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RESEARCH PAPER

New insights into human prostacyclin receptor structure and function through natural and synthetic mutations of transmembrane charged residues

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Background and purpose: The human prostacyclin receptor (hIP), a G-protein coupled receptor (GPCR) expressed mainly on platelets and vascular smooth muscle cells, plays important protective roles in the cardiovascular system. We hypothesized that significant insights could be gained into the structure and function of the hIP through mutagenesis of its energetically unfavourably located transmembrane charged residues.
Experimental approach: Within its putative transmembrane helices fourteen hydrophilic residues, both unique and conserved across GPCRs, were systematically mutated to assess for effects on receptor structure and function.
Key results: Mutations of ten of the fourteen charged residues to alanine exhibited defective binding and/or activation. Key potential interactions were identified between 6 core residues; E116$^{3.49}$-R117$^{3.50}$ (salt bridge TMIII), D274$^{7.35}$-R279$^{7.40}$ (salt bridge TMVII), and D60$^{2.50}$-D288$^{7.49}$ (H-bond network TMII-TMVII). Further detailed investigation of E116$^{3.49}$ (TMIII) with mutation to a glutamine showed a 2.6-fold increase in agonist-independent basal activity. This increase in activity accounts for a proportion (~13%) of full agonist induced activation. We further characterized two novel naturally occurring human mutations, R77$^{2.33}$C and R279$^{7.40}$C recently identified in a 1455 human genomic DNA sample screen. The R77$^{2.33}$C variant appeared to exclusively affect expression, while the R279$^{7.40}$C variant, exhibited considerable deficiencies in both agonist binding and activation.
Conclusions and implications: Transmembrane charged residues play important roles in maintaining the hIP binding pocket and ensuring normal activation. The critical nature of these charged residues and the presence of naturally occurring mutations have important implications in the rational design of prostacyclin agonists for treating cardiovascular disease.
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Keywords: prostacyclin; charged residue; polymorphism; GPCR; structure function; transmembrane

Abbreviations: Arg or R, arginine; COX-2, cyclooxygenase-2; dbSNP, SNP database; GPCR, G protein–coupled receptor; hIP, human prostacyclin receptor; PGI_2, prostacyclin

Introduction

Vascular smooth muscle relaxation and inhibition of platelet aggregation are two important roles of the human prostacyclin receptor (hIP). Dysfunctional prostacyclin (PGI_2) signaling has been implicated in the development of a number of cardiovascular diseases including thrombosis, myocardial infarction, stroke, myocardial ischemia, atherosclerosis, and systemic and pulmonary hypertension (Narumiya *et al*., 1999). Recent studies using prostacyclin receptor (IP) knockout mice have revealed increased propensities towards thrombosis (Murata *et al*., 1997), intimal hyperplasia and restenosis (Cheng *et al*., 2002), as well as reperfusion injury (Xiao *et al*., 2001). A further consequence is the recent withdrawal of selective cyclooxygenase-2 (COX-2) inhibitors, due to their discriminating suppression of COX-2-derived PGI_2 and its cardioprotective effects, leading to increased cardiovascular events, particularly in predisposed patients (Fitzgerald, 2004). PGI_2 also appears to have an atheroprotective effect, particularly in premenopausal females (Egan *et al*., 2004). Thus, orally bioavailable PGI_2 agonists may be of benefit in combating cardiovascular disease.

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As with other G protein–coupled receptors (GPCRs), the hIP is structurally characterized by an extracellular N terminus, three extracellular loops, seven membrane-spanning α-helical domains, three cytoplasmic loops and a fourth cytoplasmic loop formed by palmitoylation of the intracellular C-terminal tail (Figure 1). The extracellular domains of many GPCRs, including the hIP, contain sites for N-linked (Asn-linked) glycosylation, particularly along the N terminus and the first extracellular loop (that is, N7 and N78) (Zhang *et al*., 2001). We have identified previously a number of crucial residues within transmembrane (TM) α-helices two (TMII), three (TMIII) and seven (TMVII) that confer distinct binding interactions with ligand side-chain constituents (Stitham *et al*., 2003). Of these amino acids, a highly conserved arginine (Arg) residue (100% conserved across prostanoid receptors) located at position $279^{7.40}$ within TMVII was shown to be a crucial anchoring point for hIP receptor ligands. The cytoplasmic domain of the receptor, particularly the third intracellular loop, contains regions believed to interact with G proteins and other signal-transduction components.

The presence of charged residues within the hydrophobic TM domains of most GPCRs is relatively uncommon. This is due, in part, to the high energetic costs associated with burying polar side chains within a largely hydrophobic environment. For that reason, many such charged TM-located residues contribute to structural and functional roles, such as direct ligand binding (for example, K296 in rhodopsin forms a Schiff's base with 11-*cis*-retinal), indirect countercharges (for example, E113 neutralizes the protonated Schiff's base) (Sakmar *et al*., 1989) and/or salt-bridge formation (for example, D125 in TMIII and K331 in TMVII of the α_{1b}-adrenoceptor stabilizes the receptor in the inactive state) (Porter *et al*., 1996). In addition, charged TM residues may serve to bind prosthetic groups as well as adding to structural stabilization through hydrogen bond formation (Stojanovic *et al*., 2004). It has been postulated that charged residues at the TM boundary help orientate TM helices via inter-α-helical or helical–lipid interactions (White and von Heijne, 2005) and form ionic interaction networks needed to stabilize the receptor in the inactive state (Scheer *et al*., 1996).

Here we report the effects of selective neutralization of all charged residues within the TM domain, and also the TM-cytoplasmic and TM-extracellular junctions, of the hIP. Key charged residue associations required for both PGI_2 binding and receptor activation are $E116^{3.49}$–$R117^{3.50}$ (TMIII), $D274^{7.35}$–$R279^{7.40}$ (TMVII) and $D60^{2.50}$–$D288^{7.49}$ (TMII-TMVII). The critical $E116^{3.49}$ residue, when mutated to Gln ($E116^{3.49}Q$), is the first reported constitutively active

