Possible role for ligand binding of histidine 81 in the second transmembrane domain of the rat prostaglandin $F_{2\alpha}$ receptor

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Abstract For the five principal prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin and thromboxane A_2 eight receptors have been identified that belong to the family of G-protein-coupled receptors. They display an overall homology of merely 30%. However, single amino acids in the transmembrane domains such as an Arg in the seventh transmembrane domain are highly conserved. This Arg has been identified as part of the ligand binding pocket. It interacts with the carboxyl group of the prostanoid. The aim of the current study was to analyze the potential role in ligand binding of His-81 in the second transmembrane domain of the rat $PGF_{2\alpha}$ receptor, which is conserved among all $PGF_{2\alpha}$ receptors from different species. Molecular modeling suggested that this residue is located in close proximity to the ligand binding pocket Arg 291 in the 7th transmembrane domain. The His81 (H) was exchanged by site-directed mutagenesis to Gln (Q), Asp (D), Arg (R), Ala (A) and Gly (G). The receptor molecules were N-terminally extended by a Flag epitope for immunological detection. All mutant proteins were expressed at levels between 50% and 80% of the wild type construct. The H81Q and H81D receptor bound $PGF_{2\alpha}$ with 2-fold and 25-fold lower affinity, respectively, than the wild type receptor. Membranes of cells expressing the H81R, H81A or H81G mutants did not bind significant amounts of $PGF_{2\alpha}$. Wild type receptor and H81Q showed a shallow pH optimum for PGF2α binding around pH 5.5 with almost no reduction of binding at higher pH. In contrast the H81D mutant bound $PGF_{2\alpha}$ with a sharp optimum at pH 4.5, a pH at which the Asp side chain is partially undissociated and may serve as a hydrogen bond donor as do His and Gln at higher pH values. The data indicate that the His-81 in the second transmembrane domain of the $PGF_{2\alpha}$ receptor in concert with Arg-291 in the seventh transmembrane domain may be involved in ligand binding, most likely not by ionic interaction with the prostaglandin's carboxyl group but rather as a hydrogen bond donor.

Key words: Prostaglandin $F_{2\alpha}$ receptor; FP receptor; Prostanoid receptor; Site-directed mutagenesis; Ligand binding site; Structure-function relationship

1. Introduction

Prostanoid receptors are ectoreceptors with seven transmembrane spanning domains that couple to heterotrimeric G-proteins [1]. One receptor each exists for the prostanoids PGD_2 (DP-R), $PGF_{2\alpha}$ (FP-R), prostacyclin (IP-R) and thromboxane A_2 (TP-R), while four types of receptors are known for PGE_2 (EP1-R to EP4-R) [2]. The different prostanoid receptors couple to different G-proteins: FP-R, EP1-R and TP-R couple to G_q and increase $InsP_3$ and hence cytosolic Ca^{2+}, DP-R, EP2-R, EP4-R and IP-R couple to G_s and increase intracellular cAMP while EP3-R couples to G_i and decreases hormone-stimulated cAMP formation. The different prostanoid receptors display an overall sequence homology of merely 30%, yet some amino acids, especially in the putative transmembrane domains, are highly conserved [3]. Analysis of naturally occurring splice variants [4,5], of receptor hybrids [6–9] and of receptors in which single highly conserved amino acids had been exchanged by site-directed mutagenesis [10,11], has provided some insight into the structure-function relationship of prostanoid receptors. While the intracellular C-terminal domain has been implicated in G-protein coupling control [6,8] and agonist-induced receptor desensitization [7,9], amino acids in the transmembrane domains and the extracellular loops have been shown to be responsible for high affinity ligand binding and ligand specificity: all EP-R have a common sequence in the second extracellular loop, which is absent from any other prostanoid receptor and confers the specificity towards PGE_2 [12]. The seventh, sixth and third transmembrane domains have been implicated in ligand binding by analysis of hybrid receptors between IP-R and DP-R [8]. An Arg in the seventh transmembrane domain, which is conserved among all prostanoid receptors, has been shown to be essential for high affinity ligand binding in the EP3-R [13] and TP-R [14].

Other conserved polar amino acids in the otherwise apolar transmembrane domains are likely potential candidates for interaction with the prostanoid ligand. These include Asp and Thr in the second, a Gln in the fifth, a Cys in the sixth and an Asp in the seventh transmembrane domain which are conserved among all rat prostanoid receptors so far cloned. In the rat FP-R (rFP-R) there is a His-81 in the second transmembrane domain which is conserved among all FP-Rs from different species. Molecular modeling (see below) has revealed that this residue may be located in close proximity to the ligand binding pocket Arg-291 in the seventh transmembrane domain and might, thus, participate in $PGF_{2\alpha}$ binding. It was the purpose of the current study to analyze a possible role of this histidyl residue for high affinity $PGF_{2\alpha}$ binding.

