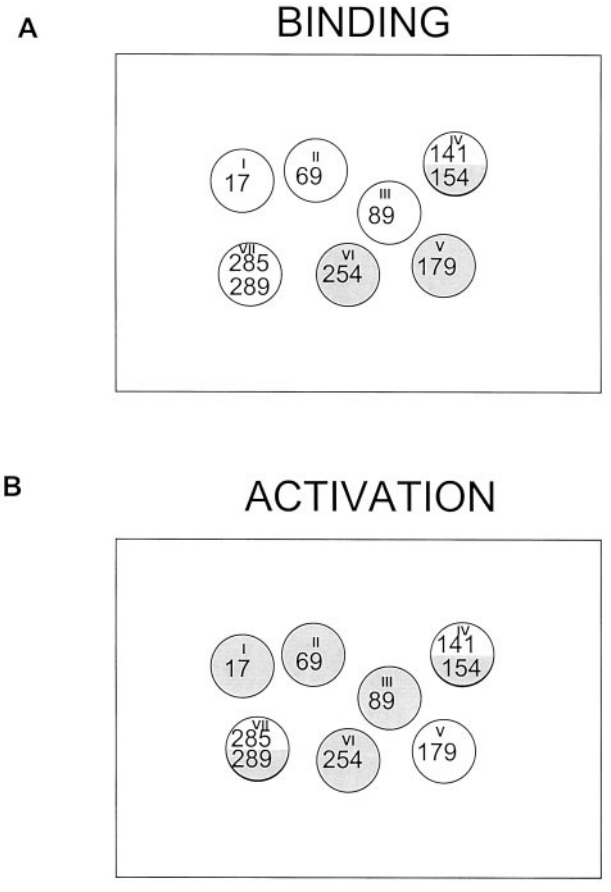

Fig. 6. Model describing the effects on binding and activation by the individual proline-to-alanine mutations. A representation of the helical bundle as observed from the cytoplasmic face is shown. Affected helices from proline mutations are shaded. A, area surrounded by TMIV, TMV, and TMVI, which affect binding when the transmembrane prolines are mutated to alanine. In TMIV, Pro-141 did not affect binding, whereas P154A was detrimental to binding. B, equivalent diagram for activation. Shaded are all the affected transmembrane domains except P141A (TMIV), P179A (TMV), and P285A (TMVII). Thus, there are mutants that affect binding but leave activation intact (P179A), those that do not affect binding but affect activation (P17A, P69A, P89A, P289A), those that disrupt both (P154A and P254A), and, finally, those that affect neither (P141A and P285A).

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