Alanine Exchanges of Polar Amino Acids in the Transmembrane Domains of a Platelet-activating Factor Receptor Generate Both Constitutively Active and Inactive Mutants*

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To determine ligand-binding sites of a platelet-activating factor (PAF) receptor, alanine-scanning mutagenesis was carried out. All 23 polar amino acids in the putative 7-transmembrane (TM) domains of a guinea pig PAF receptor were individually replaced with alanine. The ligand-binding properties of mutant receptors were determined after transient expression in COS-7 cells. Mutants in TM II (N58A, D63A), TM III (N100A, T101A, S104A) and TM VII (D289A) displayed higher PAF-binding affinities than seen with the wild-type receptor. In contrast, mutants in TM V (H188A), TM VI (H248A, H249A, Q252A), and TM VII (Q276A, T278A) showed lower affinities. Representative mutants were then stably expressed in Chinese hamster ovary cells to observe PAF-induced cellular signals (arachidonate release, phosphatidylinositol hydrolysis, adenyl cyclase inhibition). An N100A mutant with the highest affinity was constitutively active and was responsive to lysophosphatidic acid (LPA), an inactive derivative of PAF. One nanomolar PAF induced no signals in low affinity mutants, an EC50 value for the wild-type receptor. Three histidines (His-188, His-248, His-249) might form a binding pocket for the phosphate group of PAF, since zinc effectively inhibited ligand-binding. Based on these results, a three-dimensional molecular model of PAF and its receptor was generated using bacteriorhodopsin as a reference protein.

Platelet-activating factor (PAF),1 is a potent phospholipid mediator with diverse physiological actions on a wide variety of cells and tissues. PAF is thought to play important roles in allergic disorders, inflammation, shock, and some diseases and also to have effects on the reproductive, cardiovascular, and central nervous systems (1–4). Despite the highly hydrophobic structure with a glycerolphospholipid skeleton, PAF binds to a cell surface receptor, which was first cloned from a guinea pig lung cDNA library (5). The PAF receptor, with a seven-transmembrane (TM) topology like that of rhodopsin, belongs to a G protein-coupled receptor (GPCR) superfamily. PAF receptor homologs in four mammalian species (guinea pig, human, rat, and mouse) have been reported (6–12). The PAF receptors couple with various second messenger systems including activation of phospholipase A2, C, and D; activation of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and tyrosine kinases; and inhibition of adenyl cyclase, thus exerting pleiotropic effects (13–15).

To date, a large body of information regarding ligand (agonist and antagonist)-binding characteristics of GPCRs represented by β-adrenergic receptors has been obtained (16), and this greatly contributes to effective clinical applications. Various PAF receptor antagonists have been developed as antiallergic and antiinflammatory drugs, but little is known of ligand-binding sites in the cloned PAF receptors. Most GPCR agonists are considered to bind to a hydrophobic core surrounded by seven-TM α-helices with electrostatic and hydrophobic force. We designed experiments using alanine-scanning mutagenesis to replace individually all 23 polar amino acids in the putative TM domains of a guinea pig PAF receptor, the objective being to define positions involved in the recognition of PAF.

Such replacements generated both constitutively active and inactive mutant receptors with altered affinities and altered cellular responses to their ligands. These results strongly support the two-state model of GPCR activation, which extends the ternary complex model based on recent cumulative pharmacological evidence (17, 18). We describe here a systematic approach to determine PAF-PAF receptor interactions, an approach that will aid in screening inverse agonists (19). We also generated a three-dimensional molecular model of the guinea pig PAF receptor TM regions, based on our mutagenesis studies and using bacteriorhodopsin as a reference protein. Thus, a pertinent explanation was obtained for reports that zinc ions preferentially inhibit specific PAF binding to its receptor in platelets (20, 21).

buffer, 20% fetal bovine serum in Hepes-Tyrode’s buffer; CHO, Chinese hamster ovary.

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1 The abbreviations used are: PAF, platelet-activating factor; TM, transmembrane; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; IPs, insoluble phosphates; Hepes-Tyrode’s buffer, 140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 0.49 mM MgCl2, 12 mM NaHCO3, 5.6 mM d-glucose, 0.37 mM NaH2PO4, 1 mM Hepes, pH 7.4; Hepes-Tyrode’s-BSA buffer, 20% fetal bovine serum in Hepes-Tyrode’s buffer; CHO, Chinese hamster ovary.
EXPERIMENTAL PROCEDURES