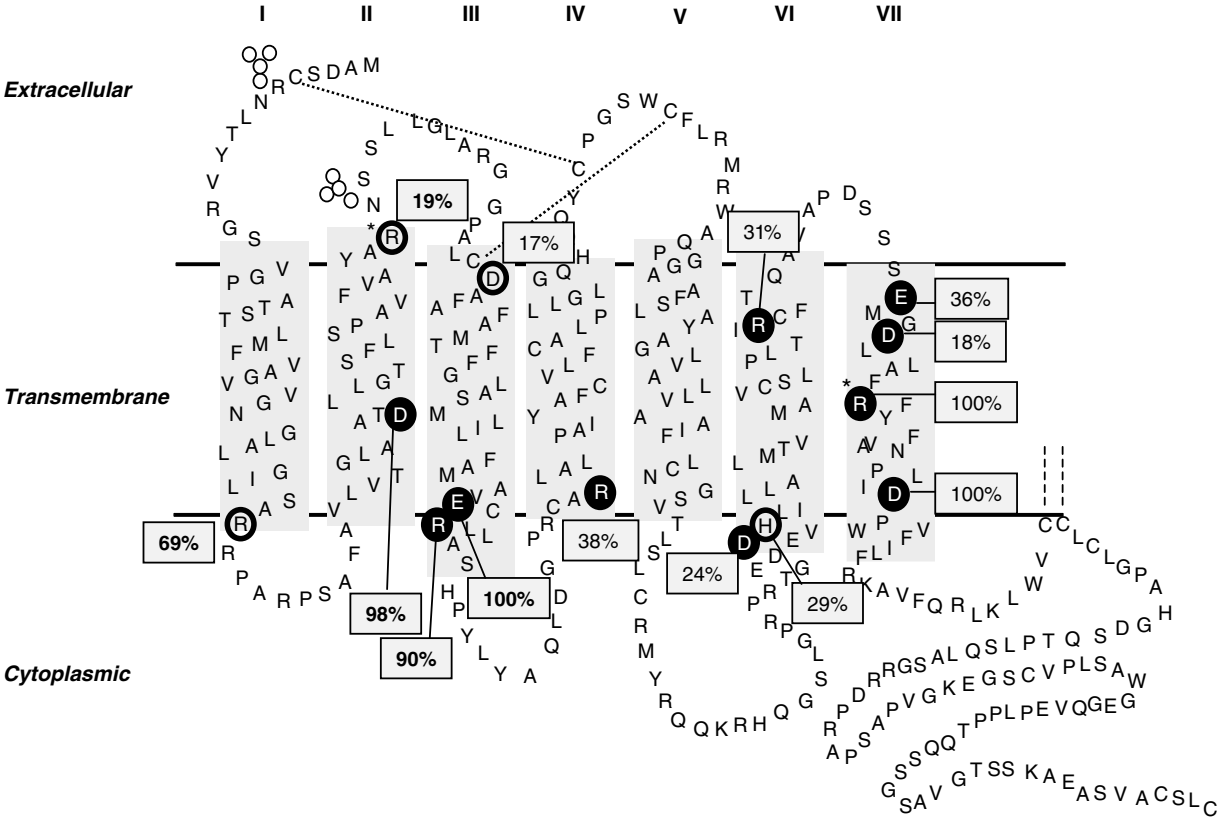

Figure 1 Secondary structure of human prostacyclin receptor. The seven transmembrane α-helical domains (shaded) are shown, along with the position of each of the 14 charged residues, highlighted by either a black ring (no binding or activation defect when mutated to alanine) or a black circle (binding or activation is affected when mutated to alanine). Naturally occurring mutations ($R77^{2.33}C$ and $R279^{7.40}C$) are denoted by an asterisk (*). Degree of amino-acid conservation (%) across 42 mammalian prostanoid receptors (GPCR database). GPCR, G-protein-coupled receptor.

mutation of the hIP. Furthermore, we report the characterization of two novel human IP variants, affecting charged TM residues R77$^{2.33}$C and R279$^{7.40}$C. Both serve differential roles in hIP expression, binding and activation.

Methods

Construction of mutant receptors
A PCR protocol was used for site-directed mutagenesis as described previously (Stitham *et al*., 2003). In brief, two complementary primers were designed extending 10–12 nucleotides 3′ and 5′ from the desired mutation site. The reaction mixture contained *Pfu* reaction buffer, DNA construct, each of two primers (sense and antisense), dNTPs and *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). DpnI (Promega, Madison, WI, USA) digested PCR product was then used to transform competent DH5α ($\sim 2 \times 10^9$ cells), followed by DNA extraction and sequencing confirmation (Dartmouth Medical School, Molecular Biology Core Facility) of selected clones.

Transfection of COS-1 cells and membrane preparations
Transfection of COS-1 cells were performed as described previously (Hwa and Perez, 1996). Twenty micrograms of DNA construct was used per 15-cm plate in a DEAE–Dextran transfection cocktail. Cells were harvested 48 h after transfection and plasma membranes were prepared (Hwa *et al*., 1995). 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, a specific agonist at IP receptors (Hwa *et al*., 1997a). The competition study involved duplicate wells containing 50 μg of membrane, HEM buffer (20 mM HEPES, pH 7.4, 1.5 mM EGTA and 12.5 mM $MgCl_2$) and 15 nM [^{3}H]iloprost, and cold iloprost, cold carbacyclin or cold PGE_1 at 11 concentrations extending from 10 μM to 0.1 nM. Nonspecific binding was determined with the addition of a 500-fold excess of cold iloprost (7.5 μM). After 1.5 h incubation at 4°C, the reactions were stopped by the addition of ice-cold 10 mM Tris–HCl buffer (pH 7.4) and were filtered onto Whatman GF/C glass filters with a Brandel cell harvester. The filters were counted in the presence of 5 ml Ecoscint™ (National Diagnostics, Atlanta, Georgia, USA). Data were analyzed using GraphPad Prism. IC_{50} values were converted to K_i using the Cheng–Prusoff equation. K_i values were expressed as a mean ± s.e. To determine approximate expression levels (B_{max} pmol receptor per mg membrane protein), saturation binding studies were performed with 5, 10, 25, 50, 75 and 100 nM [^{3}H]iloprost. Normalization of receptor numbers (to ~ 0.5 pmol mg^{-1} membrane protein) were performed by varying DNA concentrations using principles and linear regression described previously (Stitham *et al*., 2004).

cAMP determination
The wild-type and mutant constructs were analyzed for their signal-transduction capabilities. Plates (25 mm) were transfected with 2 μg DNA as described by Stitham *et al*. (2004). Basal and stimulation were performed in parallel in triplicates, in the presence or absence of 1 μM iloprost for 20 min on transfected whole cells. Concentration–response curves were determined by the addition of six different concentrations (1 μM to 10 pM) in duplicate. cAMP levels were measured using the radio-receptor competition assay (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA). In brief, [^{3}H]cAMP was used in competition for a cAMP binding protein (protein kinase A; Sigma-Aldrich, St Louis, MO, USA) against known concentrations of cold cAMP followed by determination of the unknowns. Samples were then subjected to counting with 5 ml Ecoscint™ (National Diagnostics). Results were analyzed with GraphPad Prism. Mean ± s.e.mean was calculated for basal and maximal cAMP production. For the concentration response, a best-fit concentration–response curve was calculated, and the EC_{50} was determined for wild-type and mutant constructs.