2. Materials and methods

2.1. Materials

All materials were of analytical grade and from commercial sources. [^{3}H]$PGF_{2\alpha}$ was obtained from Amersham (Braunschweig, Germany), unlabeled $PGF_{2\alpha}$ was purchased from Calbiochem-Novabiochem (Bad Soden, Germany). Cell culture media were from Gibco-BRL (Eggenstein, Germany) and primers (Table 1) were synthesized by

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Abbreviations: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; MES, 2-[*N*-morpholino]ethanesulfonic acid; PBS, phosphate-buffered saline; PG, prostaglandin; PVDF, polyvinylidene difluoride; R, receptor; UTR, untranslated region

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NAPS (Göttingen, Germany). The monoclonal antibody FLAG-M2 was obtained from Sigma (Heidelberg, Germany). The sources of other materials are given in the text.

2.2. Cloning of the rat hepatocyte FP-R and computer-directed modeling of three-dimensional receptor conformation

The cDNA for the rFP-R was cloned from rat hepatocyte cDNA by RT-PCR. Total RNA was isolated from purified hepatocytes by CsCl gradient centrifugation [15]. Poly(A)$^+$ mRNA was prepared by affinity purification using oligo-(dT) beads from Qiagen (Rathingen, Germany) according to the manufacturer's instructions. First strand cDNA was synthesized by reverse transcription using oligo-$(dT)_{12-18}$ (Pharmacia, Freiburg, Germany) as a primer. PCR was carried out using 10 ng first strand cDNA as a template and the primer pair RFP-1F and RFP-8R (Table 1). Thirty-five cycles of PCR were performed with the following temperature profile: 1 min 95°C, 1 min 57°C and 3 min 72°C. A 1350 bp fragment was amplified and cloned into pUC18 (Pharmacia). Nucleotide sequence analysis was carried out on double stranded templates using the dideoxy chain termination method. For eukaryotic receptor expression a 1.0 kb cDNA fragment encoding the translated region of the rFP-R was subcloned in the eukaryotic expression vector pSVL (Pharmacia).

The seven putative transmembrane domains of the receptor were deduced from hydropathy analysis according to Kyte and Doolittle. The sequences were entered into the SWISS-MODEL Automated Comparative Protein Modeling Server in the GPCR mode as follows: helix 1: ^{28}LSVFFSIIFMTVGIVSNSLAIAILM; helix 2: ^{66}FLLLASGLVITDFFGHLINGGIAVF; helix 3: -^{109}SVFGISMVFSGLCPLFLGSTMAI-; helix 4: --^{154}MILSGVCMFAVFVALLPILGH--; helix 5: ^{203}LFFSSLGLLALGISFSCNAVTGVTL; helix 6: -^{248}VIQLLAIMCVSCVCWSPFLVTMA-; helix 7: -^{285}TTLFALRMATWNQILDPWVY ILL-. The topology was calculated with the human NPY receptor as template.

2.3. Site-directed mutagenesis and attachment of a N-terminal Flag-epitope tag

A Flag octapeptide sequence (N-Asp-Lys-Tyr-Asp-Asp-Asp-Asp-Lys-C) recognized by the monoclonal antibody FLAG-M2 was inserted after the initiator methionine of the rFP-R by PCR using Silver Star Taq polymerase (Eurogentec, Seraing, Belgium). The forward primer was FLAG-FP-F (Table 1), which starts at the second translated codon of the rFP-R and possesses a 5′ overhanging sequence encoding a *Xho*I site, a Kozak sequence and a Flag tag. The reverse primer was FP-Xba-R. It is located in the 3′-UTR of the rFP-R cDNA and it encodes an *Xba*I site (Table 1). The PCR was performed with 10 ng template (pSVL/rFP-R) and 35 cycles of the following temperature profile: 1 min 95°C, 1 min 60°C and 2 min 72°C. The PCR product was purified, digested with *Xho*I and *Xba*I and then ligated into the *Xho*I/*Xba*I site of the eukaryotic expression vector pSVL (Pharmacia).

