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Involvement of non-conserved residues important for PGE_2 binding to the constrained EP3 eLP_2 using NMR and site-directed mutagenesis

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Abstract A peptide constrained to a conformation of second extracellular loop of human prostaglandin-E_2 (PGE_2) receptor subtype3 (hEP3) was synthesized. The contacts between the peptide residues at S211 and R214, and PGE_2 were first identified by NMR spectroscopy. The results were used as a guide for site-directed mutagenesis of the hEP3 protein. The S211L and R214L mutants expressed in HEK293 cells lost binding to [^{3}H]PGE_2. This study found that the non-conserved S211 and R214 of the hEP3 are involved in PGE_2 recognition, and implied that the corresponding residues in other subtype receptors could be important to distinguish the different configurations of PGE_2 ligand recognition sites.

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Keywords: Extracellular loop 2; Prostaglandin E_2; EP3 receptor

1. Introduction

Prostaglandin (PG) E_2 exerts its actions by acting on a group of G-protein-coupled receptors (GPCRs) which are designated as subtypes EP1, EP2, EP3 and EP4. These EPs exhibit differences in signal transduction and tissue localization [1–3] and yet share common ligands. For example, the EP3 mediates the pyrogenic response [4], EP1 and EP3 mediate release of corticotropin-releasing hormone [5], EP2 facilitates ovulation and fertilization [6] and EP2 and EP4 mediate collagen-induced arthritis. Also, prostanoids exert both pro-inflammatory and anti-inflammatory responses, through regulation of gene expression in relevant tissues [7].

The agonists to EP induce a signaling cascade inside the cell which seems to have differences and similarities and yet show different signaling outcomes. EP1 mediate signaling by activation of phospholipase C, protein kinase Cα and c-Src with upregulation of endothelial growth factor-C [8]. The EP2 and EP4 are linked to cAMP/protein kinase A and phosphoinositide-3-kinase signaling [9]. The EP3 however couples to multiple G proteins such as the Gi, resulting in the inhibition of adenylyl cyclase and Gs resulting in cAMP production [10,11]. EP3 can also activate the Ras signaling pathway leading to cancers [12].

The seven conserved residues in the second extracellular loop (eLP_2) of rabbit EP3 involved in ligand recognition has been demonstrated previously [13]. However, in this paper we hypothesize that the differences in ligand recognition and ultimately the functional effect must logically lie in the non-conserved region as these EPs have multiple common ligands showing different effects. For example, PGE_2 acts as an inflammatory molecule, whereas PGE_1 acts as an anti-inflammatory molecule and both act on the same set of EPs with different affinities [14]. Thus, in order to understand the EPs it is important to uncover how the eight prostanoid receptors can distinguish between the similar prostanoids, which are synthesized from the same precursor, PGH_2. Using the TM (transmembrane) domains of the working model for the EP3 receptor the constrained peptide mimicking the EP_3 eLP_2 was synthesized and purified. The residues in the EP_3 eLP_2 interacting with PGE_2 in solution were determined by NMR spectroscopy. The importance of this study lies in the fact that it helps us recognize the fundamental differences that give quality to a receptor and can be used for future benefit of therapeutics.

2. Materials and methods

D_2O (Cambridge Isotope Laboratories, Andover, MA), PGE_2 (Amersham Biosciences, Piscataway, NJ), HEK293 cells (ATCC, Manassas, VA), DMEM culture media (Invitrogen, Carlsbad, CA), and [^{3}H]PGE_2 and PGE_2 (Perkin–Elmer, USA).

2.1. Peptide synthesis and purification

A peptide mimicking the human EP3 eLP_2 (residues 189–227, Fig. 1B) with homocysteine added at both ends was synthesized using the solid phase method [15,16]. After cleavage with TFA, the peptide was purified by HPLC on a C4 reversed phase column with a gradient from 0% to 80% acetonitrile in 0.1% TFA. For cyclization, the purified peptide (0.02 mg/ml) was dissolved in H_2O and adjusted to pH 8.5 using triethylamine, and then stirred overnight at room temperature. It was then lyophilized and purified by HPLC on the C4 column [17].

2.2. Fluorescence spectroscopic studies

0.75 ml (0.1 mg/ml) of the peptide was dissolved in 0.01 M phosphate buffer, pH 7.2, with 0.1 M NaCl and then incubated with various concentrations of PGE_2. Fluorescence spectra were acquired with an Hitachi F-4500 spectrofluorometer using 294 nm for excitation and 300–360 nm for emission [18].

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Abbreviations: NOESY, nuclear overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; NOE, nuclear overhauser effect

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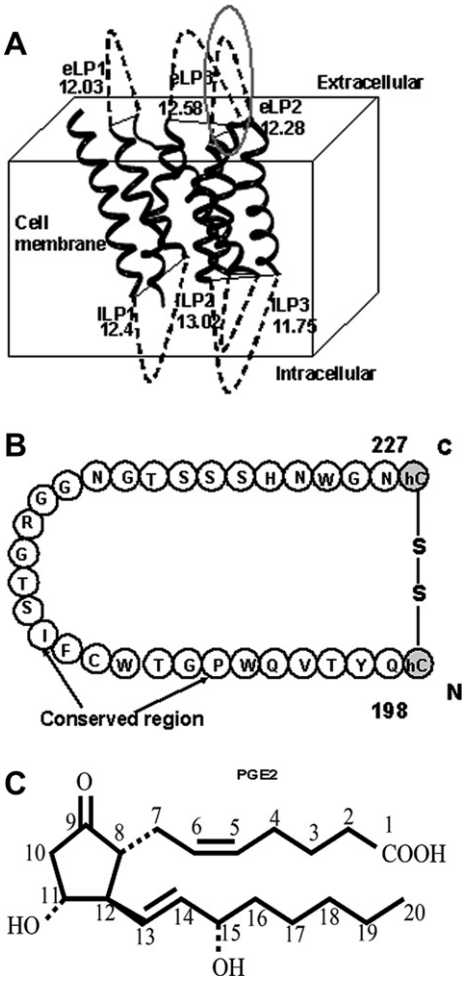

Fig. 1. (A) The homology model of the human EP3 receptor using crystal structure of β2 receptor [23]. The distance in angstroms (Å) between the transmembrane domains are shown. The synthesized peptide, EP_3 eLP_2, is marked with a circle. (B) The synthesized eLP_2 in its constrained form. (C) Structure of PGE_2.

