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# Mutation of a Putative Amphipathic $\alpha$-Helix in the Third Intracellular Domain of the Platelet-Activating Factor Receptor Disrupts Receptor/G Protein Coupling and Signaling

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## ABSTRACT

Platelet-activating factor (PAF) is a potent phospholipid mediator that interacts with G protein-coupled PAF receptors to elicit diverse physiological and pathophysiological actions. We recently demonstrated that the third intracellular domain of the rat PAF receptor (rPAFR) is a critical determinant in its coupling to phosphoinositide phospholipase C-activating G proteins. Here, we report identification of a putative amphipathic helix in the third intracellular domain of the rPAFR and the effects of mutational disruption of its amphipathic character on G protein coupling of and signaling by the rPAFR. Modeling of the third intracellular domain and adjacent transmembrane regions of the rPAFR identified a single amphipathic helix located in the amino-terminal region of the third intracellular domain of the receptor. Baby hamster kidney cells were transiently transfected with cDNAs encoding the rPAFR or rPAFR mutants in which nonconserved substitutions were made separately in the hydrophobic or polar face of this amphipathic helix. The number and affinity of binding sites for specific PAF receptor antagonist WEB2086 were identical in membranes prepared from rPAFR and amphipathic helix mutant PAFR transfectants. However, only membranes derived from rPAFR transfectants possessed high affinity PAF binding sites that were sensitive to the G protein-uncoupling effects of guanosine-5′-*O*-(3-thio)triphosphate. These results show that substitutions into either face of the amphipathic helical domain abolished the ability of the rPAFR to undergo coupling to G proteins to form a high affinity agonist/receptor/G protein ternary complex. To examine the effects of these mutations on rPAFR signaling, PAF-stimulated inositol phosphate accumulation was determined in cells transfected with cDNAs encoding the wild-type or amphipathic helix mutant PAFRs. Although PAF stimulated 10-fold increases in inositol phosphate accumulation in rPAFR transfectants, it had no effects on inositol phosphate accumulation in amphipathic helix mutant PAFR transfectants. These results suggest that an amphipathic helix located in the amino-terminal region of the third intracellular domain of the rPAFR is required for its coupling to and activation of G proteins. This study provides the first insight into the structure of the receptor interface for G protein coupling of a PAFR and suggests a conserved role of amphipathic helices in G protein coupling of receptors ranging from those for biogenic amines to the phospholipid mediator PAF.

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine) is an ether phospholipid that elicits an impressive range of physiological and pathophysiological actions (see review by Braquet *et al*., 1987). The production and release of PAF, by both circulating and established cells, enable this phospholipid to serve as an autocrine, paracrine, and hormonal mediator. Many of the pathophysiological effects of PAF are attributed to its ability to dramatically activate immune and inflammatory processes (Behrens and Goodwin, 1990; Kim *et al*., 1995; Nourshargh *et al*., 1995; Resnick *et al*., 1995). PAF also is a physiological mediator of neural, respiratory, cardiovascular, and reproductive functions (Braquet *et al*., 1987; Battye *et al*., 1993; Kato *et al*., 1994). The vast majority of effects of PAF are mediated by its specific interaction with extracellular receptors identified in a variety of cell types. The PAFR was identified as a member of the G protein-coupled family of receptors by GTP-dependent binding studies and by isolation of PAFR cDNAs (Hwang *et al*., 1986; Honda *et al*., 1991; Nakamura *et al*., 1991; Bastien and Mazer, 1994; Bito *et al*., 1994). This receptor couples primarily to pertussis toxin-insensitive, PI PLC-activating G proteins in the $G_q$ family of G proteins, although its coupling to $G_i$ also

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**ABBREVIATIONS:** PAF, platelet-activating factor; BHK, baby hamster kidney; DMEM, Dulbecco's modified Eagle's medium; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; IP, inositol phosphate; PAFR, platelet-activating factor receptor; rPAFR, rat platelet-activating factor receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction; PLC, phospholipase C; 1 i, first intracellular domain; 2 i, second intracellular domain; 3 i, third intracellular domain

has been reported (Murayama and Ui, 1985; Amatruda *et al.*, 1993; Honda *et al.*, 1994).

