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Phenylalanine 138 in the Second Intracellular Loop of Human Thromboxane Receptor Is Critical for Receptor-G-Protein Coupling

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Eicosanoid receptors exhibit a highly conserved ERY(C)XXV(I)XXPL sequence in the second intracellular loop. The carboxyl end of this motif contains a bulky hydrophobic amino acid (L,I,V, or F). In human thromboxane A2 receptor (TXA_2R), phenylalanine 138 is located at the carboxyl end of this highly conserved motif. This study examined the function of the F138 in G protein coupling. F138 was mutated to aspartic acid (D) and tyrosine (Y), respectively. Both mutants F138D and F138Y showed similar ligand binding activity to that of the wild type TXA_2R. The Kd and Bmax values of either mutant were comparable to those of the wild type receptor. However, both mutants showed significant impairment of agonist induced Ca^{2+} signaling and phospholipase C activation. These results suggest that the F138 plays a key role in G protein coupling. © 1999 Academic Press

Thromboxnae A_2 (TXA_2) is an important lipid mediator with potent platelet aggregating and vesoconstrictive actions (1). It has been implicated in many cardiovascular, renal and respiratory disorders. Its effects are mediated by activating specific cell surface receptors (TXA_2R) and subject to regulatory controls (2). TXA_2R belongs to a heptahelical G-protein coupled receptor (GPCR) superfamily. The structure of GPCRs is known to consist of seven transmembrane domains, three each of extracellular and intracellular loops, the amino extracellular head and the cytoplasmic carboxyl tail. The three intracellular loops and the carboxyl tail are thought to interact with the G-protein(s). Two forms of TXA_2R (α and β) have been identified and they are derived by alternate splicing in the carboxyl tail from a single gene (3, 4). The α form is expressed predominantly in most tissues with a M.W. around 55 kDa. Both forms appear to couple to phospholipase C which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol-1,4,5-triphosphate which activate protein kinase C and release of Ca^{2+} from intracellular stores respectively (5, 6). The significance of TXA_2R in clinical medicine was realized by the discovery of various forms of mutant receptors in patients having bleeding disorders (7–9). One of the mutant receptors described recently is related to a single amino acid mutation in the first intracellular loop (Arg^{60} to Leu^{60}) characterized by defective thromboxane receptor coupling to phospholipase C (9). The existence of this mutant thromboxane receptor points to the need of identifying structural determinants of the receptor involved in receptor G-protein coupling.

The cytoplasmic domains of the receptors have been shown to interact with the G-proteins (10, 11). Specific residues involved in G-protein coupling are beginning to uncover. Moro *et al.* (12) first noted that β_2-adrenergic and m_1-muscarinic receptors contains a highly conserved motif (DRYXXV(I)XXPL) in the second intracellular loop. The carboxyl end of this motif usually contains a bulky hydrophobic amino acid (L,I,V,M,F). Substitution of this amino acid with a polar amino acid results in strongly defective coupling, whereas another hydrophobic amino acid can replace it without losing coupling activity.

Sequence analysis of prostanoid receptors also reveals a similar motif in the second intracellular loop (13). In this study, site-directed mutagenesis was performed to examine the role of Phe-138 in the second intracellular domain of the TXA_2R in the G-protein coupling.

Abbreviations used: TXA_2R, thromboxane A_2 receptor; GPCR, G-protein coupled receptor; PCR, ploymerase chain reaction; IP, inositol phosphate; I-BOP, [15-(1,2(5Z),3-(1E,3S)4)]-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl)-7-oxa-bicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; U46619, (15S)-hydroxy-11,9-(epoxymethano)prosta-5Z,13E-dienoic acid; SQ29548, ([1S-1,2(5Z),3,4))-7-(3-(2-((phenyl-amino)-carbonyl)hydrazino)methyl)-7-oxa-bicyclo-(2.2.1)heptan-2-yl)-5-heptenoic acid]).