2.2.1. NMR sample preparation. Four milligrams of the purified and constrained peptide was dissolved in 0.5 ml, pH 5.5, 10 mM sodium phosphate buffer with 10% D_2O, pH 6.0, at a final concentration of 2.43 mM. 0.5 mg of PGE_2 was dissolved in 50 μl ethanol-d_6 and then added to 0.45 ml of sodium phosphate buffer (20 mM) containing 10% D_2O [17].

2.3. NMR experiments

NMR was performed on a Bruker AVANCE 800 MHz NMR spectrometer at 293 K. 2D Nuclear overhauser effect spectroscopy (NOESY) (300 ms mixing time), total correlation spectroscopy (TOCSY) and double quantum filtered-correlation spectroscopy for above samples were recorded. Thereafter, 2.5, 5, 10, 20 and 40 μl of PGE_2 in ethanol-d_6 solution (1 mg/100 μl) was added into the sample and the 1D proton spectra were recorded. 2D NOESY (200 ms mixing time) and TOCSY spectra were then recorded for the final mixture of EP3 eLP_2 and PGE_2. Details are mentioned in previous publications [18,21].

2.3.1. Site-directed mutagenesis. pAcSG-EP3 wild-type cDNA was first subcloned into *Eco*RI/*Xba*I sites of pcDNA3.1(+) expression vector. The EP3 receptor mutants were then constructed using standard PCR. Details in [22]. The plasmids were prepared using Midiprep kit (Qiagen) for transfection into HEK293 cells.

2.3.2. Expression of EP3 receptor wild-type and mutants in HEK293 cells. HEK293 cells were transfected with purified cDNA of pcDNA3.1(+)/EP3 wild-type and mutants with lipofectamine according to the manufacturer's instructions [22]. Western blot was performed to evaluate the protein expression (Fig. 8A).

2.3.3. Ligand binding assay. Ligand binding assay for the WT and mutant EP3 receptor was performed on whole cells in 48-well culture plates with 0.035 and 1 nM [^{3}H]PGE_2, respectively, in the presence and absence of 5 μM unlabeled (cold) PGE_2 in the 0.1-ml reaction volume of DMEM at room temperature for 60 min. The reaction was terminated by adding 1 ml of ice-cold washing buffer (25 mM Tris–HCl, pH 7.4). The ligand bound cells were dissolved in 0.5 N NaOH, which was later, neutralized with acetic acid. The procedure was modified from Ref. [20] and radioactivity detected by MicroBetaTrilux counter.

3. Results

3.1. Homology model and peptide design

The human EP3 receptor model with seven TM domains was created by homology modeling using the crystallized beta2 receptor as the template [23]. The distance between the transmembrane helices was 12–14 Å which is similar to the distance between the disulphide bonds formed by the homocysteine residues in the constrained peptide (approximately 12 Å) (Fig. 1A and B).

3.2. Sequence alignment of the eLP_2 regions from the eight human prostanoid receptors

The identical residues, QW-PGTWCF, in the eLP_2 regions are centrally located within the eLP_2.The identified residues, S211 and R214 are not conserved in other prostanoid receptor eLP_2s indicating that these residues of EP3 eLP_2 could be involved in specific ligand recognition (Fig. 2).

3.3. Fluorescence spectroscopy of PGE_2 with the peptide segment

The recorded intrinsic fluorescence signal was generated from the Trp residue, which is sensitive to the conformational change in the peptide induced by the interaction with its ligand. The fluorescence intensity of the constrained peptide increased by 11–14% (K_d 5.12) with PGE_2, which started at 3.4 μM and became saturated at 68.1 μM of the ligand (Fig. 3). However, no significant fluorescence changes were induced upon the addition of PGE_2 to the crude EP3 eLP_2. These results indicate that the constrained EP3 eLP_2 peptide

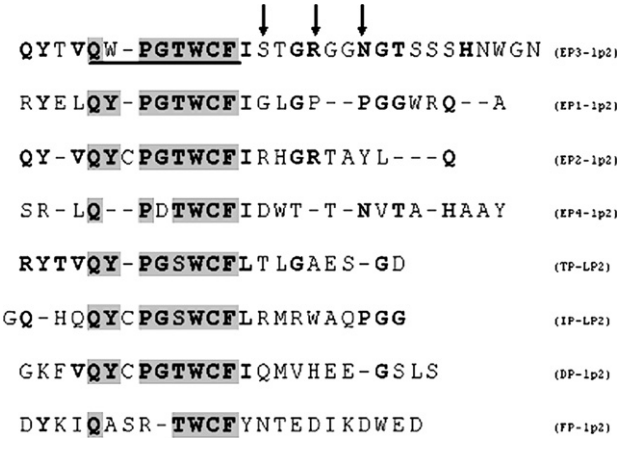

Fig. 2. Sequence alignment of eLP_2's of prostanoid receptors. The conserved residues are highlighted. Arrows indicate residues for mutation (non-conserved region).