Mutation of the His-81 of the rFP-R was performed by PCR-based site-directed mutagenesis using Silver Star Taq polymerase, pSVL/FLAG-rFP-R as template and the following PCR program: 3 min 95°C, 35×(1 min 95°C, 1 min 55°C, 2 min 72°C), 10 min 72°C. For each mutant two overlapping PCR products containing the mutation in the overlap region were synthesized and then fused by a subsequent PCR with the two flanking primers. A 296 bp 5′ fragment was generated with FLAG-FP-F as a forward primer and a reverse primer containing the H81 mutation (H81Q-R, H81D-R, H81R-R, H81G-R or H81A-R, Table 1). A 3′ 976 bp fragment was produced with the forward primer covering the mutation site (H81Q-F, H81D-F, H81R-F, H81G-F or H81A-F, Table 1) and the primer FP-Xba-R. A PCR product containing the entire open reading frame was then generated with both overlapping fragments as template and the primers FLAG-FP-F and FP-Xba-R. The PCR product was purified and digested with *Xho*I at the 5′ site and *Bst*EII at the 3′ site of the mutation. The fragment of the PCR product bearing the mutation was then ligated into pSVL/FLAG-rFP-R in which the corresponding cDNA fragment had been removed by digestion with *Xho*I and *Bst*EII. The sequences of the mutated cDNAs cloned into pSVL were verified by sequencing.

2.4. Cell culture and transfection

COS-7 cells were cultured in DMEM containing 10% (v/v) FCS and penicillin (100 U/ml) and streptomycin (100 μg/ml) as antibiotics. COS-7 cells were seeded at a density of 10^6 cells/10 cm diameter plate and transiently transfected after 24 h using the DEAE-dextran method [16] with wild type pSVL/FLAG-rFP-R or the mutant receptor constructs cloned into pSVL (5 μg/plate). Assays were performed 72 h after transfection.

2.5. Membrane isolation and $PGF_{2\alpha}$ binding assay

For membrane preparations, transfected cells were scraped into a homogenization buffer containing 25 mM Tris-HCl pH 7.5, 250 mM sucrose, 10 mM $MgCl_2$, 1 mM EDTA and 0.2 mM Pefabloc SC (Biomol, Hamburg, Germany), 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor as protease inhibitors. After homogenization of the cells in a Dounce homogenizer a crude membrane fraction was prepared by centrifugation of the homogenate at $100\,000\times g$. The resulting pellet was suspended in binding buffer containing 25 mM MES-NaOH pH 6.2, 10 mM $MgCl_2$ and 1 mM EDTA and stored at −70°C. To determine the pH optimum of binding below pH 5.5 MES was replaced in the binding buffer by 25 mM glycine. For $PGF_{2\alpha}$ binding, membranes (20–50 μg protein) were incubated with 5 nM [^{3}H]$PGF_{2\alpha}$ in 100 μl binding buffer for 1 h at 20°C. Non-specific binding was determined in the presence of 10 μM $PGF_{2\alpha}$. Bound and unbound ligand were separated by rapid vacuum filtration through GF 52 filters (Schleicher and Schüll, Dassel, Germany). Filters were washed five times with 4 ml ice-cold binding buffer. Radioactivity retained on the filter was counted in 5 ml Hydroluma (Baker, Deventer, The Netherlands). Binding constants were calculated by non-linear regression analysis (LIGAND [17]).

2.6. Protein deglycosylation with endoglycosidase F

Membrane proteins (20–50 μg) of transfected or control cells were solubilized with a 0.1 M sodium phosphate buffer pH 8.6 containing 1.2% (v/v) Nonidet-P 40, 0.2% (w/v) SDS, 2 mM EDTA, 1% (v/v) β-mercaptoethanol and 0.2 mM Pefabloc SC, 10 μg/ml leupeptin and

Table 1
Oligonucleotide primers used for RT-PCR cloning and site-directed mutagenesis

