Single Nucleotide Polymorphism of Human Platelet-activating Factor Receptor Impairs G-protein Activation*

Received for publication, August 28, 2001
Published, JBC Papers in Press, September 17, 2001, DOI 10.1074/jbc.M108288200

Kouichi Fukunaga†, Satoshi Ishii‡, Koichiro Asano‡, Takehiko Yokomizo§, Tetsuya Shiomi‡, Takao Shimizu§, and Kazuhiro Yamaguchi‡

From the †Department of Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan, the §Department of Biochemistry and Molecular Biology, Faculty of Medicine, the University of Tokyo, Tokyo 113-0033, Japan, and ¶CREST of Japan Science and Technology Corporation, Tokyo 113-0033, Japan

Various proinflammatory and vasoactive actions of platelet-activating factor (PAF) are mediated through a specific G-protein-coupled PAF receptor (PAFR). We identified a novel DNA variant in the human PAFR gene, which substitutes an aspartic acid for an alanine residue at position 224 (A224D) in the putative third cytoplasmic loop. This mutation was observed in a Japanese population at an allele frequency of 7.8%. To delineate the functional consequences of this structural alteration, Chinese hamster ovary cells were stably transfected with constructs encoding either wild-type or A224D mutated PAFR. No significant difference was observed in the expression level of the receptor or the affinity to PAF or to an antagonist, WEB2086, between the cells transfected with wild-type and mutant PAFR. Chinese hamster ovary cells expressing A224D mutant PAFR displayed partial but significant reduction of PAF-induced intracellular signals such as calcium mobilization, inositol phosphate production, inhibition of adenyl cyclase, and chemotaxis. These findings suggest that this variant receptor produced by a naturally occurring mutation exhibits impaired coupling to G-proteins and may be a basis for interindividual variation in PAF-related physiological responses, disease predisposition or phenotypes, and drug responsiveness.

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) receptor (PAFR) with seven-transmembrane domain structure belongs to the G-protein-coupled receptor superfamily (1, 2). PAFR is linked to intracellular signal transduction pathways, including turnover of phosphatidylinositol, elevation in intracellular calcium concentration, and inhibition of adenyl cyclase. Concomitantly, PAFR couples with various phospholipases, such as phospholipase A₂, Cβ, and D, and kinases, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and tyrosine kinases, thus exerting pleiotropic effects (3).

Responsiveness to PAF in vivo demonstrates a significant intersubject variation in human subjects and mice. For example, a bronchoprovocation test of aerosolized PAF induced bronchial and vascular responses in healthy subjects, but some subjects almost completely lack these responses (4). Intravenous administration of PAF changes airway responsiveness in mice, but the magnitude of this effect is extremely different among inbred strains (5). Brzustowics et al. (6) examined PAF-evoked calcium transients in immortalized human B lymphocytes and demonstrated that there is a substantial intersubject difference, suggesting that the interindividual variation of the responses to PAF occurs at the level of PAFR or its downstream signaling. Such variation can be explained by the genetic polymorphisms in the receptor itself, its cognate G-proteins, or downstream intracellular targets. Because the PAF-PAFR system is important in the physiology of reproductive, cardiovascular, respiratory, and central nervous systems and in the pathophysiology of allergy, inflammation, shock, and thromboembolic diseases (3, 7), functional variants in PAFR may act as predisposing factors for these diseases or as modifiers of the disease phenotypes and therapeutic responses. Given the above, we screened for a polymorphism within the human PAFR gene in a Japanese population and identified a novel DNA variant that predicts the amino acid substitution in the receptor protein. The substitution occurs in the putative third cytoplasmic loop and might be expected to affect the interaction with G-proteins. We therefore analyzed the ligand binding and signal transduction properties of the mutant receptor by expressing it in mammalian cell lines.

MATERIALS AND METHODS

Polymorphism Detection—For initial examination, DNA isolated from six healthy Japanese volunteers was utilized to detect a polymorphism in the human PAFR gene. The intronless human PAFR coding region (1029 bp) was amplified by polymerase chain reaction (PCR) using appropriate primers designed according to the reported nucleotide sequence (2). PCRs were performed using Taq DNA polymerase (AmpliTaq Gold; PerkinElmer) as follows: one cycle of 95 °C for 9 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and one cycle of 72 °C for 5 min. The nucleotides of these PCR products were directly sequenced using an automated sequencer (ABI 373S, Applied Biosystems Inc., Foster City, CA).