Statistical methods. Mean (± s.e.mean) values of results are shown. ANOVA and Student's *t*-test was used to determine significant differences ($P < 0.05$), between means.

Materials
[^{3}H]Iloprost (17.0 Ci mmol^{-1}), unlabeled iloprost and the cAMP radioimmunoassay system were purchased from Amersham Pharmacia Biotechnology. Carbacyclin was obtained from Sigma. PGE_1 was obtained from Biomol Research Labs. Oligonucleotides were purchased from Sigma-Genosys (St Louis, MO, USA).

Results

Essential-charged TM amino acids
All charged hIP TM residues were individually mutated to alanine and assessed for binding and activation properties. In Figure 1 are shown the locations of the charged residues and their degree of conservation based on sequence alignment of prostanoid receptors in the GPCR database (Horn *et al*., 2003). D60$^{2.50}$ (TMII) 98%, E116$^{3.49}$ (TMII) 100%, R117$^{3.50}$ (TMIII) 90%, R279$^{7.40}$ (TMVII) 100% and D288$^{7.49}$ (TMVII) 100% showed the highest degree of conservation and, not surprisingly, exhibited the greatest defects in binding (Table 1) and activation (Table 2) when mutated to alanine. Each amino acid resides within important structural–functional motifs for (1) G-protein activation (E116$^{3.49}$ and R117$^{3.50}$ part of the ERY/C motif), (2) ligand recognition (R279$^{7.40}$) and (3) TM α-helical stabilization (D288$^{7.49}$ is part of the DPXXY motif and D60$^{2.50}$ in close proximity). Such conservation and functional significance imply that these residues constitute a charged structural core for not only the hIP, but also for all prostanoid receptors, and other GPCRs, in general.

Of equal importance are the residues that characterize (that is, are more exclusive to) the IP. R137$^{4.43}$A (33%

Table 1 Ligand-binding studies for the single charged-to-alanine mutations

	Iloprost (K_i) nM	*Carbacyclin* (K_i) nM	*PGE_1* (K_i) nM	*Expression pmol mg^{-1} protein*
WT	7±1	121±16	318±28	1.2
EC				
R77$^{2.33}$A (II)	4±1	127±16	799±87	0.5
D93$^{3.26}$A (III)	5±1	152±14	374±33	1.8
R258$^{6.54}$A (VI)	8±1	225±51	763±98	0.4
E271$^{7.32}$A (VII)	19±3	105±22	158±38	0.8
MD				
D60$^{2.50}$A (II)	**>100**$^{\#}$	ND	ND	0.1
D274$^{7.35}$A (VII)	**>100**$^{\#}$	ND	ND	0.3
R279$^{7.40}$A (VII)	**>100**$^{\#}$	ND	ND	0.5
CP				
R41$^{1.60}$A (I)	5±1	163±21	482±52	0.6
E116$^{3.49}$A (III)	**>100**$^{\#}$	ND	ND	0.1
R117$^{3.50}$A (III)	**>100**$^{\#}$	ND	ND	0.2
R137$^{4.43}$A (IV)	**54±2***	**610±79**$^{+}$	1976±333	0.8
D236$^{6.33}$A (VI)	**68±4**$^{+}$	**1260±259**$^{\#}$	346±44	0.4
H237$^{6.34}$A (VI)	6±1	163±18	449±75	1.5
D288$^{7.49}$A (VII)	**74±15***	**2516±423**$^{\#}$	**2695±293**$^{+}$	0.6

Abbreviations, ND, not determined; WT, wild type.
Shown are K_i values (K_i±s.e., nM) from at least three separate experiments (duplicates of 12 different concentrations). Receptor expression was determined by saturation-binding experiments, using membrane preparations from COS-1 cells transfected with 20 μg DNA per plate. Residue position within transmembrane domain defined as EC (extracellular portion), MD (middle) and CP (cytoplasmic portion). Shown in bold are those mutations with significantly different characteristics versus wild type ($^{+}P<0.05$, $^{*}P<0.01$ and $^{\#}P<0.001$).

Table 2 cAMP dose–response studies for the single charged-to-alanine mutations

	Iloprost (EC_{50}) nM	*Ratio EC_{50} (EC_{50} mutant:EC_{50} WT)*
WT	0.5±0.1	1
EC		
R77$^{2.33}$A (II)	1.2±0.6	2
D93$^{3.26}$A (III)	1.7±0.5	3
R258$^{6.54}$A (VI)	**6.8±2.9**	**14***
E271$^{7.32}$A (VII)	**5.1±1.1**	**10***
MD		
D60$^{2.50}$A (II)	30.1±18.4	**60**$^{\#}$
D274$^{7.35}$A (VII)	6.5±1.5	**13***
R279$^{7.40}$A (VII)	130.0±109.0	**260**$^{\#}$
CP		
R41$^{1.60}$A (I)	0.5±0.2	1
E116$^{3.49}$A (III)	**58.7±40.1**	**117**$^{\#}$
R117$^{3.50}$A (III)	**63.0±13.0**	**126**$^{\#}$
R137$^{4.43}$A (IV)	2.3±0.9	5
D236$^{6.33}$A (VI)	2.2±1.2	4
H237$^{6.34}$A (VI)	0.9±0.3	2
D288$^{7.49}$A (VII)	**8.5±3.5**	**17***

Abbreviations: CP, cytoplasmic portion; EC, extracellular portion; MD, middle; WT, wild type.
EC_{50} values are shown (EC_{50}±s.e., nM) for agonist-induced activation, as well as the activation ratio of mutant to wild type (EC_{50} mutant:EC_{50} wild type). Results from at least three separate experiments (duplicates of six different concentrations). Bold numbering indicates significant fold decrease in EC_{50} compared with wild type ($^{+}P<0.05$, $^{*}P<0.01$ and $^{\#}P<0.001$).

conservation), D236$^{6.33}$A (24% conservation) (Table 1 and Figure 2) and D274$^{7.35}$A (18% conservation) (Table 1) all significantly affect receptor binding. Mutations affecting activation include R258$^{6.54}$A (31% conservation), E271$^{7.32}$A (36% conservation) and D274$^{7.35}$A (18% conservation) (Table 2 and Figure 3). Interestingly, the two mutations that predominantly affect ligand binding, namely R137$^{4.43}$A and D236$^{6.33}$A, involve residues that are more cytoplasmically located, whereas the two mutations affecting activation, R258$^{6.54}$A and E271$^{7.32}$A, are more extracellularly located. Of further interest to the hIP are mutations of the D288$^{7.49}$ to alanine (D288$^{7.49}$A), which equalizes the binding affinity of both carbacyclin and PGE_1, as well as D236$^{6.33}$A, which reverses the binding affinity of carbacyclin and PGE_1 (Table 1 and Figure 2). Such results suggest that these residues play an important role in agonist selectivity.