Name	Sequence (5′→3′)	Position in GenBank (accession number X83856)
RFP-1F (forward)	CTGGTTGCGCCATGGAACACCGGGC	1–25
RFP-8R (reverse)	TACACACAGCGGTCCAAGTGTTCCAG	1345–1319
FLAG-FP-F (forward)	gcgctcgag**ccaccatg***gactacaaggacgacgacgacaag*TCCATAAACAGTTCCAAGCAGCCG	*Xho*I recognition site (underlined), Kozak sequence (bold), Flag epitope (italic) and RFP-R 152–175
FP-Xba-R (reverse)	ggcgcgtctagaTACACACAGAGGTCCAAGTGT	*Xba*I recognition site (underlined) and RFP-R 1345–1325
H81Q-F (forward)	CTTCGGCCA**A**CTCATCAACGGAGGG	406–382 (mutation bold)
H81D-F (forward)	CTTCGGC**GA**CCTCATCAACGGAGGG	406–382 (mutation bold)
H81R-F (forward)	CTTCGGCC**G**CCTCATCAACGGAGGG	406–382 (mutation bold)
H81G-F (forward)	CTTCGGC**GG**CCTCATCAACGGAGGG	406–382 (mutation bold)
H81A-F (forward)	CTTCGGC**GC**CCTCATCAACGGAGGG	406–382 (mutation bold)
H81Q-R (reverse)	inverted complementary sequence of H81Q-F	
H81D-R (reverse)	inverted complementary sequence of H81D-F	
H81R-R (reverse)	inverted complementary sequence of H81R-F	
H81G-R (reverse)	inverted complementary sequence of H81G-F	
H81A-R (reverse)	inverted complementary sequence of H81A-F	

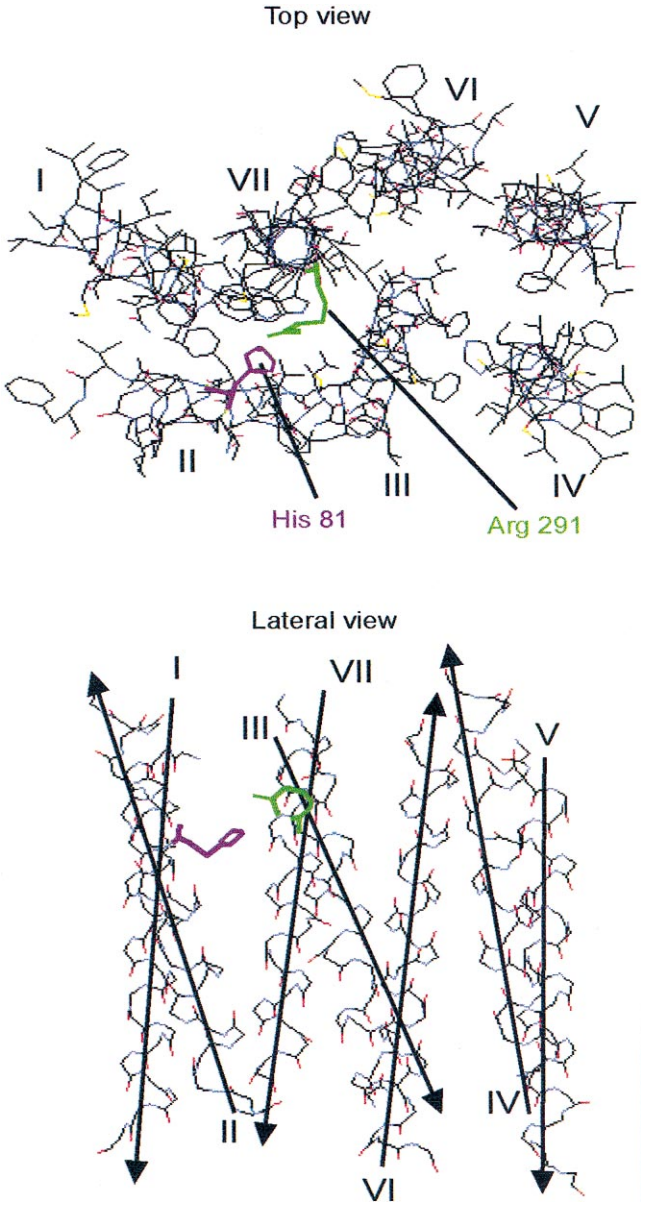

Fig. 1. Molecular modeling of a hypothetical three-dimensional arrangement of the rFP-R transmembrane domains. The seven putative transmembrane domains of the receptor were deduced from hydropathy analysis according to Kyte and Doolittle [20]. The sequences were entered into the SWISS-MODEL Automated Comparative Protein Modeling Server in the GPCR mode as follows: helix 1: ^{28}LSVFFSIIFMTVGIVSNSLAIAILM; helix 2: ^{66}FLLLASGLVITDFFGHLINGGIAVF; helix 3: -^{109}SVFGISMVFSGLCPLFLGSTMAI-; helix 4: --^{154}MILSGVCMFAVFVALLPILGH--; helix 5: ^{203}LFFSSLGLLALGISFSCNAVTGVTL; helix 6: -^{248}VIQLLAIMCVSCVCWSPFLVTMA-; helix 7: -^{285}TTLFALRMATWNQILDPWVY ILL-. The topology was calculated with the human NPY receptor as template.