As discussed below, a novel polymorphism was identified in one individual. This genetic variant results in the loss of a PstI restriction site. DNA samples from 116 healthy volunteers from the general Japanese population were analyzed by a restriction fragment length polymorphism method. PCR-amplified DNA using a sense primer (5'-CCACAGCGCCCGGCGCTTGACTGCA-3') and an antisense primer (5'-ATCGTGTTCAGCTTCTTCCTGGTC-3') and digested with PstI (New England Biolabs, Beverly, MA) at 37 °C for 2 h, and the fragments were resolved in a 3% agarose gel (NuSieve 3:1 agarose; FMC, Rockland, ME). The wild-type allele yielded 105-bp and 24-bp fragments, while the mutant allele remained undigested (129 bp).

* This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor and Welfare of Japan and by a grant from the Yamanouchi Foundation for Metabolic Disorders. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement." To whom correspondence should be addressed: Cardiopulmonary Division, Dept. of Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan; Tel.: 81-3-3353-1211; Fax: 81-3-3353-2502; E-mail: ko-asano@qa2.so-net.ne.jp.

The abbreviations used are: PAFR, platelet-activating factor receptor; PAF, platelet-activating factor; bp, base pair(s); PCR, polymerase chain reaction; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

This paper is available on line at http://www.jbc.org

Vol. 276, No. 46, Issue of November 16, pp. 43025–43030, 2001
Printed in U.S.A.
Constructs and Transfection—The mutant human PAFR cDNA (adenine at nucleotide 671) was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, the mutant human PAFR cDNA was generated by PCR with Pfu polymerase using an HA epitope (YPYDVPDYA)-tagged human PAFR cDNA as a template, a mutagenic oligonucleotide primer (5'-GTCGACGACGACGCGCAACGAGTAAGTACGCGCGG3'), and its complementary primer. PCR was performed as follows: one cycle of 95 °C for 30 s and 12 cycles of 95 °C for 30 s and 12 cycles of 65 °C for 1 min, and 68 °C for 13 min. The nucleotide sequence of the mutated cDNA was verified by direct sequencing. The wild-type or mutated PAFR cDNA was subcloned in the expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA).

Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium (Sigma) containing 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin in a 5% humidified CO2 atmosphere at 37 °C. For stable transfection, CHO cells were incubated with DNA constructs containing wild-type or mutated PAFR cDNA and Transfectum (BioSepra, Inc., Marlborough, MA). Cells were reseeded on 100-mm dishes 48 h after transfection. Clones resistant to Geneticin (1.0 mg/ml) (Wako, Osaka, Japan) isolated after a 10-day culture, were recloned by limiting dilution and were analyzed for the binding activity to 125I-hexadecyl-1,2-[3H]2-acetyl-sn-glyceryl-3-phosphorylcholine ([3H]PAF; 2157.1 GBq/mmol) (PerkinElmer Life Sciences). Six representative clones with relatively high binding activity (three clones expressing wild-type PAFR and three clones expressing mutant PAFR) were maintained in the culture medium containing 0.3 mg/ml Geneticin. For transient transfection, HEK293 cells, grown in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin, were coinubated with DNA constructs and LipofectAMINE PLUS (Invitrogen). The cells were harvested 72 h after transfection.