Recently, we demonstrated that 3 i of the rPAFR is a critical determinant in its coupling to G proteins by using intracellular domain minigenes to antagonize rPAFR signaling and receptor chimerogenesis to confer an rPAFR signaling phenotype to another receptor (Carlson *et al.*, 1996a). The small size of 3 i of the rPAFR and its lack of similarity to homologous domains of other $G_q$-coupling receptors raised the possibility that a secondary structure or structures within this domain may comprise the interface for receptor/G protein coupling. In view of studies implicating amphipathic helices in the coupling of adrenergic and muscarinic receptors to G proteins (Strader *et al.,* 1987; Cotecchia *et al.*, 1990; Bluml *et al.*, 1994; Blin *et al.,* 1995; Liu *et al.*, 1995), it seemed crucial to determine whether a similar structure could serve as a site of G protein coupling in the structurally unrelated receptor for the lipid mediator PAF. Here, we report the identification of a putative amphipathic helix in the amino-terminal region of 3 i of the rPAFR and the effects of introducing nonconserved substitutions separately into the hydrophobic and polar faces of this helix on G protein coupling and signaling by the rPAFR. Substitutions into either face of this amphipathic helical domain completely prevented rPAFR coupling to G proteins and PAF-stimulated signaling without altering rPAFR expression. These results provide the first insight into the structure of the receptor interface for G protein coupling within 3 i of the rPAFR and suggest a conserved role of amphipathic helices in G protein coupling of receptors ranging from those for biogenic amines to the lipid mediator PAF.

## Experimental Procedures

**Materials.** A cDNA encoding the rPAFR was isolated in our laboratory as we described previously (Carlson *et al.*, 1996a). Oligonucleotides used for PCR and sequencing were obtained from the University of Iowa DNA Core Facility. PAF was purchased from Bachem Biosciences (King of Prussia, PA). [$^3$H]WEB2086 ([*methyl*-$^3$H], 13.5 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA). *myo*-[2-$^3$H]Inositol (16.5 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). LipofectAMINE, OptiMEM, and inositol-free DMEM were obtained from Life Technologies (Grand Island, NY). Dowex AG1-X8 resin was obtained from BioRad (Hercules, CA). Qiagen MaxiPrep kits were from Qiagen (Chatsworth, CA). The Quick Change Mutagenesis kit was from Stratagene (La Jolla, CA). pCRIII vector was from InVitrogen (San Diego, CA). Perfect Prep plasmid isolation kits were from 5 Prime-3 Prime (Boulder, CO). Dialyzed fetal bovine serum and GTPγS were from Sigma Chemical (St. Louis, MO). Cell culture media and fetal bovine serum were obtained from the Diabetes Endocrinology Research Center (University of Iowa, Iowa City, IA). Other molecular biology reagents were from the University of Iowa DNA Core Facility (Iowa City, IA). BHK-21 cells (American Type Culture Collection, Rockville, MD) were a gift from Dr. Jeffrey Pessin (University of Iowa, Iowa City, IA).

**Construction of cDNAs encoding PAFRs with substitutions in 3 i.** cDNAs encoding rPAFRs with substitutions of three amino acids, each within the 3 i of the rPAFR (rPAFR RRQΔLLL and rPAFR ILLΔNQR), were created using the Quick Change Mutagenesis kit according to the manufacturer's protocol. Briefly, the mutant rPAFR cDNAs were generated by PCR with *Pfu* polymerase using rPAFR cDNA in pCRIII vector as template and two mutagenic oligonucleotide primers for each mutant (5′-CTCACGCTGCCTGTGCTGCTGCAGCGC-3′ and its complement for rPAFR RRQΔLLL cDNA, and 5′-GTCATCAACCACACGCAGCGCACGCGG-3′ and its complement for the rPAFR ILLΔNQR cDNA). PCR was performed using 18 cycles of 95° for 30 sec, 55° for 1 min, and 68° for 12 min 24 sec. Mutations were verified by automated fluorescent dideoxynucleotide double-stranded sequencing of Qiagen MaxiPrep-purified cDNAs.

**Cell culture and transfections.** BHK cells were cultured in DMEM containing 10% fetal bovine serum and 50 μg/ml gentamicin in a 5% humidified $CO_2$ atmosphere at 37°. Cells were plated onto 24-well tissue culture dishes at a density of $7.5 \times 10^4$ cells/well and allowed to grow for 24 hr before transfection. For transient transfection of wild-type and mutant rPAFR cDNAs, cells were transfected with pCRIII containing DNA encoding the rPAFR, rPAFR RRQΔLLL, or rPAFR ILLΔNQR (0.5 μg/well) using LipofectAMINE (5 μl/μg DNA) according to the manufacturer's protocol. Lipofection was performed for 16 hr at 37° and terminated by replacement of the transfection cocktail with culture medium.