Materials—[3H]WEB 2086, acetyl-<sup>3H</sup>IPAF C16, [3H]arachidonic acid, myo-[1,2-<sup>3H</sup>]inositol, and 125I-labeled sheep anti-mouse IgG F(ab')<sub>2</sub> fragment were purchased from DuPont NEN, 1-O-Hexadecyl-2-O-acetyl-sn-glycero-3-phosphocholine (PAF C16), 1-O-octadecyl-2-O-acetyl-sn-glycero-3-phosphocholine (PAF C18), 1-O-hexadecanoyl-sn-glycero-3-phosphocholine (lyso-PAF C16) were obtained from Cascade Biotech. 1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphocholine (lyso-PAF C18) were kindly donated from Takeda Chemical Industries (Osaka, Japan). Geneticin and inositol-free Dulbecco's modified Eagle's medium were purchased from Life Technologies, Inc. Anti-FLAG M2 (Osaka, Japan). Geneticin and inositol-free Dulbecco's modified Eagle's medium were purchased from Life Technologies, Inc. Anti-FLAG M2 monoclonal antibody was from Kodak. All other reagents were of analytical grade and were from Wako Chemicals (Osaka) or Sigma.

Construction of Mutant Receptor cDNAs—Twenty-three point mutations were introduced into pbLueScript SK(−) (Stratagene) containing the entire coding region of a guinea pig PAF receptor cDNA (5). The Transformam Site-Directed Mutagenesis Kit (Clontech) was used to exchange polar amino acids with alanine. For construction of epitope-tagged receptors, an 8-aminoo acid peptide (FLAG) sequence (DYKDDDDK) was inserted between the N-terminal initiator methionine and the second amino acid of the wild type and mutant receptors by polymerase chain reaction, as described (22). The coding regions of all constructs were sequenced on both strands using an ABI 373 DNA Sequencer (Perkin Elmer) and were subcloned into the mammalian expression vectors pcDNA I and pcDNA I Neo (Invitrogen) for transient expression in COS-7 cells and stable expression in CHO cells, respectively.

Expression of Mutant Receptors in COS-7 and CHO Cells—COS-7 and CHO cells were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium, respectively. COS-7 cells were plated in 12-well dishes, transfected with constructs encoding the wild type and mutants using TRANSFECTAM (BioSERA, Inc., Marlborough, MA) according to the manufacturer’s protocol, and used for assay after 72 h. For construction of stable transfomers, CHO cells were transfected in the same manner and after 48 h were resedeed into 100-mm dishes. Clones resistant to Geneticin (1 mg/ml) were isolated were transfected in the same manner and after 48 h were reseeded into 12-well dishes. Clones resistant to Geneticin (1 mg/ml) were isolated were transfected in the same manner and after 48 h were reseeded into 12-well dishes. Clones resistant to Geneticin (1 mg/ml) were isolated.

Radioligand Binding Assay—CHO cells were seeded into 12-well dishes 24 h prior to assay. Transfected COS-7 cells (described above) and Chinese hamster ovary (CHO) cells were washed twice with Hepes-Tyrode’s buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.49 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.6 mM NaHCO<sub>3</sub>, 0.37 mM MgCl<sub>2</sub>), and incubated for 1 h with 20% fetal bovine serum in PBS(−). The cells were then incubated for 20 min in Hepes-Tyrode’s-BSA buffer containing 1% BSA and 100 mM HEPES, pH 7.4 containing 0.1% (v/v) of fatty acid-free bovine serum albumin (Hepes-Tyrode’s-BSA buffer) and incubated for 1 h at 25 °C with [3H]WEB 2086 or [3H]IPAF C16 in the presence (for the nonspecific binding) or absence (for the total binding) of 10 μM unlabeled WEB 2086 in the same buffer. The concentrations for radioligands varied with the experiments. The cells were then washed twice with the buffer and solubilized with 1% Triton X-100. The radioactivity associated with the cells was measured by liquid scintillation counting. In experiments to determine the effects of divalent cations on specific [3H]PAF binding, CaCl<sub>2</sub> and MgCl<sub>2</sub> were excluded from the buffer.

Quantitation of Expression Levels of FLAG-tagged PAF Receptors in COS-7 Cells—Imunochemical detection of the cell surface-expressed FLAG-tagged PAF receptors was performed by Western blotting and densitometry. Transfected COS-7 cells in 12-well dishes were washed twice with Ca<sup>2+</sup>-<sup>3H</sup>-free phosphate-buffered saline (PBS(−)), fixed for 10 min in 2% freshly prepared paraformaldehyde in PBS(−), washed twice with PBS(−), and incubated for 1 h with 20% fetal bovine serum in the Hepes-Tyrode’s buffer (FBS–Hepes-Tyrode’s buffer) to reduce the nonspecific binding. The cells were incubated for 20 min with/without 10 μg/ml of anti-FLAG antibody in FBS–Hepes-Tyrode’s buffer and washed three times with PBS(−), incubated for 30 min with 1 μl/cell 125I-labeled sheep anti-mouse IgG F(ab')<sub>2</sub> fragment in FBS–Hepes-Tyrode’s buffer, washed three times with PBS(−), solubilized with 0.1 N NaOH, and subjected to gamma counting. The anti-FLAG antibody-dependent binding was used to show the expression levels of the receptors.