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EXPERIMENTAL PROCEDURES

Materials. Native *pfu* DNA polymerase and QuickChange Site-Directed Mutagenesis Kit were purchased from Stratagene. pcDNA3 expression vector was from Invitrogen (San Diego, CA). T4 DNA ligase, BamHI and XhoI were from New England Biolabs, Inc. Human Embryo Kidney (HEK) 293 cell was obtained from American Type Culture Collection. *Taq* DNA polymerase, heat inactivated fetal bovine serum, antibiotic-antimycotic and geneticin selective antibiotic (G418) were from GIBCO-BRL. The QIAprep Spin Plasmid Miniprep Kit, QIAquick PCR Purification Kit, QIAquick Gel Extraction Kit and Effectene Transfection Reagent were from QIAGEN. [^{3}H]-SQ29,548 (specific activity 48 Ci/mmol) was from NEN Life Science Products, Inc. I-BOP and SQ29,548 were from Cayman Chemical. Fura-2/AM was purchased from Calbiochem-Novabiochem Corporation. Oligonucleotide primers were synthesized by Intergrated DNA Technologies. Dulbecco's modified Eagle's medium (DMEM) and other chemicals were from Sigma. Myo-[2-^{3}H]inositol (specific activity 20 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. AG1-X8 (formate form), 100-200 mesh was from Bio-Rad. Antiserum against N-terminal sequence (MWPNGSSLGPCFRPTN ITLEERRLIASPW) of human TXA_2R was generated in rabbit using GST-fusion protein as an antigen.

Subcloning of human TXA_2R cDNA. Human TXA_2R (α form) cDNA was amplified by PCR using *Taq* DNA polymerase as described prevously (14). The oligonucleotides used for PCR were designed based on the amino terminal sequence (5′-CGGGATCCATG TGGCCCAACGGCAGTTC-3′) and carboxyl terminal sequence (5′-TGGTCTCGAGCTACTGCAGCCCGGAGCG-3′) with extra BamHI and XhoI sites on the ends, respectively. The PCR product was subcloned into the mammalian expression vector pcDNA3 at BamHI and XhoI sites. The insertion of the $TXA_2R\alpha$ cDNA was confirmed by DNA sequencing.

Site-directed mutagenesis. Mutagenesis of the TXA_2R was performed using QuikChange Site-Directed Mutagenesis kit. Phenylalanine 138 was changed to aspartic acid (D) or tyrosine (Y). The sequences of the mutagenic primers were listed below:

F138D Forward	5′-CACCCGGCCCGACTCGCGCCCGGC-3′
F138D Reverse	5′-GCCGGGCGCGAGTCGGGCCGGGTGATA-3′
F138Y Forward	5′-CACCGGGCCCTACTCGCGCCCGGC-3′
F138Y Reverse	5′-GCCGGGCGCGAGTAGGCCGGGTGATA-3′

Culture and transfection of human embryo kidney cell line (HEK293). HEK293 cells were grown in 90% DMEM supplemented with 10% heat-inactivated fetal bovine serum, gentamicin, and antibiotic-antimycotic at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were subcultured every three days after becoming confluent by use of 0.25% trypsin with 1 mM EDTA and plated at a density of 1×10^5 cells/ml.

To create cell lines stably expressing wild type or mutant TXA_2R, pcDNA3 expression vector containing either the wild type or mutant constructs was transfected into HEK293 cells using Effectene Transfection Reagent according to the manufacturer's directions. Briefly, 5×10^5 cells were plated per 60 mm dish in 5 ml of completed medium the day before transfection. One μg of DNA was first mixed with DNA-condensation buffer to a total volume of 150 μl, then add 8 μl of Enhancer. After incubation at room temperature for 5 min, 25 μl of Effectene Transfection Reagent was added to the DNA-Enhancer mixture, mixed and incubated at room temperature. After 15 min, 1 ml of cell culture medium was added to the transfection complexes. This solution was mixed and added to 60 mm dishes containing washed HEK293 cells. Cells were incubated with the complexes at 37°C and 5% CO_2 to allow for gene expression.