E116$^{3.49}$Q exhibits increased basal activity

To date, there have been no descriptions of constitutively active mutations of the hIP receptor. We performed parallel basal (no iloprost) and agonist-induced efficacy (1 μM iloprost activation) experiments for wild type and charged-to-alanine-mutated hIP at equivalent levels of receptor expression (~0.5 pmol mg^{-1} membrane protein; Figure 4). Results showed similar levels of basal activity for the mutants (Figure 4a). However, as expected, differences in efficacy were observed with iloprost-induced activation (Figure 4b). Such differences may arise from different signal-transduction efficiency, differential cell surface localization or differential receptor desensitization. This difference was particularly evident for the highly conserved D60$^{2.50}$A, E116$^{3.49}$A and R117$^{3.50}$A mutations. Saturation binding (Figure 4c) reconfirmed wild-type binding affinity (K_D) of 10.8±3.2 nM, and D60$^{2.50}$A, E116$^{3.49}$A and R117$^{3.50}$A mutations of >100 nM at equivalent expression levels of approximately 0.5 pmol mg^{-1} membrane protein. Of these mutants, the E116$^{3.49}$A did

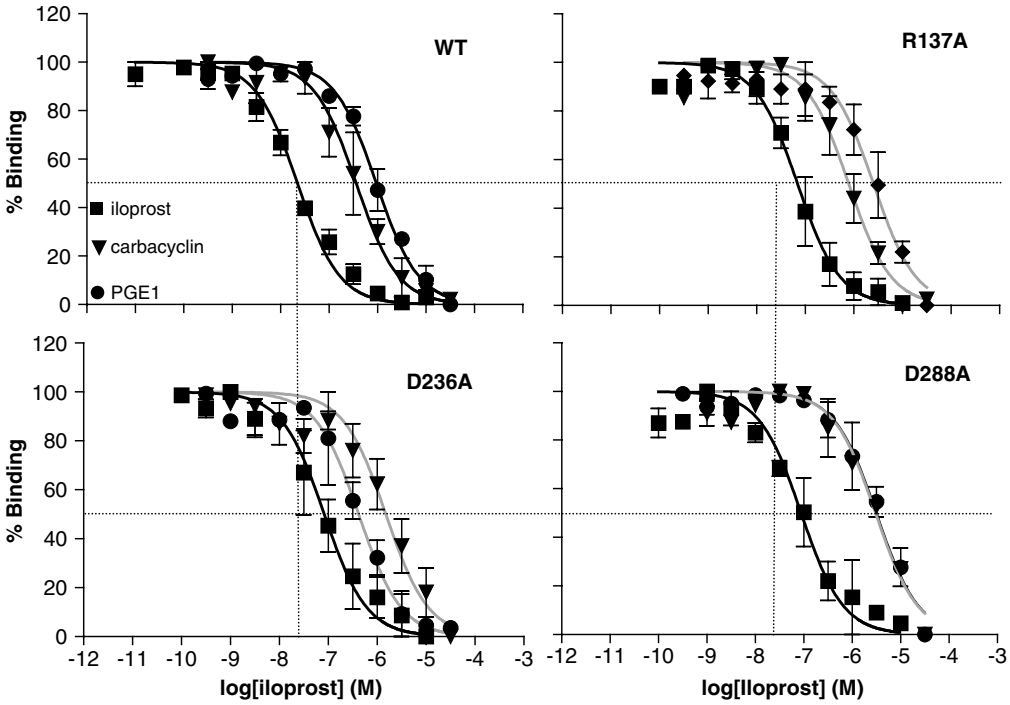

Figure 2 Competition binding curves for wild-type hIP and R137$^{4.43}$A, and D236$^{6.33}$A and D288$^{7.49}$A. Binding curves are shown for wild-type hIP and three mutant constructs for which a binding curve could be determined, R137$^{4.43}$A, D236$^{6.33}$A and D288$^{7.49}$A. For each construct, cold iloprost, carbacyclin or PGE_1 was used in competition for 15 nM [^{3}H]iloprost. Percent binding on the *y* axis is determined by dividing specific counts for each concentration, by total counts minus background in the absence of cold competitor. For wild type, the ratio of specific to nonspecific counts was 3.2 ± 0.3 ($n = 7$). Each curve was derived from at least three repetitions, each in duplicate. The broken line shows the IC_{50} for wild-type protein. Significant differences between individual K_i values are shown in Table 1. hIP, human prostacyclin receptor; PGI_1, prostacyclin.

show signs of increased basal activity (1.71 ± 0.16 pmol, $n = 3$), although not statistically significant when compared to wild-type basal activity (1.21 ± 0.16 pmol, $n = 12$, Figure 3). However, based on the absolute conservation (100%) across the prostanoid receptors, along with evidence from studies of rhodopsin (Kim *et al*., 2004) and the α_{1b} adrenoceptor (Scheer *et al*., 1996), showing that a change to glutamine (Q) at equivalent positions is constitutively active, we opted to construct and test the E116$^{3.49}$Q for constitutive activity. Interestingly, there was no significant difference in competition binding (wild-type $K_i = 14.7 \pm 1.7$ nM, $n = 3$, versus E116$^{3.49}$Q $K_i = 13.4 \pm 2.1$ nM, $n = 3$; Figure 5a) or activation (wild-type $EC_{50} = 0.79 \pm 0.34$ nM, $n = 3$ versus E116$^{3.49}$Q $EC_{50} = 0.51 \pm 2.5$ nM, $n = 4$, using equivalent DNA concentrations of 1 μg ml^{-1}; Figure 5b). However, on detailed assessment of basal activity, using transfections with different DNA concentrations (to correct for receptor numbers), in addition to basal cAMP activity (pmol), the slope of the line indicated that there was a significant degree of increased basal activity (nmol basal cAMP per pmol receptor; Figure 5c). For wild-type receptor, basal activity was 4.2 ± 0.3 nmol cAMP per pmol receptor, whereas the E116$^{3.49}$Q mutant was 2.6-fold higher at 11.0 ± 2.9 nmol cAMP per pmol receptor) ($P < 0.01$). Full wild-type receptor activation showed a 19.7-fold increase in cAMP production (4.6 pmol cAMP activated per 0.23 pmol cAMP basal). The 2.6-fold increase in basal activity strongly suggests that this motif in the hIP is only one of many constraints and motifs that contribute to full receptor activation. Based on our results, and assuming a linear proportional relationship, E116$^{3.49}$–R117$^{3.50}$ contributes approximately 13% (2.6-fold divided by 19.7-fold) to receptor activation.