Arachidonate Release Assay—CHO cells in 12-well dishes were pre-labeled with [3H]arachidonic acid (0.1 μCi/well) for 24 h in serum-free medium. The cells were washed twice with Hepes-Tyrode’s-BSA buffer and stimulated with ligands in the same buffer. After a 6-min incubation, the radioactivity released into the buffer, and that incorporated into the cells were measured as described. The activity was expressed as percentages of the released radioactivity against the incorporated one.

Phosphoinositol Hydrolysis Assay—Two assay methods were used. To detect the short term (15-s) response, an IP<sub>3</sub> receptor assay kit (DuPont NEN) was used. Briefly, CHO cells in 100-mm dishes were collected with PBS(−) containing 1 mM EDTA, and after centrifugation, the cells were suspended on ice in Hepes-Tyrode’s-BSA buffer at a density of 4 × 10<sup>6</sup> cells/ml. An aliquot of 800 μl of cell suspension was incubated for 15 s with 1 mM forskolin in the presence of ligands for 20 min. The reaction was halted, and the extraction was done using 200 μl of ice-cold 100% (v/v) trichloroacetic acid. IP<sub>3</sub> in the extract was determined according to the manufacturer’s instructions. To examine the long term (10-min) response, we used a myo-[3H]inositol labeling method. CHO cells in 12-well dishes were pre-labeled with myo-[3H]inositol (0.2 μCi/well) for 24 h in inositol-free Dulbecco’s modified Eagle’s medium. The cells were then incubated for 20 min in Hepes-Tyrode’s-BSA buffer containing 10 mM LiCl and stimulated by ligands. After the indicated time, the reaction was terminated by aspirating the supernatant and adding 500 μl of ice-cold 0.4 M HClO<sub>4</sub>. After standing on ice for 20 min, the 400-μl supernatant was neutralized with 200 μl of 0.72% KOH/0.6 M KHCO<sub>3</sub>. The precipitated material was removed by centrifugation, and the supernatant was assayed to an AG-1X8 (200-400 mesh from Dowex) column. Inositol phosphates (IPs) were eluted with the stepwise gradients of ammonium formate, as described (24).

cAMP Determination—CHO cells were seeded in 24-well dishes 24 h before experiments. After the medium was aspirated, the cells were incubated in Hepes-Tyrode’s-BSA buffer containing 0.5 mM 3-isobutyl-1-methylxanthine for 20 min at 37 °C. Next, the cells were stimulated with 1 μM forskolin in the presence of ligands for 20 min. The reaction was terminated by aspiration of the supernatant and addition of 200 μl of 0.1 N HCl. After a 20-min incubation, the supernatant was collected. cAMPs in the supernatant were determined using a radioimmunoassay kit from Yamasa (Chiba, Japan).

Molecular Modeling of the Guinea Pig PAF Receptor Transmembrane Regions—Three-dimensional structures of bacteriorhodopsin and its ligand retinal (25) were obtained from Brookhaven Protein Data Bank (26) (PDB ID-code: 1BRD). The modeling was performed with the chimera molecule system running on a NEC EWS 4800/330 work station, and a restrained and full conjugate gradient geometry optimization was carried out using a KOPT program (27). The united atom AMBER force field parameter (28) was used with the cutoff for nonbonded interactions of 10 Å and the distance-dependent dielectric constant. The conjugate gradient was optimized with the termination threshold of maximum component of energy gradient of 0.1 kcal/mol Å.<sup>2</sup>

RESULTS

Search for Polar Amino Acids in the Transmembrane Domains Involved in PAF Binding—To explore the role of electrostatic interactions between PAF and its receptor, alanine substitution was individually introduced into a guinea pig PAF receptor (Fig. 1). Wild-type and mutant receptors were transiently expressed in COS-7 cells, and ligand-binding properties were investigated (Table I). The wild-type receptor displayed a dose-dependent binding of agonist [3H]PAF C16 and antagonist [3H]WEB 2086 (data not shown). There was no detectable specific binding of both ligands in untransfected or vector (pcDNA1)-transfected cells (data not shown). To determine whether these mutations alter PAF binding affinity, a concentration of 3 nM near the K<sub>d</sub> value (3.6 nM in the wild-type receptor) was chosen for [3H]PAF binding. On the other hand, a 20 nM concentration near 7-fold of the K<sub>d</sub> value (2.9 nM in the wild type) was selected for [3H]WEB 2086 binding, since this was thought to reflect the expression level of the receptor; differences in K<sub>d</sub> values with these working conditions (1.25-200 nM) were not of interest. In the wild-type PAF receptor, the relative specific binding was expressed as percentages of the specific binding, with that of the wild type as 100. The ratio between PAF and WEB 2086 binding was also calculated to represent the relative PAF binding affinity, compared with that observed with the wild-type receptor. The binding ratios in Table I indicate that some mutations resulted in an apparent gain or loss of PAF binding, as char-
characterized by their localization among seven TM domains. PAF preferentially bound to some mutants such as N58A, D63A, T69A, N100A, T101A, S104A, and D289A (with ratios over 1.40) in which substituted residues mainly cluster in TM II and III. In contrast, PAF scarcely bound to other mutants such as H188A, H248A, H249A, Q252A, Q276A, T278A, and S283A (with ratios under 0.30) among TM V, VI, and VII domain mutants.