To isolate permanent transfectants, G418-resistant cells were selected in complete culture medium containing 500 μg/ml G418. After G418 selection, individual clones were screened for [^{3}H]-SQ-29,548 binding as described below.

Preparation of HEK293 cell membranes. Confluent cultures of HEK293 cells were incubated with 0.25% trypsin and 1 mM EDTA. After detachment, cells were immediately centrifuged at 1,000 $\times$ g for 5 min. The cell pellet was washed with phosphate-buffered saline and then resuspended in homogenization buffer (10 mM Tris-HCI, pH 7.4, 50 μg/ml phenylmethyl sulfonyl fluoride). The mixture was then sonicated 4 times 10 s each with an ultrasonicator at a setting of 4. The homogenate was centrifuged at 100,000 $\times$ g for 30 min at 4°C. The pellet was resuspended in ice cold binding buffer.

Ligand binding assay. Ligand binding assay was conducted in 50 mM Tris-HCI (pH 7.4) buffer with 5 mM $CaCl_2$ as described previously (14). For each assay, 100 μg of cell membrane fraction was incubated with 3 nM of [^{3}H]-SQ29,548 in a 100 μl reaction volume at room temperature for 60 min. The reaction was terminated with the addition of 1 ml of ice-cold washing buffer (50 mM Tris-HCI, 150 mM NaCI, pH 7.4). The solution was filtered under vacuum through a Whatman GF/C glass filter and the filter was then washed with 10 ml of washing buffer. The radioactivity retained on the filter was counted in 10 ml scintillation cocktail. The nonspecific binding was determined by adding 10 μM unlabeled SQ29,548.

Measurement of intracellular calcium signaling. Intracellular calcium signaling was measured by fluorescence excitation of cells loaded with the fluorescent probe Fura-2/AM as described previously (15). Briefly, the HEK 293 cells stably transfected with TXA_2R were collected and washed with Fura-2 assay buffer (4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 4.3 mM K_2HPO_4, pH 6.8, 113 mM NaCl, 5 mM D-glucose, 1 mM $CaCl_2$, and 0.5% BSA). The cells were resuspended in the same buffer at a concentration of 1×10^7 cells/ml. The suspension was incubated with 10 μM Fura-2/AM at 37°C for 1 h, pelleted by centrifugation at 1,000 $\times$ g for 10 min, washed with the Fura-2 assay buffer but without Ca^{2+} twice. The loaded cells were resuspended at approximately 5×10^6 cells/ml in the Fura-2 assay buffer. The Ca^{2+} signal induced by $TXA_2R\alpha$ agonist I-BOP was examined by Fura-2 fluorescence in a Hitachi F-2000 Fluorescence Spectrophotometer at 37°C with excitation and emission wavelengths at 340 and 510 nm respectively. The reaction was initiated by adding I-BOP. The final concentration of I-BOP was 100 nM.

Measurement of [^{3}H]inositol phosphate (IP) generation. Inositol phosphates were measured as described previously using anion exchange chromatography (16). HEK293 cells expressing wild-type or mutant TXA_2R in 6-well (2 ml/per well) culture plates were labeled with myo-[2-^{3}H]inositol in inositol-free DMEM (1 μCi/ well) for 24 h. Cells were washed twice and incubated with serum-free, inositol-free DMEM containing 20 mM LiCl for 10 min, and were then incubated with or without agonist. After 10 min of incubation at 37°C, the medium was aspirated and the reaction was terminated by the addition of 0.75 ml of 10 mM formic acid. Samples were neutralized by 3 ml of 5 mM NH_4OH. Total [^{3}H]IPs were isolated by anion exchange column chromatography using AG1-X8 (10.7 $\times$ 2 cm). The column was washed with 40 mM formic acid/ammonium formate (pH 5) and the [3H] IPs were eluted with 4 ml of 2 M formic acid/ammonium formate (pH 5). The radioactivity of the last elute was determined in a liquid scintillation counter. Agonist-stimulated inositol phosphate formation was expressed as a percentage of the nonstimulated sample.