Radioligand Binding Assay—Competition binding curves were determined for the membrane fraction from CHO cells or HEK293 cells expressing either wild-type or mutant PAFR. The cells were disrupted by sonication (100 watts, 30 s, three times) in Hepes-sucrose buffer (25 mM Hepes/NaOH, pH 7.4, 0.25 M sucrose, 10 mM MgCl2) containing a protease inhibitor mixture (Complete; Roche Molecular Biochemicals), and the cell lysates were centrifuged at 9000 g for 1 h. The membrane fraction was resuspended in Hepes-sucrose buffer and stored at −20 °C until use. The radioligand binding assay was performed in total 200 μl of Hepes-sucrose-bovine serum albumin (0.1%; fatty acid-free) buffer containing the membrane fraction and [3H]PAF or [3H]WEB2086 (370 GBq/nmol) (PerkinElmer Life Sciences) with or without PAF or WEB2086 as the competitor. The reaction mixture was incubated on ice for 1 h and then was filtered through a GF/C glass fiber filter (Packard Instrument Co.), which was dried at 50 °C for 3 h. The remaining radioactivity in the filter was measured in a Top-count scintillation counter (Packard Instrument Co.). The equilibrium dissociation rate constants (Kd) and maximal concentrations of binding sites (Bmax) were determined by Scatchard plots.

Intracellular Calcium Mobilization—Cells, scraped off with 2 mM EDTA, were centrifuged (200 × g, 5 min) and resuspended in a modified Hepes-Tyrode buffer (140 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 5.6 mM d-glucose, 0.49 mM MgCl2, 0.37 mM NaH2PO4, 25 mM Hepes/NaOH, pH 7.4, 0.1% bovine serum albumin). Fura-2/AM (Dojin, Kumamoto, Japan) was added at a final concentration of 3 μM from a stock solution of 1 mM in dimethyl sulfoxide. After incubation in a 5% humidified CO2 atmosphere at 37 °C for 1 h, the cells were centrifuged (200 × g, 5 min) and were resuspended in a modified Hepes-Tyrode buffer containing 1 mM CaCl2 at 2 × 105 cells/ml. Fluorescence was measured at 37 °C using a CAF110 system (Jasco, Tokyo, Japan) with dual excitations at 340 and 380 nm and emission recording at 510 nm. The fluorescence was measured before and after the cells were exposed to PAF (0.01–100 nM) or thrombin (10 units/ml, Sigma) as a control. Each cuvette contained 0.5 ml of the cell suspension with constant stirring at 800 rpm. The intracellular calcium concentration was calculated as reported previously (8).

Inositol Phosphate Accumulation—CHO cells stably expressing PAFR were incubated with myo-[3H]inositol (Amersham Pharmacia Biotech) at 5 μCi/ml in Dulbecco's modified Eagle's medium (high glucose, without inositol) for 24 h at 37 °C in 5% CO2 atmosphere. In some experiments, cells were treated with 100 ng/ml pertussis toxin (List Biological Laboratories, Campbell, CA) for 12 h. Subsequently, cells were washed and incubated with phosphate-buffered saline (PBS) for 30 min, followed by 30 min of incubation with 20 μM LiCl in PBS. Cells were then treated with either PBS alone, 0.1–100 nM PAF, or 10 units/ml thrombin for 10 min, and inositol phosphates were extracted and analyzed by TLC and autoradiography.

**TABLE I**

| Receptor | [3H]PAF Kd (nM) | [3H]WEB2086 Kd (nM) |
|----------|----------------|-------------------|
| Wild-type PAFR | 1.60 ± 0.13 | ns |
| A224D mutant PAFR | 1.40 ± 0.10 | 44.6 ± 4.3 |

**TABLE II**

| Receptor | [3H]WEB2086 Bmax (pmol/mg) | Kd (nM) |
|----------|---------------------------|--------|
| Wild-type PAFR | 11.8 ± 1.2 | 5.4 ± 5.4 |
| A224D mutant PAFR | 12.0 ± 2.1 | 26.0 ± 6.4 |
as described by Martin (9). Following separation on AG1-X8 columns (Bio-Rad), total inositol phosphates were eluted with a solution containing 0.1 M formic acid and 1 M formate.

**Radioligand Binding Assay**—CHO cells expressing PAFR toward ligands were measured using an established protocol (10). Framed polycarbonate filters with 8-μm pores (Neuroprobe, Gaithersburg, MD) were coated with 10 μg/ml fibronectin (Wako, Tokyo, Japan) in PBS and placed in a 96-well chemotaxis chamber (Neuroprobe). The lower blind wells were filled with various concentrations of PAF, and the cells at a density of 2.0 × 10^5 cells/ml were applied to the upper wells. After incubation for 4 h at 37 °C, the filters were scraped free of cells on the upper side and were stained with Diff-Quick staining kit (International Reagents Corp., Kobe, Japan). The number of cells that migrated to the lower side of the filters was quantified by measuring optical densities at 595 nm using a spectrophotometer (model 3550; Bio-Rad). The chemotaxis index was calculated as the ratio of absorbance at 595 nm for ligands and that for medium alone.