To accurately estimate the expression levels, N-terminal FLAG-tagged PAF receptors were constructed and analyzed. It was previously demonstrated that the epitope-tagged PAF receptor functions comparably to the nontagged receptor in COS-7 cells (8, 23). The binding parameters were calculated as described above, and the receptor expression levels were quantitated using an anti-FLAG monoclonal antibody (data not shown). The specific PAF binding per FLAG expression revealed that FLAG-tagged mutants showed binding characteristics similar to those of the corresponding nontagged mutants; those of N58A, D63A, N100A, T101A, S104A, and D289A (with ratios over 1.40) in which substituted residues mainly cluster in TM II and III. In contrast, PAF scarcely bound to other mutants such as H188A, H248A, H249A, Q252A, Q276A, and T278A (with ratios under 0.30) among TM V, VI, and VII domain mutants.

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Characterization of Ligand-binding Properties of Mutant Receptors in CHO Transformants—To further characterize mutant receptors, we constructed and cloned CHO transformants stably expressing the nontagged wild type and representative mutant receptors: N100A, T101A, and S104A in high affinity mutants; and H188A, H248A, and Q276A in low affinity mutants. In all transformants, the saturation experiments with [3H]PAF C16 and [3H]WEB 2086 revealed the existence of a single class of binding site noted in the Scatchard plot analysis (data not shown). Each mutation of Asn-100, Thr-101, or Ser-104 caused a 3-fold increase in the PAF binding affinity, and that of His-188, His-248, or Gln-276 resulted in an apparent loss of the PAF binding (Table II). Next, displacements of specific [3H]WEB 2086 binding with different concentrations of various PAF receptor ligands were compared between mutant receptors (Fig. 2). Two natural agonists (PAF C16 and PAF C18) displaced [3H]WEB 2086 at lower concentrations in high affinity mutants and at higher concentrations in low affinity mutants than at those in the wild-type receptor (Fig. 2, A and B). Methylcarbamyl-PAF C16, a synthetic analog of PAF resistant to PAF acetylhydrolases, displaced in a similar manner as seen with PAF C16 and PAF C18 (Fig. 2C), indicating that the altered binding profiles among mutants were not caused by the enzymatic activities. Lyso-PAF C16, an inactive derivative of PAF, displaced only in the two highest affinity mutants, N100A and S104A (Fig. 2D). In contrast, binding affinities of two structurally distinct receptor antagonists, WEB 2086 and CV-6209, were almost indistinguishable between the wild type and mutants (Fig. 2, E and F). Similar results were obtained using other PAF receptor antagonists such as BN-50730, SM-12502, CV-3988, and TCV-309 (data not shown).

Signal Transduction via Mutant Receptors in CHO Transfor-
High and Low Affinity Mutants of the PAF Receptor

**TABLE I**

Ligand-binding properties observed in COS-7 cells transiently expressing mutant PAF receptors