Western blotting analysis. The cell membranes (50 μg) prepared from HEK293 cells expressing the wild type and the mutant receptors were subjected to SDS-PAGE on 10% polyacrylamide gel. The proteins were then electrophoretically transferred onto PVDF membranes. The membrane was blocked with 5% non-fat milk in 30 mM Tris-HCl, pH 7.4 containing 120 mM NaCl and 0.05% Tween-20 (TBST) at room temperature for 1 h. It was then incubated for 2 h at room temperature with a rabbit antiserum against the N-terminal sequence of the TXA_2R in TBST with 5% non-fat milk (1:400 dilution), followed by incubation with HRP-linked Protein A (1:5,000 dilution in TBST with 5% non-fat milk) for one h at room temperature. The membrane was washed with TBST 6 times. The immuno-

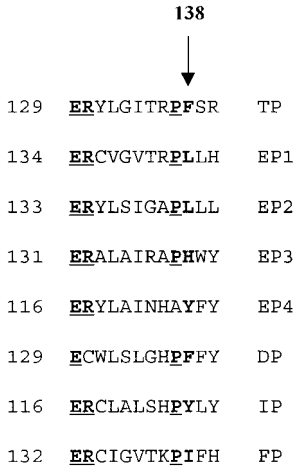

FIG. 1. Amino acid sequence alignment of the second intracellular loop of several human prostanoid receptors. TP: Thromboxane A_2 receptor; EP1: Prostaglandin E_2 receptor isoform I; EP2: Prostaglandin E_2 receptor isoform II; EP3: Prostaglandin E_2 receptor isoform III; EP4: Prostaglandin E_2 receptor isoform IV; DP: Prostaglandin D receptor; IP: prostaglandin I_2 receptor; FP: Prostaglandin $F_{2\alpha}$ receptor.

reactive bands were detected with ECL$^+$ Plus Western blotting detection system.

RESULTS AND DISCUSSION

Moro *et al.* (12) first observed that a hydrophobic residue at the carboxyl end of the highly conserved motif (DRYXXV(I)XXPL) in the second intracellular loop of the muscarinic and adrenergic receptors is essential to the receptor G-protein coupling. A similar motif was also found in the eicosanoid receptor family as shown in Fig. 1. We initiated to examine if the corresponding Phe-138 in the $TXA_2R\alpha$ was critical for receptor G-protein coupling. The Phe-138 of the TXA_2R was mutated to a slightly polar tyrosine and to a negatively charged aspartate by site-directed mutagenesis. Both F138Y and F138D mutants were expressed in similar amount to that of the wild type in HEK293 cells as shown in Western blot (Fig. 2). Either mutant also exhibited comparable Kd and Bmax values to those of the wild type as shown in Table I. This indicates that the ligand binding properties of the mutant receptors were not significantly altered except for higher Bmax with F138Y mutant. The effect of point mutation on the agonist induced Ca^{2+} release and phospholipase C activation was further examined. Figure 3 shows that I-BOP at 100 nM induced significant Ca^{2+} signal in the HEK293 cells expressing wild type TXA_2R. However, I-BOP at the same concentration induced greatly reduced Ca^{2+} signal in the HEK293 cells expressing F138Y mutant receptor. The Ca^{2+} signal was virtually lost with F138D mutant receptor. This indicates that the signal transduction system that leads to Ca^{2+} release is defective after point mutation of the Phe-138 of the TXA_2R to a more polar residue in the second intracellular loop. Presumably this is primarily due to a defective coupling of the TXA_2R to the G-protein involved. In order to further confirm the defective coupling induced by point mutation, agonist induced phospholipase C activation was examined. Figure 4 shows that I-BOP at 100 nM induced nearly 3 fold increase in inositol phosphate production in the HEK 293 cells expressing the wild type TXA_2R. However, I-BOP failed to activate phospholipase C in the HEK293 cells expressing either F138Y or F138D mutant receptor since no additional inositol phosphates was generated following agonist stimulation. These results clearly indicate that Phe-138 of TXA_2R is essential for receptor G-protein coupling.