10 μg/ml soybean trypsin inhibitor as protease inhibitors for 1 h at 37°C and 10 min at 60°C under vigorous shaking. Proteins were then deglycosylated with 0.2 U endoglycosidase F (Boehringer Mannheim, Mannheim, Germany) for 16 h at 37°C.

2.7. Western blotting

Membrane proteins (20–50 μg) of transfected or control cells were solubilized in Laemmli sample buffer under reducing conditions for 60 min at 37°C and 10 min at 60°C with vigorous shaking, separated on 10% SDS-polyacrylamide gels and transferred to PVDF membrane (Millipore, Bedford, MA, USA) by semi-dry blotting. The PVDF membrane was blocked with 5% (w/v) skim milk in PBS, 0.1% (v/v) Tween 20 (PBS-T). Flag-tagged receptor proteins were detected by incubating immunoblots overnight with 0.3 μg/ml monoclonal antibody FLAG-M2 in 1% (w/v) skim milk in PBS-T at 4°C followed by horseradish peroxidase-conjugated anti-mouse IgG (1:20 000 dilution, Bio-Rad, Munich, Germany) for 60 min at room temperature. Antigen-antibody complexes were visualized with the ECL system (Amersham, Braunschweig, Germany) according to the instruction of the company. To quantify receptor expression the intensity of FLAG-FP-R bands was determined densitometrically using the ImageQuant system (Molecular Dynamics, USA).

3. Results and discussion

3.1. Molecular modeling-based hypothesis

A cDNA for a rFP receptor was cloned from rat hepatocyte cDNA by RT-PCR based on the known sequence of the mouse ovary FP-R [18]. The cDNA was sequenced and the deduced 366 amino acid peptide sequence was found to be 96% identical to the mouse FP-R and 100% identical to the FP-R cloned from rat astrocytes [19]. Sequence homologies to human FP-R, rabbit FP-R and bovine FP-R were 85%, 80% an 81%, respectively. Hydrophobicity analysis according to Kyte and Doolittle [20] revealed the existence of seven putative transmembrane domains of 21–25 amino acids in length. The spatial arrangement of these transmembrane domains was calculated with the SWISS-MODEL Automated Comparative Protein Modeling Server in the GPCR mode [21–23] (Fig. 1). According to this model, the second and seventh transmembrane domains are close neighbors. Arg-291, which is the homologue of the Arg in the ligand binding pocket of the EP3-R and the TP-R, faces toward a cleft between the second and the seventh transmembrane domains, which is supposed to harbor the carboxyl group of the prostanoid. Interestingly, in such a model the His-81 in the second transmembrane domain comes into close proximity to the Arg-291 in the seventh transmembrane domain both in the lateral and

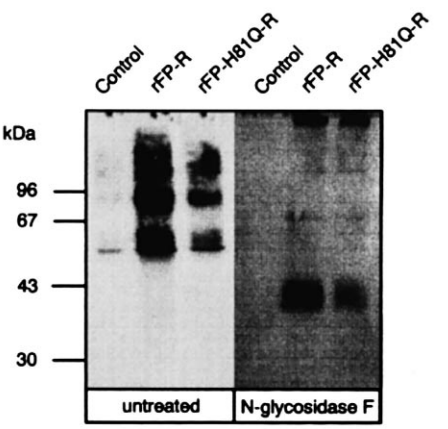

Fig. 2. Detection of Flag-tagged rFP-R and rFP-H81Q-R by immunoblotting with the FLAG-M2 antibody. Membrane proteins (20–30 μg/lane) of control and transfected cells were resolved by SDS-PAGE without or after pretreatment with endoglycosidase F and transferred to a PVDF membrane by semi-dry blotting. Receptor proteins were detected with a sandwich of the monoclonal antibody FLAG-M2 and horseradish peroxidase-conjugated anti-mouse antiserum visualized with enhanced chemiluminescence as described in Section 2.

in the vertical dimension (Fig. 1). The His-81 might thus also participate in the interaction with the carboxyl group of the $PGF_{2\alpha}$ in the FP-R binding pocket. To substantiate this hypothesis, the His-81 was exchanged to Gln, Asp, Arg, Gly and Ala and the binding characteristics of the resulting receptor mutants were analyzed.