| Receptor        | Relative specific binding | WEB 2086 binding | Binding ratio |
|-----------------|---------------------------|------------------|---------------|
|                 | % of WT                    | PAF binding      | WEB 2086 binding |
| WT              | 100.0 ± 4.9               | 100.0 ± 10.6     | 1.00          |
| 1st             | S23A                       | 163.6 ± 6.2      | 164.4 ± 3.0   | 1.00          |
|                 | N33A                       | 39.3 ± 11.4      | 46.6 ± 2.9    | 0.84          |
| 2nd             | N58A                       | 28.8 ± 2.4       | 18.0 ± 13.3   | 1.60          |
|                 | T60A                       | 172.7 ± 11.9     | 232.6 ± 13.3  | 0.74          |
|                 | D63A                       | 94.9 ± 5.1       | 48.3 ± 2.9    | 1.96          |
|                 | T68A                       | 117.7 ± 7.6      | 28.5 ± 1.5    | 4.14          |
| 3rd             | N100A                      | 307.3 ± 14.1     | 168.7 ± 11.2  | 1.92          |
|                 | T101A                      | 240.0 ± 5.8      | 168.8 ± 7.4   | 1.42          |
|                 | S104A                      | 235.5 ± 13.2     | 113.8 ± 6.2   | 2.07          |
|                 | T112A                      | 292.8 ± 5.2      | 265.1 ± 11.2  | 1.10          |
| 4th             | S138A                      | 211.9 ± 11.7     | 189.4 ± 7.2   | 1.12          |
|                 | S190A                      | 249.6 ± 13.7     | 230.3 ± 11.5  | 1.08          |
| 5th             | H188A                      | 1.3 ± 2.1        | 150.3 ± 9.0   | 0.01          |
| 6th             | T236A                      | 197.8 ± 12.4     | 313.8 ± 22.0  | 0.63          |
|                 | H248A                      | -4.9 ± 1.6       | 101.0 ± 2.7   | <0            |
|                 | H249A                      | 12.4 ± 3.4       | 48.7 ± 2.0    | 0.26          |
|                 | Q252A                      | 61.4 ± 5.4       | 215.7 ± 8.3   | 0.29          |
| 7th             | Q276A                      | 1.8 ± 3.7        | 142.5 ± 4.0   | 0.01          |
|                 | T278A                      | 14.2 ± 3.7       | 258.5 ± 10.6  | 0.06          |
|                 | S283A                      | 63.6 ± 2.7       | 224.2 ± 10.6  | 0.28          |
|                 | T284A                      | 286.2 ± 17.6     | 335.0 ± 11.0  | 0.85          |
|                 | N285A                      | 273.5 ± 14.7     | 244.2 ± 10.2  | 1.12          |
|                 | D289A                      | 268.8 ± 13.3     | 189.6 ± 7.0   | 1.42          |

**TABLE II**

Ligand-binding parameters in CHO cells expressing mutant PAF receptors

| Receptor        | Kd (nM) | Bmax (fmol/well) | Kd (nM) | Bmax (fmol/well) |
|-----------------|---------|------------------|---------|------------------|
|                 |         |                  |         |                  |
| (No transfection)| ND      |                  | ND      |                  |
| WT              | 2.52 ± 0.40 | 929 ± 165       | 2.92 ± 0.28 | 1362 ± 202     |
| N100A           | 0.83 ± 0.12 | 619 ± 141       | 8.08 ± 0.75 | 712 ± 146     |
| T101A           | 0.90 ± 0.06 | 569 ± 69        | 8.85 ± 0.85 | 688 ± 83      |
| S104A           | 0.74 ± 0.12 | 491 ± 84        | 5.96 ± 0.30 | 466 ± 78      |
| H188A           | ND       |                  | 4.21 ± 0.66 | 184 ± 39      |
| H248A           | ND       |                  | 6.22 ± 0.83 | 1372 ± 288    |
| Q276A           | ND       |                  | 9.12 ± 0.32 | 541 ± 173     |

a ND, not detectable.

To elucidate functional properties of mutant receptors, signal transduction in CHO transformants was investigated (Fig. 3). First, PAF-induced arachidonate release was measured; it was time dependent within 15 min (data not shown) and dose dependent (Fig. 3A). The dose-dependent responses during a 6-min incubation in high affinity mutants displayed patterns similar to that observed in the wild type, even though the release was more prominent at higher concentrations. In contrast, the dose-dependent curves in low affinity mutants were shifted right by 1 order of magnitude. Finally, PAF-dependent inhibition of forskolin-induced cAMP accumulation was determined (Fig. 3C). Maximum effects of PAF differed between transformants reflecting different expression levels of the receptors as shown in Table II, and the activities were expressed as percentage responses. The dose-dependent profiles in these mutants were basically the same seen for the arachidonate release or the IP₃ production. Parental CHO cells showed no response, regardless of the signals tested (data not shown). Lyso-PAF induced arachidonate release in a dose-dependent manner only in the N100A and S104A transformants (Fig. 3D). Lyso-PAF also elicited IP₃ production and inhibited forskolin-activated adenyl cyclase in these transformants (data not shown).

The basal arachidonate release in the N100A transformant was twice as large as those of other transformants (Fig. 3, A...
and D). To determine whether the N100A mutant would be constitutively active, the effects of antagonist WEB 2086 on basal activities in arachidonate release, inositol phosphate (IPs) production, and adenylyl cyclase inhibition were examined (Fig. 4). Micromolar concentrations of WEB 2086 greatly reduced the basal arachidonate release in the N100A transformant, but not in the wild type (Fig. 4A). Because the basal amounts of IP₃ were too minute to assess quantitatively, myo-[³H]inositol labeling was done to detect any constitutive activation in phosphatidylinositol hydrolysis. The radioactivity of IPs (IP₁ + IP₂ + IP₃) in the N100A transformant was five times as large as that in the wild-type transformant. This phenomenon was also observed in COS-7 cells transfected with the N100A mutant but not with the wild-type receptor (data not shown). Further, IPs production decreased slightly but significantly after a 10-min exposure to WEB 2086 in the N100A transformant (Fig. 4B). WEB 2086 augmented forskolin-induced cAMP accumulation in the N100A but not in the wild-type transformant, indicating that adenylyl cyclase was inhibited in the steady state in the N100A transformant, and this inhibition was released by WEB 2086 (Fig. 4C). Thus, the N100A mutant proved to be a constitutively active receptor.