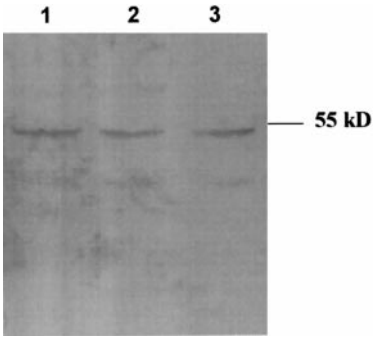

FIG. 2. Western blotting of the mutant TXA_2 receptors expressed in HEK293 cells by stable transfection. The cell membranes (50 μg) prepared from HEK293 cells expressing the wild type and the mutant receptors were respectively subjected to 10% SDS-PAGE and transferred onto PDF membrane as described under Experimental Procedures. Lane 1: wild type TXA_2R; Lane 2: TXA_2R F138D mutant; Lane 3: TXA_2R F138Y mutant.

The involvement of the second intracellular loop in receptor G-protein coupling has been recognized in several GPCRs. The m_1-muscarinic and β_2-adrenergic receptors mentioned above and another unrelated receptor, rhodopsin receptor also possess a similar motif and

TABLE I

Kd and Bmax Values of Wild Type, F138D, and F138Y Mutant TXA_2 Receptor Expressed in HEK293 Cells

TXA_2R	Kd (nM)	Bmax (pmol/mg protein)
Wild type	4.66 ± 0.81	1.10 ± 0.06
F138D	5.59 ± 1.00	1.02 ± 0.06
F138Y	4.24 ± 0.65	1.88 ± 0.09

Note. The HEK293 cell membranes (100 μg) stably transfected with the wild type and mutant TXA_2Rs were incubated with various concentrations of ^{3}H-SQ29,548. The nonspecific binding was determined in the presence of 1 μM unlabeled SQ29,548. The ^{3}H-SQ29,548 binding was assayed as described under Experimental Procedures. The Kd and Bmax values were determined by Scatchard Plot using GraphPad software.

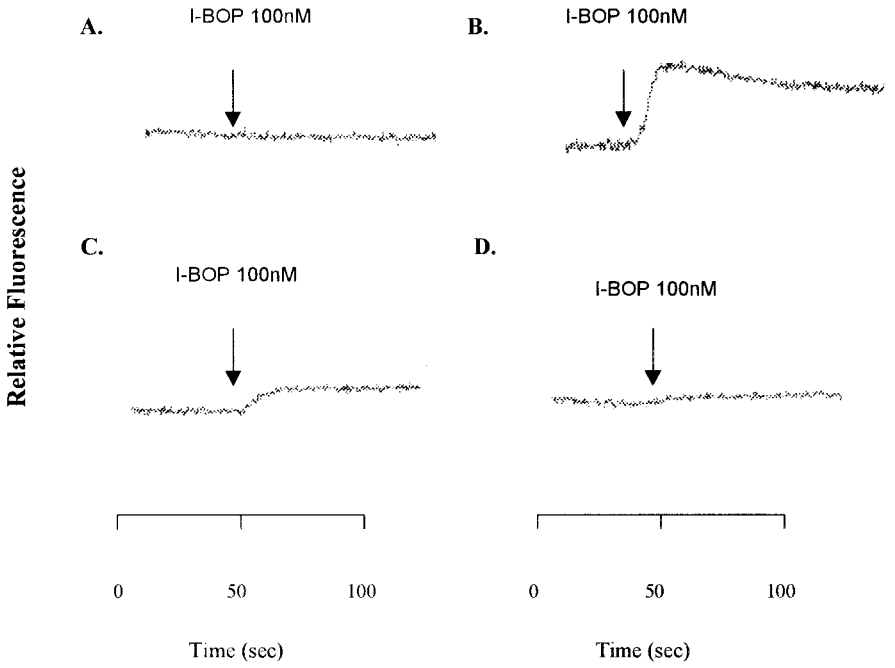

FIG. 3. I-BOP induced Ca^{2+} release in the HEK293 cells expressing wild type TXA_2R and mutant TXA_2Rs (F138Y and F138D). The intracellular Ca^{2+} signaling was examined by Fura-2/AM fluorescence in a Hitachi F-2000 Fluorescence Spectrophotometer as described under Experimental Procedures. The final concentration of I-BOP was 100 nM. (A) HEK293 cells stably transfected with pcDNA3. (B) HEK293 cells stably transfected with pcDNA3-TXA_2R cDNA. (C) HEK293 cells stably transfected with pcDNA3-TXA_2R mutant F138Y. (D) HEK293 cells stably transfected with pcDNA3-TXA_2R mutant F138D.