**Statistical Analysis**—Data are provided as means ± S.E. The Hardy-Weinberg equilibrium for genotype distribution of PAFR was tested by χ^2 test. Comparisons of the results from biochemical studies were conducted by Student’s t test or two-way analysis of variance followed by post hoc Scheffe’s test. p < 0.05 was considered significant.

**RESULTS**

**Polymorphism Detection**—During an initial screening of six volunteers, we identified a novel DNA variant of the human PAFR gene in one Japanese subject. This variant, which converts a cytosine to an adenine at nucleotide 671 of the open reading frame, predicts an amino acid substitution from alanine to aspartic acid at position 224 (A224D) in the putative third cytoplasmic loop of the receptor protein (Fig. 1). Among 116 subjects from Japanese general population, 16 subjects (13.8%) were heterozygous and one subject (0.9%) was homozygous for this variant allele, and the estimated allele frequency was 7.8%. The observed genotype distribution was compatible with that predicted from the Hardy-Weinberg equilibrium.

**Radioligand Binding Assay**—To determine the biological significance of this PAFR variant A224D, we first examined whether the amino acid substitution changes the receptor affinity to an agonist (PAF) or to an antagonist (WEB2086). The K_d values of wild-type and mutant receptors to [3H]alkyl-PAF or [3H]WEB2086 were examined in mammalian cell lines stably or transiently expressing recombinant receptors (Tables I and II). Representative CHO cell clones stably expressing either wild-type or mutant PAFR demonstrated equivalent K_d values to PAF (1.60 ± 0.13 nM for wild-type receptors versus 1.40 ± 0.10 nM for mutant receptors, n = 3 for each) or to WEB2086 (4.46 ± 4.3 nM for wild-type receptors versus 4.14 ± 2.0 nM for mutant receptors, n = 3 for each) (Table I). Transiently transfected HEK293 cells also demonstrated no significant difference between wild-type and mutant PAFR in the affinity to [3H]WEB2086 (Table II). The receptor densities (B_max) in transiently transfected HEK293 cells were also equivalent between the cells expressing wild-type receptors (11.8 ± 1.2 pmol/mg of protein, n = 3) and those expressing mutant receptors (12.0 ± 2.1 pmol/mg protein, n = 3, Table II).

**Intracellular Calcium Mobilization**—Because the amino acid substitution A224D occurs in the putative third cytoplasmic

---

**FIG. 2.** Calcium mobilization in CHO cells expressing PAFR. CHO cells expressing the wild-type PAFR (○) or mutant PAFR (●) were stimulated with various concentrations of PAF (0.01–100 nM) or 10 units/ml thrombin. The data are presented as percentages of maximum response of wild-type PAFR to PAF or thrombin. CHO cells expressing mutant PAFR displayed a significant decrease in calcium mobilization to PAF but not to thrombin. Results are from three independent experiments, each performed in duplicate. Values shown are mean ± S.E. (n = 3); *, p < 0.01 compared with wild-type.

**FIG. 3.** Total inositol phosphate accumulation in CHO cells expressing PAFR. Total inositol phosphates were quantified using CHO cells expressing wild-type receptor (○) or mutant receptor (●) after the 10-min stimulation of various PAF concentrations (0–100 nM) or 10 units/ml thrombin. The data are presented as percentages of maximum response of wild-type to PAF or thrombin. CHO cells expressing mutant PAFR displayed a significant decrease in inositol phosphate accumulation by PAF but not by thrombin. Results are from three independent experiments, each performed in duplicate. Values shown are mean ± S.E. (n = 3); *, p < 0.001 compared with wild type.
loop that could be essential for the interaction with G-proteins (11), we examined the effect of this mutation on the intracellular signal transduction. The analyses were carried out in CHO cell clones stably expressing either wild-type or mutant PAFR with a nearly equivalent receptor density (2.56 ± 0.22 pmol/mg protein versus 3.41 ± 0.68 pmol/mg protein, n = 3 for each). We first examined PAF (0.01–100 nM)-induced intracellular calcium mobilization (Fig. 2). The magnitude of calcium response plateaued at the PAF concentration of 10–100 nM, and the maximal responses in the cells expressing mutant receptors were slightly but significantly decreased compared with those of wild-type receptors at 100 nM PAF (27.1 ± 3.0% decrease, n = 3, p < 0.01). In contrast, calcium response to thrombin (10 units/ml) was not different regardless of the types of PAFR expressed.