Possible Involvement of the Transmembrane Histidines in Zinc Binding to the PAF Receptor—Some reports indicated that zinc ions preferentially inhibit PAF binding and PAF-evoked cellular responses in rabbit and human platelets (20, 21). We attempted to elucidate involvement in zinc binding of two histidines (His-188 and His-248) in TM V and VI domains, highly characteristic to the PAF receptors. First, the effects of various naturally occurring divalent cations on specific [³H]PAF binding to the wild-type receptor were examined in CHO cells (Fig. 5A). Zinc as well as copper and cadmium effectively inhibited specific PAF binding at mM concentrations. Calcium but not magnesium or iron showed a slight inhibition. Because specific [³H]WEB 2086 binding was also inhibited by zinc, the effects of zinc on WEB 2086 binding were compared between the wild-type and low affinity mutants (Fig. 5B). Even though the Kₐ values of [³H]WEB 2086 binding in H188A and H248A mutants were higher than that of the wild type (Table II), higher concentrations of zinc were needed to inhibit specific WEB 2086 binding in these mutants. Thus, His-188 and His-248 were assumed to be involved in zinc binding.

Molecular Modeling of the Transmembrane Domains of the PAF Receptor—Construction of a three-dimensional model of the TM domains of the guinea pig PAF receptor was based on the following criteria. 1) Hydropathicity analysis with the Kyte-Doolittle parameters (29) was used to define regions in the PAF receptor as putative TM domains. 2) A model for the structure of bacteriorhodopsin based on high resolution electron cryomicroscopy (25) was used as a template to position the 7 TM domains. 3) The TM α-helices were oriented so that polar residues highly conserved between GPCRs and which we found to be important for high affinity PAF binding would face the central cleft of the receptor, as observed in GPCRs. 4) PAF C16 was situated to the position similar to that of retinal in bacteriorhodopsin. 5) Energy minimization was carried out for PAF and the side chains of the PAF receptor with the main chains fixed. According to this putative molecular model, PAF C16...
penetrates through the central cleft of the receptor in an oblique direction across the plasma membrane (Fig. 6). The phosphate moiety in a PAF molecule is surrounded by three histidines (His-188, His-248, and His-249) in TM V and VI. Positively charged nitrogen atoms in imidazole rings of each histidine are oriented toward negatively charged oxygen atoms of the phosphate group of PAF, all within 2.5–3.5 Å. His-248, which is essential for high affinity PAF binding (Tables I and II), is situated as a wedge between the oxygen atom in the phosphate group and the carbonyl oxygen atom of PAF. These three histidines can coordinate a zinc ion. Gln-252 exists near and interacts with His-248. The polar residues, the alanine exchanges of which generated high affinity mutants, are mostly positioned to surround the long alkyl chain of PAF (pink color in Fig. 6). The polar residues for which exchanges did not result in apparent changes of PAF binding are all located at a distance over the van der Waals contacts from PAF.

**DISCUSSION**

GPCRs are integral membrane proteins characterized by hydrophobic seven-TM segments, despite a wide heterogeneity of their ligand structures. Site-directed mutagenesis and molecular modeling studies of GPCRs have contributed to a better understanding of the ligand-receptor interaction, and domains that are likely involved in the ligand recognition have been identified in some GPCRs (17, 30). We investigated PAF-PAF receptor interactions with alanine-scanning mutagenesis and molecular modeling of a guinea pig PAF receptor.

**Mutations Altered PAF-binding Affinity in Transfected COS-7 Cells**—From previous extensive studies of structure-activity relationships using various PAF analogs, the polarity in a PAF molecule seems to be crucial for its biological activities, as reviewed (31). In its glycerol-backboned structure, PAF has an alkyl-ether linkage at the sn-1 position, an acetyl moiety at sn-2, and the phosphocholine residue with both positive and negative charges at sn-3, which are all important for its biological activities and receptor binding. PAF receptor homologs of four mammalian species show a high identity (72.5%) in the deduced amino acid sequences (Fig. 1A). The identity in the putative TM I, II, III, VI, and VII domains are especially high (90.5%) compared with that of other portions. All 23 polar amino acids clustered in the putative seven TM regions are