**IP production.** For measurement of IPs, transfected BHK cells were allowed to recover for 32 hr after terminations of transfections and then labeled for 16 hr with [$^3$H]inositol (2 μCi/ml) in inositol-free DMEM containing 10% dialyzed fetal bovine serum. Labeled cells were rinsed with Earle's balanced salt solution, preincubated in Earle's balanced salt solution containing 10 mM LiCl for 20 min at 37°, and stimulated with vehicle or PAF for 20 min. Incubations were terminated by removing the medium and adding 1 ml of methanol. Total IPs were extracted after the addition of chloroform (1 ml) and water (0.5 ml) and then separated on Dowex AG1-X8 columns as we described previously (Carlson *et al.*, 1996a). Total IPs were eluted from columns using 1 M ammonium formate/0.1 M formic acid. IP accumulation is expressed as dpm of IPs/$10^5$ dpm in the lipid fraction.

**[$^3$H]WEB2086 binding.** Competition binding studies were performed to determine the number, affinity, and G protein-coupling state of wild-type and mutant rPAFRs in BHK cell membranes. These studies were performed in parallel with the IP studies but in six-well tissue culture dishes using the transfection protocol described above, with adjustment of the amount of DNA and LipofectAMINE for four times as many cells. Binding studies were performed 48 hr after transfection. Cells were washed twice with $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate-buffered saline and then incubated in this same buffer for 1 hr at 37° to detach cells from the culture dish. The suspended cells were pelleted by centrifugation at $2500 \times g$ for 5 min at 25°, resuspended in 5 ml of HEPES-Tyrode's buffer (Honda *et al.*, 1994) containing 0.1% bovine serum albumin, and then homogenized with a Teflon-glass homogenizer. The resulting cell homogenates were centrifuged at $2500 \times g$ for 10 min at 4°, and the resulting supernatants were centrifuged at $20{,}000 \times g$ for 30 min. at 4°. The crude membrane pellets were resuspended in 0.5 ml of HEPES-Tyrode's buffer containing 0.1% bovine serum albumin. Binding assays were performed with 10 μl of resuspended membranes in 0.25 ml of the HEPES-Tyrode buffer containing 10 nM [$^3$H]WEB2086 alone or with varying concentrations of unlabeled WEB2086, unlabeled PAF, or unlabeled PAF plus GTPγS (10 μM) for 2 hr at 25°. Binding reactions were terminated by centrifugation at $15{,}000 \times g$ for 30 sec. Bound radioactivity was determined by scintillation counting of pelleted membranes. The resulting competition binding data were transformed to a Scatchard plot using a least-squares regression analysis to determine the number and affinity of WEB and PAF binding sites in membranes from BHK transfectants. The binding data were normalized per milligram of membrane protein used in the individual binding assays. Significance of differences between conditions was determined by analysis of variance followed by Fisher's *post hoc* analysis.

## Results

Recently, we demonstrated that 3 i of the rPAFR is a primary structural determinant for its coupling to phosphoinositide phospholipase C-activating G proteins (Carlson *et*

*al*., 1996a). For those members of the G protein-coupled receptor family in which 3 i has been implicated in G protein coupling, there is little sequence conservation that would enable the identification of G protein-coupling sites. However, studies of the $G_q$-coupled m3 and m5 muscarinic and $\alpha_{1B}$-adrenergic receptors (Cotecchia *et al*., 1990; Bluml *et al*., 1994; Blin *et al*., 1995), $G_i$-coupled m2 muscarinic receptor (Liu *et al*., 1995), and $G_s$-coupled $\beta_2$-adrenergic receptor (Strader *et al*., 1987) have provided evidence that an amphipathic helix structure within 3 i of these receptors mediates their coupling to G proteins. Therefore, helical wheel models of the rPAFR 3 i were constructed to determine whether such an intradomain structure could exist within the 3 i of the rPAFR. Fig. 1 illustrates the primary sequence and predicted topological arrangement of the rPAFR based on hydrophobicity analysis and comparison with the deduced structure of bacteriorhodopsin. We examined all possible permutations of 3 i of the rPAFR for the presence of an amphipathic helix, spanning from F200 located within transmembrane V to V237 located within transmembrane VI. Of these 38 possible models of amphipathic helices within this region of the rPAFR, only one model conformed to an amphipathic helix. This putative amphipathic helix encompasses the sequence from I209 to Q220 in the amino-terminal region of 3 i of the rPAFR and is shown in a helical wheel representation in Fig. 1. As shown, the hydrophobic face of the helix is comprised of two leucines, two isoleucines, one valine, and one proline, and the hydrophilic face is comprised of two arginines, two threonines, one glutamine, and one histidine. This putative amphipathic helix has a hydrophobic moment comparable to that of the G protein-coupling amphipathic helix present in 3 i of the $G_q$-coupled m3 muscarinic receptor (Blin *et al*., 1995). Fig. 2 shows the putative amphipathic helix as a ball-and-stick model (*top*), a molecular surface showing lipophilic potential (*middle*), and a molecular surface showing electrostatic potential (*bottom*). Representations are rotated about the helical axis to illustrate the membrane-facing side (Fig. 2a) and solvent-facing side (Fig. 2b) of the helix. The molecular surface is colored to show the electrostatic (*E*) potential of amino acids in the putative amphipathic helix in 3 i of the rPAFR. Images were prepared using the molecular modeling package Sybyl 6.2 from Tripes (St. Louis, MO). As shown, this region of the rPAFR can assume an $\alpha$-helical conformation with a hydrophobic, neutral surface (Fig. 2a) and a hydrophilic, charged surface (Fig. 2b) oriented on opposite sides of the helix. The presence of a proline (P217) four amino acids from the carboxyl-terminal region of the helix suggests possible interactions of this group with residues outside of the helix to accommodate its unsatisfied hydrogen bond. Together, these analyses suggest that an amphipathic helix is a likely structure in the region of the rPAFR encompassing I209 to Q220.