Differential effects of novel naturally occurring charged residue variants

Upon sequencing the coding region of human prostacyclin gene (*PTGIR*) from 1455 volunteers, two mutations in TM-located charged residues were detected (R77$^{2.33}$C and R279$^{7.40}$C) (Stitham *et al*., 2007). Both of these changes involved significant substitutions from a basic, positively charged Arg amino acid to a small, relatively neutral-charged Cys (CGC to TGC codon changes). Furthermore, both R77$^{2.33}$ and R279$^{7.40}$ reside within binding and activation critical regions of the hIP receptor (Figure 1). The R77$^{2.33}$C receptor variant was identified within an Asian (oriental) individual of a general (multiracial) population (1/127). The R77$^{2.33}$C did not exhibit any functional abnormalities with respect to agonist-binding affinity (R77$^{2.33}$C $K_i = 6.4 \pm 1.1$ versus wild-type $K_i = 7.9 \pm 1.7$ nM) (Figure 6a) or receptor-mediated cAMP production (R77$^{2.33}$C $EC_{50} = 0.9 \pm 0.3$ versus wild-type $EC_{50} = 1.1 \pm 0.1$ nM) (Figure 6b). However, a decrease in receptor expression was observed for the R77$^{2.33}$C ($B_{max} = 0.32 \pm 0.04$ pmol mg^{-1} membrane protein) compared to that of the wild-type hIP ($B_{max} = 1.05 \pm 0.06$ pmol mg^{-1} membrane protein, $P < 0.001$). The R279$^{7.40}$C receptor

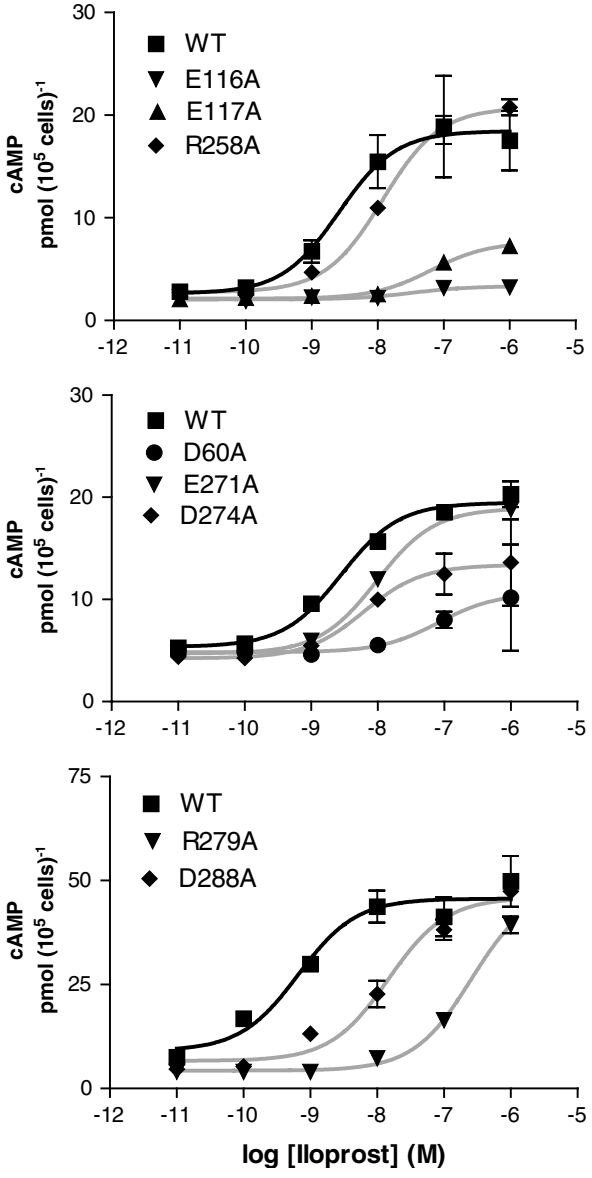

Figure 3 Dose–response (iloprost) for wild-type hIP and eight charged-to-alanine mutations. Dose–response curves are shown for wild-type hIP and eight mutant constructs with significant effects on potency. Experiments were performed in parallel, and each curve represents the combination of at least three separate experiments performed in duplicate. Values for EC_{50} and significant differences from wild type are shown in Table 2. hIP, human prostacyclin receptor.