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**Fig. 3.** Signal transduction via the wild-type and six mutant receptors in CHO cells in response to PAF C16 (A–C) and lyso-PAF C16 (D). A, PAF-induced arachidonate release. Cells prelabeled with [3H]arachidonic acid were incubated with PAF for 6 min. Data are shown as percentages of the released radioactivity with the incorporated as 100. B, PAF-induced IP₃ production. Amounts of IP₃ produced after a 15-s stimulation by PAF were quantified with a radioreceptor assay kit. Data are presented as percentages of the response with that at 1 μM PAF as 100. C, PAF-dependent inhibition of forskolin-induced cAMP accumulation. Cells were stimulated for 20 min by 1 μM forskolin with PAF. Amounts of cAMP accumulated were quantified using a radioimmunoassay kit, and data are expressed as percentages of the response with that at 1 μM PAF as 100. D, lyso-PAF-induced arachidonate release in 6 min. In all figures, the receptors studied are indicated using the same symbols as for Fig. 2, and data are the means of duplicate samples and are representative of three independent experiments.
High and Low Affinity Mutants of the PAF Receptor

perfectly conserved, including not only residues highly con-
served between members of the GPCR superfamily such as
Asp-63 and Asn-285 (32), but also highly characteristic resi-
dues such as the three histidines in TM V and VI domains (Fig.
1B). We reasoned that electrostatic interactions between PAF
and these polar residues are likely to be important for binding
and selected these residues as targets of alanine substitution.

An alanine-scanning search was carried out using both non-
tagged and FLAG-tagged receptors, and similar results were
obtained concerning ligand-binding properties. Mutations of
N58A, D63A, N100A, T101A, S104A, and D289A generated
high affinity mutants, as determined both by the binding ratio
and the specific PAF binding per FLAG expression (data not
shown). The results agree well with the finding that mutations
of Asp-63 (D63N) and Asp-289 (D289A) in the human PAF
receptor augmented PAF binding affinity (33, 34). Mutants of
N100A, T101A, and S104A were chosen for further analysis,
because PAF binding affinities of these mutants were much
higher than that of D289A and the expression levels of the
receptors were more constant than those of N58A and D63A.
On the other hand, mutations of H188A, H248A, H249A,
Q252A, Q276A, and T278A impaired PAF binding, indicating
that these six residues may be involved in direct or indirect
physical interactions with PAF. Mutations of H188A, H248A,
and Q276A were selected, because these residues in targets of alanine substitution.

Ligand-binding Profiles and Functional Properties of Mutant
Receptors in CHO Transformants—The CHO transfectants
showed essentially similar characteristics in PAF binding to
those of transfected COS-7 cells (Table II). In ligand displace-
ment experiments (Fig. 2), high and low affinity mutants ex-
hibited largely altered affinities only for agonists, but much
less for six distinct antagonists. Next, signal transduction via
mutant receptors was examined. In all signals tested, dose-de-
pendent curves in high affinity mutants were more similar to those
for the wild-type receptor, whereas those in low affinity mu-
tants were all shifted right by 1 or 2 log units (Fig. 3). Although
PAF-induced IP₃ production and inhibition of adenylyl cyclase
are mediated by different types (pertussis toxin insensitive and
sensitive, respectively) of G proteins (14), both of the dose-de-
pendent responses were affected in the same manner by recep-
tor mutations. One of the highest affinity mutants, N100A, was
constitutively active and was responsive to lyso-PAF (Figs. 3D
and 4). In contrast, three low affinity mutants were practically
inactive with 1 nM PAF, but with higher concentrations their
responses were observed.

According to the two-state model of GPCR activation, recep-
tors exist in an equilibrium between the two conformational
states, the inactive (R) and the active (R*) (19). In the resting
state, the equilibrium is largely shifted to R, and the amounts
of R* are under a threshold to trigger a productive receptor-G
protein coupling in the absence of agonists. Agonists bind to R'
with a higher affinity than to R and shift the equilibrium to R*
by stabilizing it, which in turn activates G proteins and trans-
mits the intracellular signals. In contrast, neutral antagonists
do not distinguish the two states, bind to the both states of the
receptor with the same affinity, and do not shift the equilib-
rium. In our experiments, the equilibrium might be shifted to R'
in high affinity mutants (N100A, T101A, and S104A), and to
R in low affinity mutants (H188A, H248A, and Q276A), com-
pared with that in the wild type. Previous studies have indi-
cated that mutations in ligand-binding sites, G protein-cou-
pling domains, and intracellular C-terminal tails of various
GPCRs confer constitutive activity on receptors (35–38). It was
recently described that mutations in the third intracellular
loop speculated as a G protein-coupling domain of the human
PAF receptor generated also both constitutively active and
inactive mutants (38). Some of the constitutively active recep-
tors were accompanied by increased affinities for agonists but
not for antagonists (17, 36, 38). In the N100A mutant, the
equilibrium might be overshifted to R* enough to generate a
constitutive activation. Lyso-PAF seems to bind efficiently only
to the active state of the PAF receptor.