Inositol Phosphate Accumulation—We next explored the functional consequence of PAFR A224D variant on the phosphatidylinositol turnover. The accumulation of total inositol phosphates in response to PAF (0.1–100 nM) was significantly different depending on the type of PAFR (Fig. 3). The inositol phosphate production by 100 nM PAF in the cells expressing mutant receptors was reduced by 49.7 ± 3.0% compared with those expressing wild-type receptors (n = 3, p < 0.001). In our system, wild-type and mutant PAFR-mediated inositol phosphate production was partially but significantly ablated by pertussis toxin (Fig. 4A), suggesting the interaction of PAFR with pertussis toxin-insensitive (probably Gq/11) and pertussis toxin-sensitive (probably Gi/Go) G-proteins. A224D substitution reduced both the pertussis toxin-sensitive and -insensitive inositol phosphate production by 29.2 ± 3.7 and 56.1 ± 1.5%, respectively (n = 3, p < 0.001) (Fig. 4B).

cAMP Assay—Because Gq/11-associated Gβγ protein stimulates phospholipase Cβ as above and Gαi inhibits adenylyl cyclases, we examined the impact of A224D substitution on the}

---

**Fig. 4.** A. effect of pertussis toxin treatment on inositol phosphate accumulation in CHO cells expressing PAFR. CHO cells expressing either wild-type PAFR (open column) or mutant PAFR (shaded column) were preincubated with or without pertussis toxin (100 ng/ml) for 12 h prior to the measurement of inositol phosphate accumulation in response to PAF (100 nM). The data are presented as a percentage of the response of wild-type PAFR in the absence of pertussis toxin. Wild-type and mutant PAFR-mediated inositol phosphate production was significantly reduced by pertussis toxin. Results are from three independent experiments, each performed in duplicate. Values shown are mean ± S.E. (n = 3); *, p < 0.001. B. pertussis toxin-insensitive and -sensitive inositol phosphate accumulation. Pertussis toxin-insensitive accumulation corresponds to the value obtained in the presence of pertussis toxin in A. Pertussis toxin-sensitive accumulation is calculated by subtracting the value in the presence of pertussis toxin from that in the absence of pertussis toxin in A. The cells expressing mutant PAFR demonstrated a significant decrease in both pertussis toxin-insensitive and pertussis toxin-sensitive PAF-induced inositol phosphate accumulation. Values shown are mean ± S.E. (n = 3); *, p < 0.0001.

**Fig. 5.** Inhibition of forskolin-induced cyclic AMP production by PAF. CAMP production was measured after forskolin stimulation. CHO cells expressing either wild-type PAFR (open column) or mutant PAFR (shaded column) were exposed to vehicle or 100 nM PAF for 30 min at 37°C. Results are from four independent experiments, each performed in triplicate. Values shown are mean ± S.E. (n = 4); *, p < 0.05 compared with wild-type.

**Fig. 6.** PAF-induced chemotaxis of CHO cells expressing PAFR. Chemotactic activity of CHO cells expressing wild-type PAFR (○) and mutant PAFR (●) in response to various concentrations of PAF was measured as described under “Materials and Methods.” The chemotaxis index was calculated as the ratio of absorbance at 595 nm for ligands and that for vehicle. CHO cells expressing mutant PAFR displayed a significant decrease in chemotaxis to PAF. Results are from five independent experiments, each performed in triplicate. Values shown are mean ± S.E. (n = 5); *, p = 0.02 compared with wild-type.
PAFR-mediated inhibition of the forskolin-induced cAMP accumulation. 100 nM PAF inhibited the forskolin-induced cAMP accumulation by $27.6 \pm 5.2\%$ in the cells expressing wild-type PAFR and by $7.8 \pm 2.0\%$ in the cells expressing mutant PAFR $(n = 4, p = 0.01, \text{Fig. 5})$.