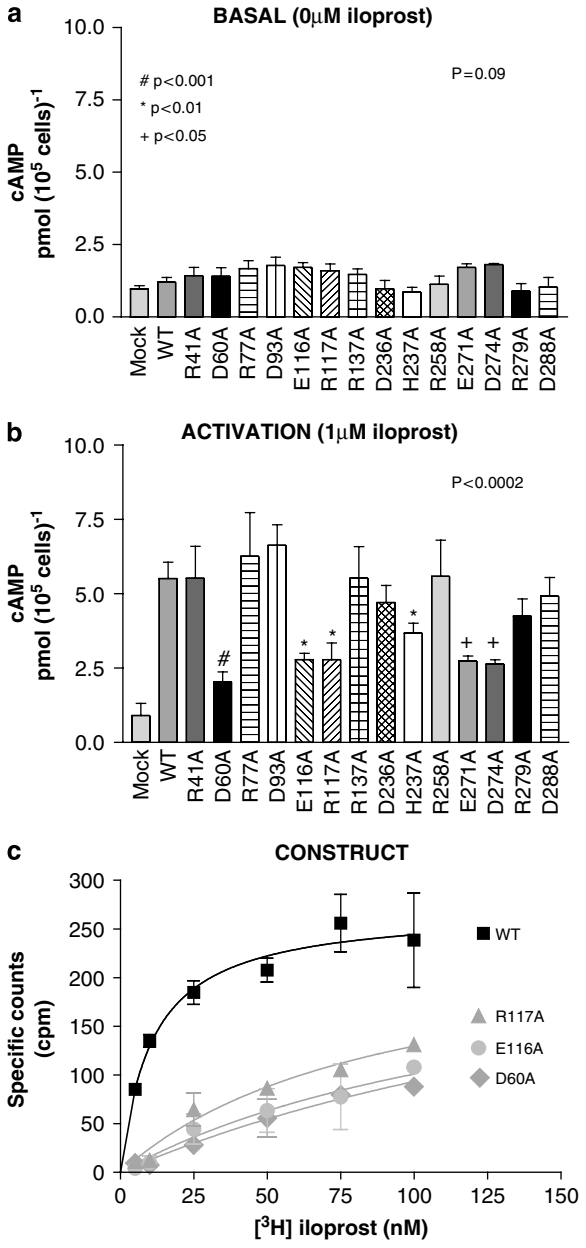

Figure 4 Basal and iloprost-activated cAMP activity. The results shown are for basal and agonist-induced cAMP production for wild-type and mutated hIP receptors, as well as mock-transfected cells. (**a**) Basal activation in the absence of iloprost for mock, wild-type hIP and alanine mutants. Cells were transfected to a level of receptor expression that approximately equals 0.5 pmol mg^{-1} membrane protein. ANOVA showed no significant difference between each of the results. (**b**) Iloprost-induced (1 μM) cAMP production for mock, wild-type hIP and alanine mutants. Significant differences in levels of cAMP were observed (ANOVA $P<0.0002$). (**c**) Saturation binding studies using 0.5 μg DNA per ml transfection solution for wild type and 2.0 μg DNA per ml for the mutations, D60$^{2.50}$A, E116$^{3.49}$A and R117$^{3.50}$A, resulting in approximately equivalent expression levels of 0.5 pmol receptor per mg membrane protein. The results shown are the raw specific counts (c.p.m.) for increasing concentrations of iloprost (nM). hIP, human prostacyclin receptor.

variant was also detected with low frequency (Hispanic, 1/100). As with the R77$^{2.33}$C, the codon changes were CGC to TGC. The R279$^{7.40}$ residue is fully conserved (100%) across the prostanoid receptors and has been proposed to be a key binding-pocket residue, interacting with the C1-carboxyl group of hIP ligands (Stitham *et al*., 2003). When expressed in a COS-1 cell system, the R279$^{7.40}$C exhibited significant defects in iloprost-binding affinity (R279$^{7.40}$C $K_i>100$ nM versus wild-type $K_i=7.9\pm1.7$ nM, $P<0.001$) (Figure 6c), underscoring the necessity of this Arg within the binding pocket. Receptor-mediated activation (as measured by iloprost-induced cAMP production) was also defective in the R279$^{7.40}$C mutant compared to wild-type hIP (R279$^{7.40}$C $EC_{50}=34.0\pm3.0$ versus wild-type $EC_{50}=1.2\pm0.1$ nM, $P<0.001$) (Figure 6d). Compared to the wild-type receptor ($B_{max}=1.05\pm0.06$ pmol mg^{-1} membrane protein), the

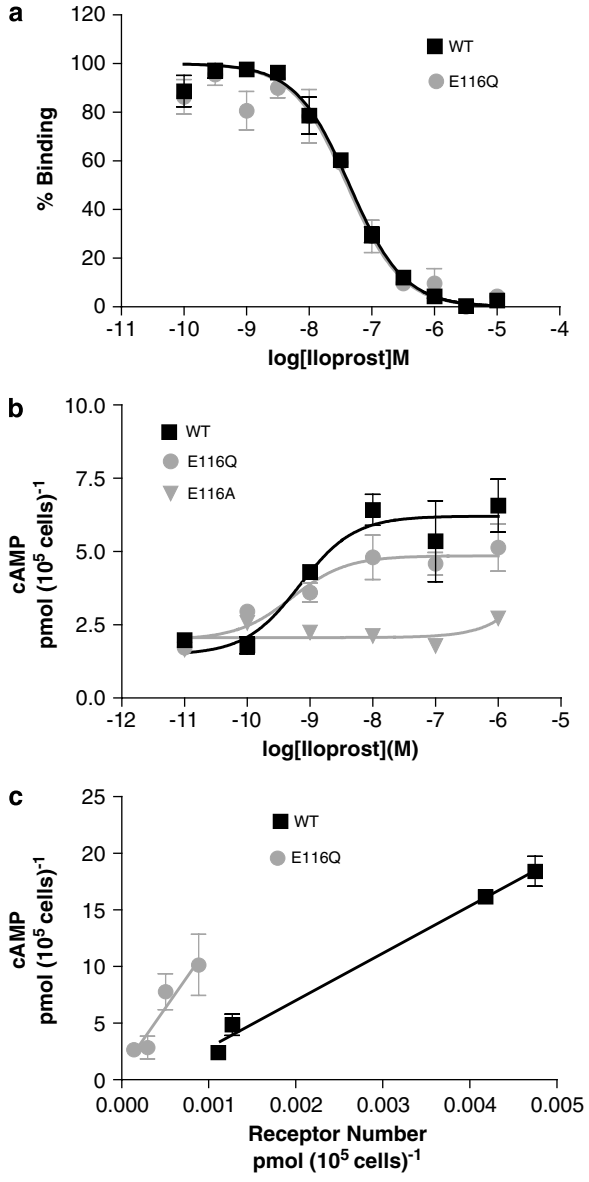

Figure 5 Study of constitutively active E116$^{3.49}$Q mutant. Results shown are from competition binding and iloprost-induced cAMP production for E116$^{3.49}$Q. (**a**) Competition binding for wild type and E116$^{3.49}$Q. Graphs were compiled from at least three separate experiments performed in duplicate. (**b**) Dose–response for wild type, E116$^{3.49}$Q and E116$^{3.49}$A. Results were obtained from at least three separate experiments performed (using equivalent DNA transfections of 1 μg ml^{-1} transfection solution) in duplicate. (**c**) Basal activity for wild type and E116$^{3.49}$Q. Results reflect cAMP production per 10^5 cells and receptor numbers per 10^5 cells. The respective slopes were calculated to determine pmol cAMP per pmol receptor.

R279$^{7.40}$C variant expressed approximately 4-fold less ($B_{max} = 0.27 \pm 0.02$ pmol mg^{-1} membrane protein, $P < 0.001$).

Discussion and conclusions

Charged residues within the TM domain of GPCRs can serve diverse roles, such as structural stabilization through strong ionic, inter-α-helical interactions, assisting in receptor constraint in the native and unbound state (with breakage of this constraint contributing to receptor activation) (Porter *et al*., 1996; Scheer *et al*., 1996; Kim *et al*., 2004). Charged amino acids may also interact directly with ligand or prosthetic groups, such as zinc (Hwa *et al*., 1997b; Stitham *et al*., 2003; Stojanovic *et al*., 2004), as well as with polar head groups of lipids (White and von Heijne, 2005). Furthermore, many of these interactions may not be direct at all, but rather facilitated through an intervening water molecule (for example, solvent-accessible charged residues). The goal of the current study was to identify and distinguish such residues within the hIP. This is particularly important in light of its emerging role in cardioprotection and the need for stable, orally active hIP agonists for the treatment of various cardiovascular diseases. Ligands may be designed to target such charged residues in improving receptor activation.