3.2. Expression of the rFP-H81X receptor proteins

The codons for the His residue in the second transmembrane domain were replaced by codons for Gln, Asp, Arg, Ala and Gly by site-directed mutagenesis. Additionally the translated region of the cDNA was extended at the 5′ end by 27 nucleotides encoding a start Met and an octapeptide that represents the epitope for the monoclonal FLAG-M2 antibody and allows the immunological detection of the mutant receptor proteins. The modified cDNAs were cloned into the eukaryotic expression vector pSVL and expressed transiently in COS-7 cells. In membranes of transfected cells the wild type rFP-R protein was detected as a group of bands above 53 kDa, which were absent from membranes of untransfected cells (Fig. 2). An identical pattern was seen with the rFP-H81Q-R (Fig. 2) and all other mutant receptors (not shown). These bands most likely represent receptor proteins in which the three potential glycosylation sites in the N-terminal domain and the third extracellular loop are glycosylated to different extents. The bands with an apparent molecular weight above 90 kDa most likely represent aggregates of receptor proteins. Such aggregates are frequently found with GPCRs. If membranes of transfected cells were pretreated with endoglycosidase F prior to electrophoretic separation only one broad band appeared in the range of 41 kDa (Fig. 2), the calculated molecular mass of the non-glycosylated receptor protein. The expression levels of the wild-type rFP-R and the five mutant receptors were estimated by densitometric quantification of the Western blots (Fig. 3C). Expression levels reached 50–80% of the receptor protein level found in membranes transfected with the wild type receptor cDNA (Fig. 3B).

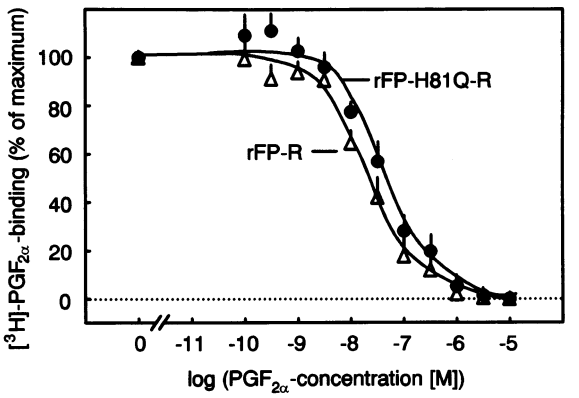

Fig. 4. Determination of the affinity of the rFP-R and the rFP-H81Q-R by competition binding of [^{3}H]$PGF_{2\alpha}$ to membranes transfected COS-7 cells. Binding of 5 nM [^{3}H]$PGF_{2\alpha}$ to membranes of transfected cells was measured after 1 h at 20°C in the presence of the concentrations of unlabelled $PGF_{2\alpha}$ indicated. [^{3}H]$PGF_{2\alpha}$ binding in the presence of 10 μM $PGF_{2\alpha}$ was defined as unspecific binding. Maximal specific binding was set at 100%. Values are means ± S.E.M. of three different experiments performed in triplicate.