Chemotaxis—To determine the biological significance of PAFR A224D variant, chemotactic activities of PAFR-transfected CHO cells to PAF were compared between wild-type and variant PAFR. The chemotactic response of CHO cells demonstrated a bell-shaped dose-response curve to PAF, and the maximum chemotactic activity was observed at the PAF concentration of 5 nM in either cells expressing wild-type or mutant receptor (Fig. 6). However, the CHO cells expressing A224D variant receptor showed a 65.5 $\pm 7.9\%$ decrease in the chemotactic index compared with those expressing the wild-type receptor $(n = 5, p = 0.02)$ (Fig. 6).

**DISCUSSION**

PAFR activates multiple signaling pathways in response to its agonist PAF and exhibits numerous biological activities (3, 12, 13). We analyzed the human PAFR gene in search for the host genetic factors that modify the biological phenotypes related to PAF-PAFR system and identified a single amino acid substitution (A224D) in the third cytoplasmic loop of human PAFR. This DNA variant was relatively common in the Japanese population with an allele frequency of 7.8%. The *in vitro* analysis of this variant demonstrated significant impairment of the receptor functions in terms of intracellular calcium mobilization, phosphatidylinositol hydrolysis, and inhibition of adenylyl cyclase. Furthermore, the cells expressing the variant receptor showed decreased chemotactic activity to PAF compared with the cells expressing the wild-type receptor.

The third cytoplasmic loop has been considered essential for receptor-G-protein coupling in various seven-transmembrane receptors (14, 15). Previous papers demonstrated that the PAFR also confers its biological activities through the interaction with multiple G-proteins including G$_s$ and G$_q$ protein chains and that the third cytoplasmic loop is important for the interaction (11, 16–18). Carlson *et al.* (19) demonstrated that the expression of the minigene homologous to the third cytoplasmic domain, but not to first or second domain, of PAFR inhibited PAF-stimulated inositol phosphate production, suggesting that the third cytoplasmic loop is essential for the coupling of PAFR with G$_{s/ q}$ protein. Using a mutagenesis approach, two separate portions of the third cytoplasmic loop of PAFR have been identified as the region possibly involved in G-protein interaction and activation/inactivation of the receptor (20). Carlson *et al.* (19) proposed the presence of an amphipathic $\alpha$-helix encompassing the amino acids 209–220 in the third cytoplasmic domain of PAFR and proposed that the disruption of either the polar or hydrophobic faces of this putative amphipathic $\alpha$-helix completely diminished PAF-induced inositol phosphate accumulation. Another candidate region for the coupling of PAF with G-proteins is the C-terminal portion of the third cytoplasmic loop. Studies of the $\alpha_{1}$-, $\alpha_{2}$-, and $\beta_{2}$-adrenergic receptors have demonstrated that the substitution of a specific amino acid residue in this region resulted in the constitutively active receptors (21–23). Human PAFR with an artificial mutation at position 231 from leucine to arginine in the C terminus of the third cytoplasmic loop is also constitutively active with the higher affinity to PAF (20). In contrast, substitution of alanine at position 230 to glutamic acid of human PAFR caused unresponsiveness to PAF assessed by phosphatidylinositol hydrolysis and a marked decrease in the receptor affinity to the agonist (20). Since the naturally occurring A224D variant identified in the Japanese population is located between the putative amphipathic $\alpha$-helix and the C-terminal portion of the third cytoplasmic loop, this residue is unlikely to be directly associated with G-protein coupling. The alanine 224 is conserved among human, mouse, and guinea pig PAFR and is replaced with noncharged proline in rat PAFR. Thus, the introduction of negatively charged aspartic acid in A224D variant may modify the conformation of the N-terminal amphipathic $\alpha$-helix or the C-terminal portion or both, resulting in exerting an indirect effect on the PAFR coupling to G-proteins. Furthermore, our data showed that this mutation attenuated both PAFR couplings to pertussis toxin-sensitive (probably G$_{i/o}$) and -insensitive (probably G$_{q/o}$) G-proteins. Calcium transient, inositol phosphate accumulation, and cAMP production by the mutant PAFR suggest the reduction of PAF-evoked $\alpha$ subunit activity. The impaired chemotactic activity of mutant PAFR, however, may be due to an inefficient PAF-triggered dissociation of the $\beta\gamma$-subunit complex from the $\alpha$ subunit, because release of the $\beta\gamma$-subunit from the heterotrimeric G-proteins is reported to be absolutely required for the G-protein-coupling receptor-mediated chemotaxis (24).