Essential structural core of conserved charged residues

Four polar residues, D60$^{2.50}$, D274$^{7.35}$, D288$^{7.49}$ and R279$^{7.40}$, when changed to alanine have a profound effect on both binding and activation. The R279$^{7.40}$ in TMVII may be the counterion for the C1-carboxylate group (COO^-) of PGI_2. Previous mutagenesis studies performed on the EP3 (Boie *et al*., 1995; Chang *et al*., 1997) and EP2 (Kedzie *et al*., 1998) receptors, as well as hIP (Stitham *et al*., 2003), have strongly supported this hypothesis. EP1 receptor studies have confirmed that the primary interaction between this residue and ligand constituents is ionic (electrostatic), rather than hydrogen bonding, as modification to various esters resulted in a greatly reduced affinity and potency (Ungrin *et al*., 2001). The role of D274$^{7.35}$ within the hIP is unclear. However, there is some indirect evidence from our rhodopsin-based IP model that suggests residue D274$^{7.35}$ may act as a counterion, forming a salt bridge with R279$^{7.40}$ (Figures 7a and b). This is analogous to E113 (TMIII) in rhodopsin, which neutralizes the protonated Schiff's base (Sakmar *et al*., 1989). Furthermore, in the α_{1b}-adrenoceptor, D125 in TMIII and K331 in TMVII stabilize the receptor in the inactive state and are broken upon activation by agonist (Porter *et al*., 1996).

The D288$^{7.49}$ is fully conserved (100%) across the prostanoid receptors, and resides within the conserved NPXXY motif (DPXXF in case of the hIP). Loss of D288$^{7.49}$ (via alanine mutagenesis) also significantly affected both binding and activation. The NPXXY motif has been postulated to contribute an indirect conformational effect (induced protein folding) that facilitates high-affinity ligand binding. Furthermore, it has been demonstrated that disruption of the NPXXY motif, particularly the proline, results in abnormal activation in numerous GPCRs, including the rat M_3 muscarinic receptor (Wess *et al*., 1993), the C5A receptor (Kolakowski *et al*., 1995) and the LH/CG receptor (Fernandez and Puett, 1996; Hong *et al*., 1997). Previous evidence and our current study has shown that D60$^{2.50}$ within the hIP may play a similar stabilizing role, along with D288$^{7.49}$ and N31$^{1.50}$, as all three amino acids have been shown to be vital to proper hIP binding and activation (Stitham *et al*., 2002a) (Figures 7a and c).

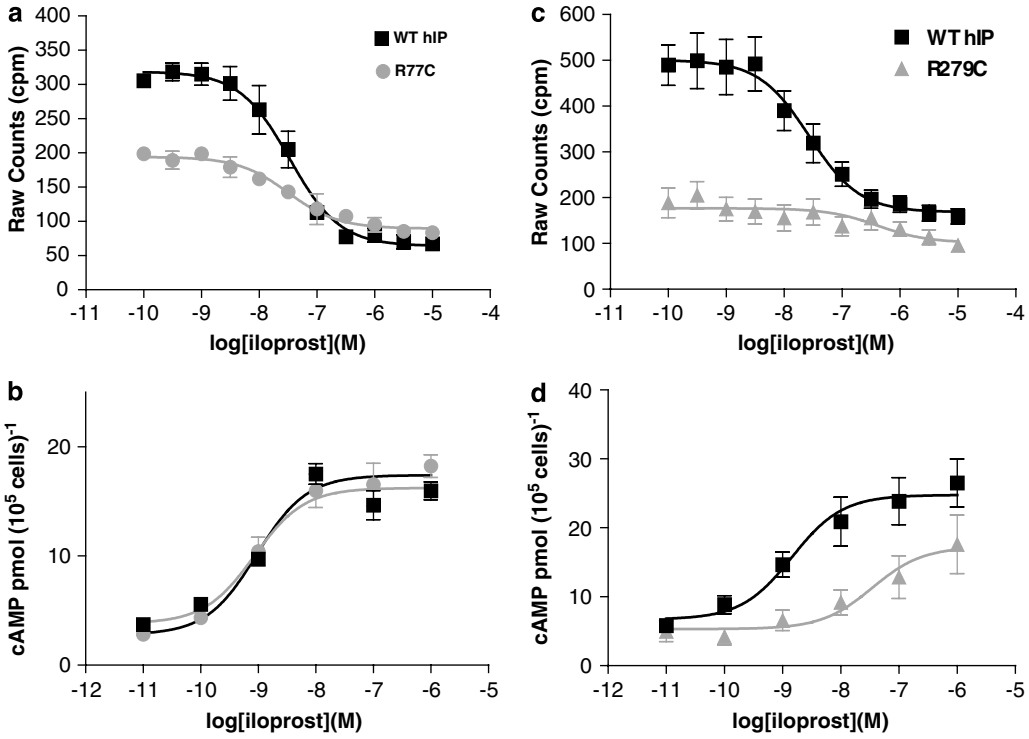

Figure 6 Analysis of naturally occurring variants R77$^{2.33}$C and R279$^{7.40}$C. Results are from competition binding and iloprost-induced cAMP production for R77$^{2.33}$C and R279$^{7.40}$C variant receptors. (**a**) Competition binding curves for wild type and R77$^{2.33}$C. (**b**) Dose–response for wild type and R77$^{2.33}$C. (**c**) Competition binding comparing wild type with R279$^{7.40}$C. (**d**) Iloprost dose–response for wild type with R279$^{7.40}$C. All graphs are composites from at least three separate experiments all performed in duplicate.