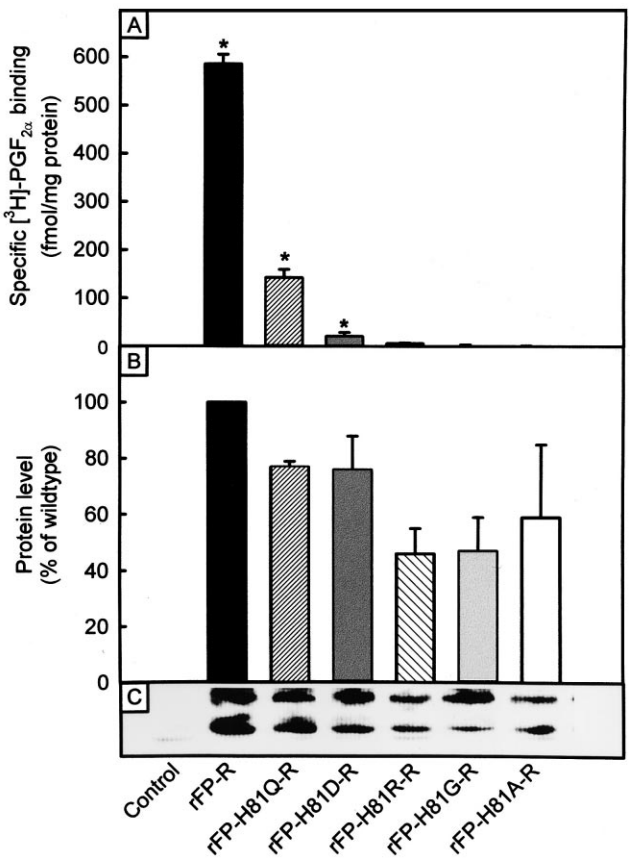

Fig. 3. $PGF_{2\alpha}$ binding and protein expression level of wild type rFP- and rFP-H81X mutant receptors. A: Membranes of cells transfected with the wild type rFP-R or the mutant receptors were incubated with 5 nM [^{3}H] $PGF_{2\alpha}$ for 1 h at room temperature. Bound and unbound ligand were separated by rapid vacuum filtration. Unspecific binding was determined in presence of a 2000-fold excess of unlabelled $PGF_{2\alpha}$. B: Receptor expression determined densitometrically with ImageQuant. Expression of wild type rFP-R was set at 100%. Data are means ± S.E.M. from three independent transfection experiments. C: Wild type and mutant receptors were detected as described in the legend to Fig. 2.

3.3. $PGF_{2\alpha}$ binding to the rFP-H81X receptors

Membranes of cells transfected with the wild type rFP-R or the mutant receptors were incubated with 5 nM [^{3}H]$PGF_{2\alpha}$ for 1 h at room temperature. Bound and unbound ligand were separated by rapid vacuum filtration. Unspecific binding was determined in the presence of a 2000-fold excess of unlabeled $PGF_{2\alpha}$. Under these conditions, membranes of cells expressing the wild type rFP-R bound 586 fmol [^{3}H]$PGF_{2\alpha}$/mg membrane protein (Fig. 3A). Despite being expressed to a comparable protein level (Fig. 3B) rFP-H81R-R, rFP-H81G-R and rFP-H81A-R did not bind $PGF_{2\alpha}$ at all. Only rFP-H81Q-R and rFP-H81D-R bound $PGF_{2\alpha}$ to a significant extent; however, [^{3}H]$PGF_{2\alpha}$ binding was merely 24% and 4%, respectively, of the wild type receptor and thus was much less than would have been expected from their protein expression level of about 80% of the wild type receptor protein (Fig. 3B). Reduced binding can most likely be attributed to a reduced affinity of the receptor proteins. The K_d of the wild type receptor was determined to be about 22 nM (Fig. 4). Taking into account the specific binding at 5 nM $PGF_{2\alpha}$ and the protein expression level determined by Western blot analysis,

one can estimate the K_d values of the mutant receptors to be roughly 80 nM for the rFP-H81Q receptor and 540 nM for the rFP-H81D receptor[(1)]. Due to the very low amount of radioactivity bound to the rFP-H81D receptor it was not possible to determine its affinity by competition binding. Therefore, a competition binding analysis was performed for the rFP-H81Q mutant only. The K_d value of 48 nM determined by non-linear regression analysis of competition binding [17] (Fig. 4) agrees reasonably well with the value of 80 nM estimated from the single measurement at 5 nM $PGF_{2\alpha}$. Both the rFP-R and the rFP-H81Q-R bound $PGF_{2\alpha} > PGD2 > PGE_2$. The relative affinities were comparable, the absolute affinities of the rFP-H81Q-R always appeared to be two- to threefold lower (not shown).

3.4. pH optimum of $PGF_{2\alpha}$ binding to rFP-R, rFP-H81Q-R and rFP-H81D-R

It was unexpected that the rFP-H81D-R mutant bound $PGF_{2\alpha}$ to a significant extent. The Asp side chain carboxyl group is largely dissociated at pH 6.2 and hence negatively charged. Assuming that the carboxyl group of the prostanoid should interact with Arg-291 in the seventh transmembrane domain and therefore according to the calculated model should come into contact with His-81 in the second transmembrane the negatively charged Asp in position 81 should electrostatically repel the also negatively charged prostanoid from the hypothetical binding pocket. Shifting the pH of the binding buffer to acid pH values, where both the Asp side chain and the prostanoid carboxyl group are undissociated to a significant extent, should, therefore, increase $PGF_{2\alpha}$ binding to the rFP-H81D-R mutant. In line with such a hypothesis binding to the rFP-H81D-R mutant was strongly increased when the pH was decreased to 4.5 (Fig. 5), a pH that corresponds to the p*K* of the Asp side chain carboxyl group. In contrast to the H81D mutant the wild type rFP-R and the rFP-H81Q-R mutant showed a shallow pH optimum around pH 5 with almost no decrease of binding at higher pH values and a significant decrease of binding at lower pH values (Fig. 5). These data indicate that the amino acid at position 81 of the rFP-R most likely is part of the binding pocket and presumably interacts with the carboxyl group of the prostanoid. However, probably there is no need for a positively charged amino acid side chain in position 81 of the second transmembrane domain of the FP receptor that could interact via ionic interactions with the negatively charged carboxyl group of the prostanoid, but rather a hydrogen bond donor is required in this position that forms hydrogen bonds with the carboxyl group oxygens of the prostanoid.