The biological significance of the PAF-PAFR system has been well established using genetically engineered mice. Overexpression of PAFR in mice resulted in bronchial hyperreactivity to methacholine and increased endotoxin lethality (25). Mice carrying a disrupted PAFR gene showed attenuated anaphylactic responses to allergen exposure and pulmonary damage induced by acid (26, 27). The diversity in the PAF-PAFR system in the human population may be correlated with interindividual variability in physiological responses, susceptibility and clinical phenotypes of PAF-related diseases, and responses to drugs. Interestingly, genetic deficiency of plasma PAF acetylhydrolase, a PAF-degrading enzyme, has been identified in the Japanese population (28). Substitution of valine 279 with phenylalanine (V279F) of plasma PAF acetylhydrolase impairs the extracellular secretion of this protein and also completely abolishes the enzyme activity (29). This polymorphism, which may strengthen the PAF-PAFR signals, is associated with coronary artery disease (30), stroke (31), renal diseases (32), and possibly asthma (33, 34). In contrast, the impaired PAFR function caused by A224D variation may be protective to these PAF-related diseases.

In summary, we delineated the signaling phenotypes of a polymorphism of PAFR with a substitution of an aspartic acid for an alanine residue in the third intracellular loop. This polymorphism had a significant impact on agonist-promoted signaling to calcium mobilization, inositol phosphate accumulation, and inhibition of adenylyl cyclase and also on cell physiology such as chemotaxis. This phenotype may be considered a basis for interindividual variation in physiological response, disease predisposition or modification, and drug responsiveness. To our knowledge, this is the first naturally occurring mutation in the human PAFR gene to affect G-protein-mediated signaling pathways.

**Acknowledgments**—We thank M. Ito (The University of Tokyo) for technical assistance, Dr. I. Ishii (University of California, San Diego) for instruction in determining inositol phosphate levels, and other laboratory members (The University of Tokyo and Keio University School of Medicine) for valuable discussions.