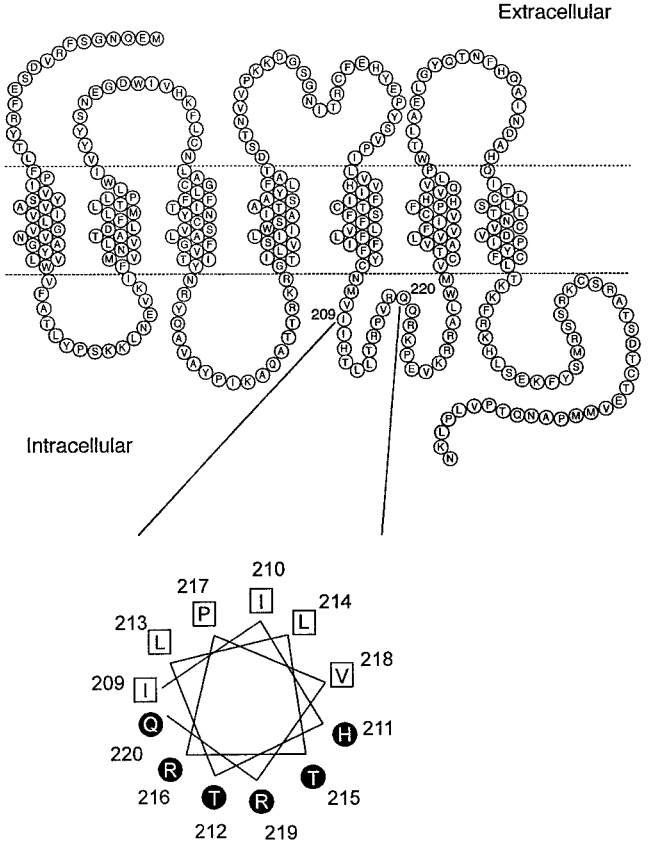


**Fig. 1.** Structure of the rPAFR and helical wheel representation of the putative amphipathic helix in 3 i of the rPAFR showing the amino acid sequence and putative membrane topology of the rPAFR and the location of the amphipathic helix within 3 i of the rPAFR. The amphipathic helix encompasses I209 to Q220, located in the amino-terminal region of 3 i of the rPAFR. ●, polar amino acids; □, hydrophobic amino acids. I209, L213, P217, I210, L214, and V218 comprise the hydrophobic face of the amphipathic helix, and Q220, R216, T212, R219, T215, and H211 comprise its polar face.