3.5. Conclusion

The experimental data support the hypothesis that the second transmembrane domain of the rFP-R is part of the ligand binding pocket. The data furthermore favor the assumption

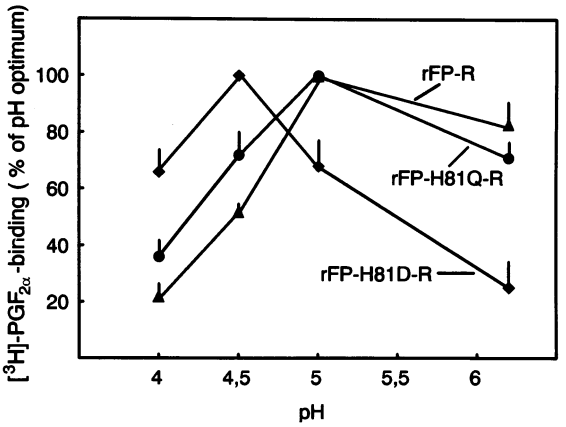

Fig. 5. pH optimum of $PGF_{2\alpha}$ binding to rFP-R, rFP-H81Q-R and rFP-H81D-R. [^{3}H]$PGF_{2\alpha}$ binding to membranes of COS-7 cells expressing the respective receptor protein was determined as described in legend to Fig. 3 except that membranes were suspended and binding assays were performed in a glycine-buffered binding buffer of the pH indicated. Values are means ± S.E.M. of three independent transfections in triplicate.

that the His-81 faces towards the interior of this binding pocket and might together with the Arg-291 in the seventh transmembrane domain interact with the carboxyl group of the prostaglandin. In the EP3-R the interaction between the prostaglandin's carboxyl group and the seventh transmembrane Arg has been characterized in detail. It was found that both PGE_2 and its uncharged methyl ester, which is a hydrogen bond acceptor, were potent agonists for the EP3-R while the complete lack of a carboxyl group led to a loss of agonist activity. This indicates that agonist binding to the EP3-R requires the carboxyl group but does not depend on an ionic interaction of the carboxyl group with amino acids in the binding pocket but is rather due to hydrogen bond formation [24] between constituents of the binding pocket and the carboxyl group of the prostaglandin. Assuming that the interaction of the rFP-R with the carboxyl group of $PGF_{2\alpha}$ is similar to that of the EP3-R with PGE_2, Arg-291 should form hydrogen bonds with the carboxyl group of the prostaglandin. The imidazole ring of the His-81 in the rFP-R could act as an additional hydrogen bond donor in such a constellation. The Gln side chain, which has a similar size as the His side chain, could in part replace the His as a hydrogen bond donor. This could be shown for the seventh transmembrane Arg of the EP3α-R where mutation of the Arg residue to Gln did not affect high affinity PGE_2 binding whereas substitution to Leu abolished PGE_2 binding by the EP3-R [24]. The Asp side chain in its uncharged form could probably still fulfil the hydrogen bond donor function in position 81 to some extent. In contrast, the bulky and charged residue of a second Arg in the rFP-H81R-R mutant probably distorts the binding pocket thereby preventing $PGF_{2\alpha}$ from binding. The lack of $PGF_{2\alpha}$ binding to the rFP-H81A-R and rFP-H81G-R mutants is in line with the inability of the small aliphatic or missing side chains to act as hydrogen bond donors.

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(1) The estimate for the K_d of the mutant receptor (K_{dmt}) was calculated according to the following equations: (1) binding to the wild type (wt) and mutant (mt) receptor at a given lingand concentration: $B_{wt} = B_{maxwt} \times [L]/(K_{dwt}+[L])$ and $B_{mt} = B_{maxmt} \times [L]/(K_{dmt}+[L])$, respectively. (2) Known parameters: K_{dwt} (determined by saturation binding assays), $B_{mt} = n \times B_{wt}$ (values determined in the single point binding assay), $B_{maxmt} = m \times B_{maxwt}$ (B_{max} was assumed to be proportional to the receptor protein level found by Western blot). (3) Since the ligand concentration in both experiments was the same, the equations can be combined to calculate K_{dmt}: $K_{dmt} = m/n \times ([L]+K_{dwt})-[L]$.

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