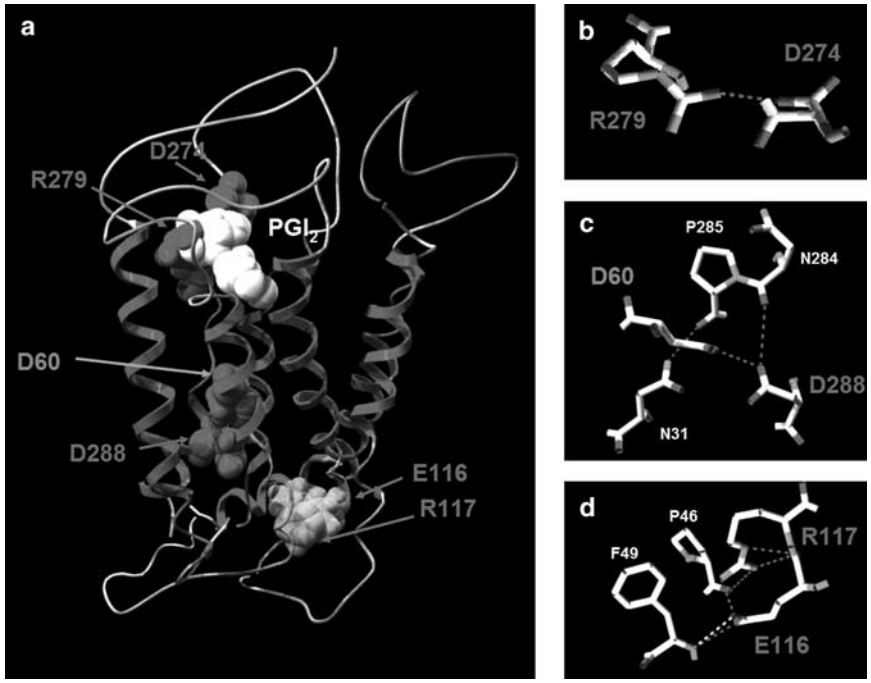

Figure 7 Rhodopsin-based homology model of the hIP. The positions of six crucial transmembrane amino acids are indicated. (**a**) Side (transmembrane) view of PGI_2-bound hIP. Essential structural core of conserved charged residues (space-filled molecules) reside in TM domains II (D60$^{2.50}$), III (E116$^{3.49}$ & R117$^{3.50}$) and VII (D274$^{7.35}$, R279$^{7.40}$ and D288$^{7.49}$) at or in line with PGI_2-binding region. (**b**) Proposed salt-bridge formed between D274$^{7.35}$ and R279$^{7.40}$ in the unbound state (blue represents amine groups, whereas red corresponds to carboxyl groups). (**c**) Hydrogen bond network involving D60$^{2.50}$ and D288$^{7.49}$. (**d**) Proposed salt-bridge formed between E116$^{3.49}$ and R117$^{3.50}$, as well as hydrogen bond interactions with P46$^{1.65}$and F49$^{1.68}$. hIP, human prostacyclin receptor; PGI_1, prostacyclin.

Unique hIP-charged structural components

The R137$^{4.43}$A and D236$^{6.33}$A mutations are located on the cytoplasmic surface of TMIV and TMVI, respectively, and have adverse effects only on binding affinity, with no significant effect on activation. According to our 3-D rhodopsin-based homology model of the hIP, these residues are unlikely to form a salt bridge with one another. However, given their positions near the cytoplasmic interface, they may serve as stabilization factors through interactions with surrounding lipids. This may also correspond to the network of cytoplasmic interactions proposed by Scheer *et al*. (1996) to be required for receptor stabilization. In contrast to the R137$^{4.43}$ and D236$^{6.33}$, residues R258$^{6.54}$ and E271$^{7.32}$, which are located on the extracellular interface of the hIP, were found to significantly affect activation upon mutation, with no effect on binding. This lends more evidence to the theory of a network of ionic interactions on the protein–lipid interface (extracellular and cytoplasmic) of the hIP, which remain important for proper receptor activity. However, only with precise biophysical-structural information, gathered via techniques such as NMR or X-ray crystallography, the exact roles of such residues be determined.

The first 'constitutively active' hIP mutation provides insights into hIP activation

In rhodopsin, the highly conserved ERY region at the end of TMIII (E134–R135–Y136) exhibits a slight deviation from the regular α-helix (Palczewski *et al*., 2000), and a salt bridge, between E134 and R135, is believed to be the cause of this structural divergence (Teller *et al*., 2001). Studies using site-directed mutagenesis have also implied that both these charged residues are critical for $G_t(\alpha)$ interaction (Franke *et al*., 1992; Yang *et al*., 1996). Thus, during photoactivation of rhodopsin, it is believed that the E134 may become protonated (neutralized), thus, breaking the proposed salt bridge and allowing for proper activation (Arnis *et al*., 1994). The importance of this conserved ERY motif has been extended to many GPCRs, with numerous studies showing varying effects on binding, activation and expression upon disruption (Franke *et al*., 1992; Zhu *et al*., 1994; Acharya and Karnik, 1996; Burstein *et al*., 1998; Alewijnse *et al*., 2000; Scheer *et al*., 2000; Chung *et al*., 2002). Interestingly, in the prostanoid-related TPα (thromboxane, TxA_2) receptor, mutations of E129–R130 in this motif showed no constitutive activity, but only a loss-of-function phenotype (Capra *et al*., 2004). These important principles were explored in the hIP, where the motif consists of an ERC (E116$^{3.49}$–R117$^{3.50}$–C118$^{3.51}$). Our modeling of the IP receptor supports a salt-bridge formation analogous to rhodopsin (Figures 7a and d). Interestingly, despite a 2.6-fold increase in basal activity, there were no shifts in either the competition binding or dose–response curves—two common features of constitutive activity. The approximated 13% contribution (fold increase in basal activity from the E116$^{3.49}$Q versus total fold increase activity upon receptor activation by iloprost) by the putative E116$^{3.49}$–R117$^{3.50}$ salt-bridge breakage is relatively small, but significant. This is supported by recent spin-labeled studies (Kim *et al*., 1997, 2004), where E134–R135 in rhodopsin appears to provide a small but significant contribution to rhodopsin activation (Kim *et al*., 1997). There are likely to be additive, synergistic or cooperative interactions with other constraints and motifs in both rhodopsin and the hIP.

Pharmacogenetic implications of novel naturally occurring charged residue mutations

In this study, we have characterized a number of novel Arg mutations within functionally important regions of the hIP. Our initial biochemical molecular characterization of the first two non-synonymous, single-nucleotide polymorphisms identified within the hIP, namely V25M and R212H, revealed an inherent defect in receptor activation associated with the R212H variant (Stitham *et al*., 2002b), which is located within the important G protein–interacting third cytoplasmic loop. We now report differential defects associated with a further two detected hIP variants (R77$^{2.33}$C and R279$^{7.40}$C). Owing to the fairly low frequencies of many of these Arg-to-Cys mutations, large-scale genetic screening studies will be required to determine true population prevalence, ethnic propensities and correlation with cardiovascular disease.

Collectively, these data suggest that receptor stabilization, through ionic interactions, is critical for normal hIP function. The E116$^{3.49}$ residue appears to form a localized structural constraint that contributes to overall receptor activation, whereas the R279$^{7.40}$ serves as the positive counterion for the negatively charged C1-carboxylate group (COO^-) of PGI_2 and other hIP ligands. The additional discovery of an R279$^{7.40}$C, naturally occurring variant, has the potential for significant implications on cardiovascular disease development.

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Conflict of interest

The authors state no conflict of interest.

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