Molecular Modeling of the Transmembrane Domains of the
PAF Receptor—No member of the GPCR family has been sub-
ject to x-ray crystallography, but diffraction data of bacterior-
hodopsin (25) provide structural information that may apply to
GPCRs. Although bacteriorhodopsin is a light-driven proton
pump but not a GPCR, it has a structure with hydrophobic
seven-TM α-helices. Bovine and frog rhodopsins have been
analyzed at a low resolution (6–9 Å) also by electron cryomi-
croscopy (39, 40), but information on the receptor structure is
yet limited.

Because zinc ions inhibited ligand binding to the PAF recep-

Fig. 4. Constitutive activation in signal transduction via the N100A mutant receptor. The effects of antagonist WEB 2086 on basal
cellular activities were examined in the wild type (open columns) and the N100A mutant (closed columns) expressed in CHO cells. A, basal
arachidonate release in 6 min in the presence of WEB 2086; B, basal accumulation of inositol phosphates in 10 min. Cells prelabeled with
myo-[3H]inositol were incubated with WEB 2086, and radioactivity of IPs was measured. C, forskolin-induced cAMP accumulation in 20 min was
measured in the presence of WEB 2086. Data were expressed as percentages of the accumulation with that in the absence of WEB 2086 as 100.
In all figures, data shown are the means ± S.E. of triplicate samples and are representative of two individual experiments. The effects of WEB 2086
were significant in the N100A transformant (*, p < 0.06; **, p < 0.02; ***, p < 0.01; two-tailed, unpaired t test).
tor (Fig. 5), TM V and VI domains were aligned so that the three histidine residues (His-188, His-248, and His-249) should adjoin and form a zinc-binding pocket. Zinc is an essential component of many catalytic enzymes and transcription factors, all of which contain homologous domains involving zinc-binding sites (41). A consensus sequence consisting of three contiguous histidines has been observed among zinc-binding proteins such as carbonic anhydrases. Recently, it was reported that artificial introduction of three histidines into appropriate positions among seven-TM segments of the tachykinin NK-1 receptor and the \( \kappa \)-opioid receptor gave rise to high affinity zinc-binding sites (42, 43). IC\( _{50} \) of zinc was 1 mM in the wild-type receptor (Fig. 5A), a value higher than that in human platelets (21) but comparable with that in rat Kupffer cells (44). Divalent cations of zinc, copper, and cadmium but not iron inhibited specific PAF binding, indicating that coordination numbers might be four but not six. These three histidines and a water molecule may occupy the four coordination sites of a zinc ion with a distorted tetrahedron geometry (41). Even though the physiological importance of zinc in PAF-mediated signal transduction in vivo is not obvious, our findings support the idea that these three histidines would interact with a zinc ion as well as the phosphate group of a PAF molecule.

In this putative model, the long alkyl chain of PAF and polar side residues in Asp-63, Asn-100, Ser-104, and Asp-289 are within the van der Waals contacts and are repulsive in hydrophobic interactions. The alanine exchange of these residues may increase the hydrophobic interactions causing increased affinity for PAF. The three histidines, His-188, His-248, and His-249, seem to be involved directly in PAF binding, and Gln-252 might associate with His-248 and stabilize the conformation of His-248. The roles of Asn-58, Thr-101, Gln-276, and Thr-278 in PAF binding remain to be clarified. However, mutations of these residues distant from the ligand-binding site may cause the steric effects.

**Conclusion**—We obtained evidence that alanine exchanges of polar amino acids in the putative TM domains of the PAF receptor generated both constitutively active and inactive mutants. Natural mutations of various GPCRs cause serious diseases because of their constitutive activity or inactivity (45–47). Overexpression of the PAF receptor in transgenic mice elicited abnormal reproduction, bronchial hyperreactivity, in-
increased endotoxin-induced lethality, and tumorigenesis in skin (48). Further, deficiency of plasma PAF acetylhydrolase was associated with severe respiratory symptoms in asthmatic children (49, 50). These studies indicate that an excess of PAF receptor signaling causes various disorders. It is, therefore, intriguing to screen the natural mutations of the PAF receptor (49, 50). These studies indicate that an excess of PAF receptor, using these high and low affinity mutants. Inverse agonists are expected to be more agonists of PAF receptor, using these high and low affinity mutant receptors. Inverse agonists are expected to be more effective drugs than neutral antagonists for diseases in which PAF is involved. Finally, our molecular model would aid in predicting PAF-PAF receptor interactions for a rational drug design, even though a better understanding of the interactions awaits physicochemical analysis of the receptor structure.

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