**REFERENCES**

1. Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T., and Shimizu, T. (1991) *Nature* **349**, 342–346
2. Nakamura, M., Honda, Z., Isumi, T., Sakanaka, C., Mutoh, H., Minami, M., Bito, H., Seyama, Y., Matsumoto, T., Noma, M., and Shimizu, T. (1991) *J. Biol. Chem.* **266**, 20400–20405
3. Ishii, S., and Shimizu, T. (2000) *Prog. Lipid Res.* **39**, 41–82
4. Rodriguez-Boulan, R., Pérez, M. A., Chung, K. F., Barbera, J. A., Wagner, P. D., Cobos, A., Barnes, P. J., and Roca, J. (1994) *J. Clin. Invest.* **93**, 188–194
5. Longphre, M., Zhang, L. Y., Paquette, N., and Kleeberger, S. R. (1996) *Am. J. Respir. Cell Mol. Biol.* **14**, 461–469
6. Brzustowicz, L. M., Gardner, J. P., Hopp, L., Jeanclolos, E., Ott, J., Yang, X. Y., Fekete, Z., and Avic, A. (1997) Hypertension 29, 158–164
7. Prescott, S. M., Zimmerman, G. A., Stafforini, D. M., and McIntyre, T. M. (2000) Annu. Rev. Biochem. 69, 419–445
8. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
9. Martin, T. F. (1983) J. Biol. Chem. 258, 14816–14822
10. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., and Shimizu, T. (1997) Nature 387, 620–624
11. Honda, Z., Takano, T., Gotoh, Y., Nishida, E., Ito, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 2307–2315
12. Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1990) J. Biol. Chem. 265, 17381–17384
13. Hanahan, D. J. (1986) Annu. Rev. Biochem. 55, 483–509
14. Strader, C. D., Dixon, R. A., Cheung, A. H., Candelore, M. R., Blake, A. D., and Sigal, I. S. (1987) J. Biol. Chem. 262, 16439–16443
15. Blin, N., Yun, J., and Wess, J. (1995) J. Biol. Chem. 270, 17741–17748
16. van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Koch, W. J., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 1266–1269
17. Ali, H., Richardson, R. M., Tomhave, E. D., DuBose, R. A., Haribabu, B., and Snyderman, R. (1994) J. Biol. Chem. 269, 24557–24563
18. Shi, L. C., Wang, H. Y., Horwitz, J., and Friedman, E. (1996) J. Neurochem. 67, 1478–1484
19. Carlson, S. A., Chatterjee, T. K., Murphy, K. P., and Fisher, R. A. (1998) Mol. Pharmacol. 53, 451–458
20. Parent, J. L., Le Gouill, C., de Brum-Fernandes, A. J., Rola-Pleszczynski, M., and Stanekova, J. (1996) J. Biol. Chem. 271, 7949–7953
21. Small, K. M., Forbes, S. L., Brown, K. M., and Liggett, S. B. (2000) J. Biol. Chem. 275, 38518–38523
22. Small, K. M., Forbes, S. L., Rahman, F. F., Bridges, K. M., and Liggett, S. B. (2000) J. Biol. Chem. 275, 23059–23064
23. Jin, T., Zhang, N., Long, Y., Parent, C. A., and Devreotes, P. N. (2000) Science 287, 1034–1036
24. Ishii, S., Nagase, T., Tashiro, F., Ikuta, K., Sato, S., Waga, I., Kume, K., Miyazaki, J., and Shimizu, T. (1997) EMBO J. 16, 133–142
25. Nagase, T., Ishii, S., Kume, K., Otsuki, N., Izumi, T., Ouchi, Y., and Shimizu, T. (1999) J. Clin. Invest. 104, 1071–1076
26. Ishii, S., Kuwaki, T., Nagase, T., Maki, K., Tashiro, F., Sunaga, S., Cao, W. H., Kume, K., Fukuchi, Y., Ikuta, K., Miyazaki, J., Kuma, M., and Shimizu, T. (1998) J. Exp. Med. 187, 1779–1788
27. Miwa, M., Miyake, T., Yamanaka, T., Sugatani, J., Suzuki, Y., Sakata, S., Araki, Y., and Matsumoto, M. (1988) J. Clin. Invest. 82, 1983–1991
28. Stafforini, D. M., Satoh, K., Atkinson, D. L., Tjoelker, L. W., Eberhardt, C., Yoshida, H., Imazumi, T., Takamatsu, S., Zimmerman, G. A., McIntyre, T. M., Gray, P. W., and Prescott, S. M. (1996) J. Clin. Invest. 97, 2784–2791
29. Yamada, Y., Ichihara, S., Fujimura, T., and Yokota, M. (1998) Metabolism 47, 177–181
30. Hiramoto, M., Yoshida, H., Imazumi, T., Yoshimizu, N., and Satoh, K. (1997) Stroke 28, 2417–2420
31. Tanaka, R., Iijima, K., Xu, H., Inoue, Y., Murakami, R., Shirakawa, T., Nishiyama, K., Miwa, M., Shiozawa, S., Nakamura, H., and Yoshikawa, N. (1999) Am. J. Kidney Dis. 34, 289–295
32. Stafforini, D. M., Numao, T., Tsodikov, A., Vaitkus, D., Fukuda, T., Watanabe, N., Fueki, N., McIntyre, T. M., Zimmerman, G. A., Makino, S., and Prescott, S. M. (1999) J. Clin. Invest. 103, 989–997
33. Satoh, N., Asano, K., Nakai, K., Fukunaga, K., Iwata, M., Kanazawa, M., and Yamaguchi, K. (1999) Am. J. Respir.Crit. Care Med. 159, 974–979