a hydrophobic amino acid residue at the C-terminus of this motif (12). The involvement of the second intracellular loop in G-protein coupling has been indicated. The hydrophobic site appears to be rather essential since 64 out of 70 mammalian GPCRs have a bulky lipophilic amino acid at this site (17). This site could represent a major coupling site and might interact with conserved residues of the carboxyl terminal domain of Gα subunit which also contain conserved lipophilic amino acids (18). However, we can not rule out the possibility that this hydrophobic residue is essential to the folding of the second intracellular loop before eliciting G-protein coupling. Nevertheless, identification of this key residue in the second intracellular loop of the TXA_2R provides a molecular basis of defective coupling in the signal transduction of this important eicosanoid receptor.

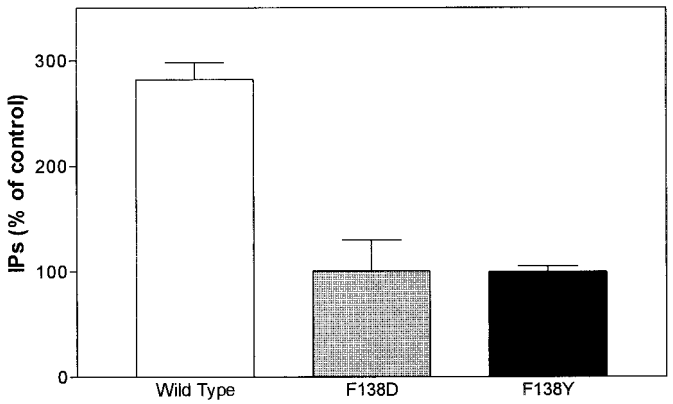

FIG. 4. I-BOP-stimulated production of IPs in HEK293 cells expressing wild type and F138 mutant TXA_2R. HEK293 cells expressing wild type, F138D mutant, and F138Y mutant receptors were respectively incubated with or without 100 nM I-BOP as described under Experimental Procedures. IPs production was expressed as a percentage increase in stimulated sample over non-stimulated sample (control). Duplicate samples were run for each mutant or wild type receptor.

Although the second intracellular of the TXA_2R appears to be essential to G-protein coupling, other intracellular domains of the TXA_2R have also been found to be involved in G-protein coupling. Hirata *et al.* (9) identified the first intracellular loop of TXA_2R being involved in G-protein coupling in their study of platelet TXA_2R in a patient having dominantly inherited bleeding disorder. This patient's platelets exhibited mutation of Arg^{60} to Leu^{60} in the TXA_2R. The mutation was found to significantly decrease agonist induced inositol phosphate production in platelets and to result in defective platelet response to TXA_2. The involvement of the third intracellular loop of the TXA_2R in the signal transduction was first described by D'Angelo *et al.* (19) who found that mutation of Cys^{223} in this loop to Ser^{223} resulted in a receptor essentially devoid of calcium signaling ac-

tivity. The C-terminal tail of the TXA_2R was also reported to contribute to G-protein coupling as the mutant receptor which lacked 22 amino acids in the C-terminus resulted in 50% less inositol phosphate production than the wild type receptor when stimulated with the U-46619 (20). Taken together, these data suggest that multiple receptor determinants are involved in the coupling of the TXA_2R to its effector systems. Nevertheless, mutation of a specific residue in any of the cytoplasmic domains that results in defective coupling as described in this study is of great significance in our understanding of the molecular basis of receptor function.

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