Initially, we assessed the possible role of the putative amphipathic helix in 3 i of the rPAFR in its coupling to G proteins by examining the binding characteristics of rPAFR mutants in which substitutions were made separately in the hydrophobic and hydrophilic faces of the putative amphipathic helix. Agonist binding to G protein-coupled receptors induces receptor coupling to G proteins and formation of an agonist/receptor/G protein ternary complex in which the receptor binds agonists with high affinity. Uncoupling of receptors from G proteins, a process normally initiated by GTP binding to G protein $\alpha$ subunits, shifts the receptor back to the low affinity binding state. Antagonist ligands bind to G protein-coupled receptors without inducing their coupling to G proteins and do not discriminate between G protein-coupled and -uncoupled receptor forms. Therefore, one way to assess the ability of a receptor to undergo coupling to G proteins is to determine the affinity of agonist binding to the receptor and its sensitivity to the G protein-uncoupling effects of GTP or its stable analogues. Fig. 3 illustrates the substitutions that were made in two rPAFR mutants to disrupt the putative amphipathic helix in 3 i of the rPAFR. In one mutant, termed rPAFR ILLΔNQR, three hydrophobic leucines were substituted for polar amino acids (R216, R219, Q220) on the hydrophilic face of the helix. We made a corresponding mutant of the hydrophobic face of the helix, termed rPAFR ILLΔNQR, by substituting three polar residues for three hydrophobic amino acids (I210, L213, L214). These amino acid substitutions in the polar and hydrophobic faces of the putative amphipathic helix of 3 i of the PAFR were selected based on mathematical calculations for how these substitutions perturb the amphipathic character of the helix as described by Jones *et al*. (1992). The mutations made in the two amphipathic helix mutants of the PAFR are predicted to disrupt both the respective face of the amphipathic

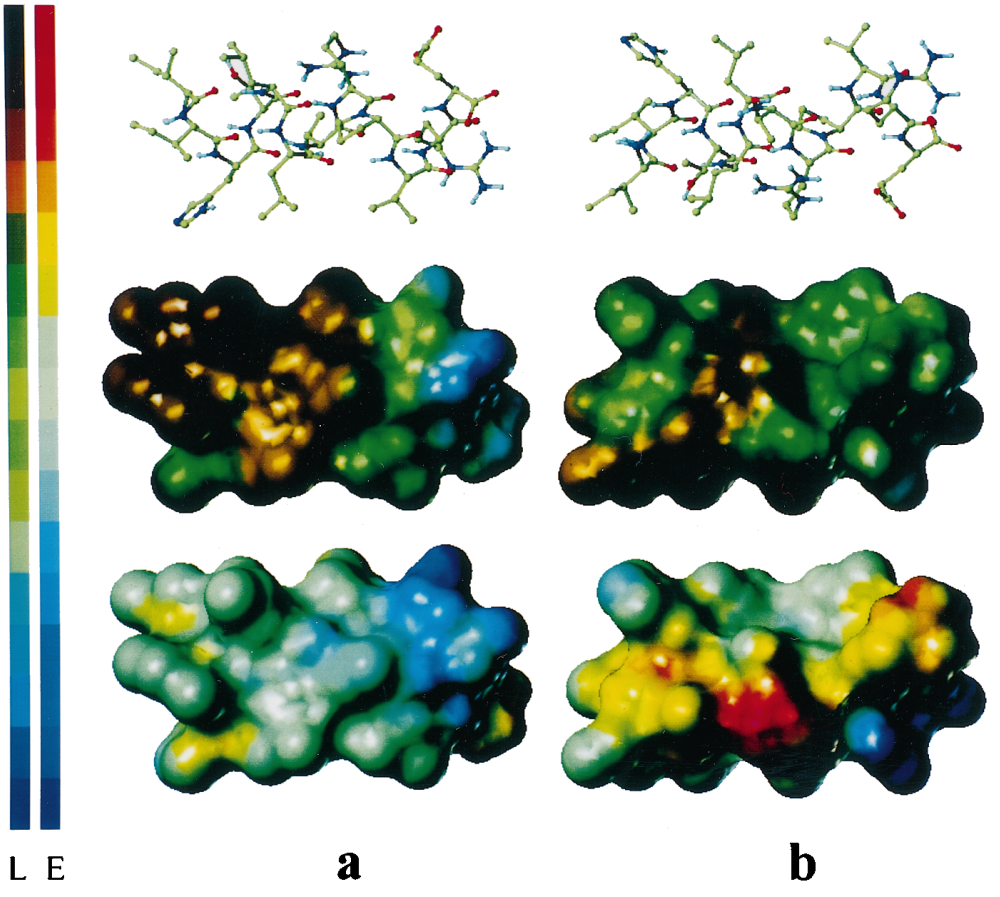


**Fig. 2.** Molecular modeling of the putative amphipathic helix in 3 i of the rPAFR. *Top*, ball-and-stick model of the putative amphipathic helix in 3 i of the rPAFR. *Middle*, molecular surface colored to show the lipophilic (*L*) potential of amino acids in the putative amphipathic helix in 3 i of the rPAFR. *Bottom,* for the electrostatic potential (*E*), *purple* indicates electronegative and *red* indicates electropositive. For the lipophilic potential (*L*), *blue* indicates lipophobic (i.e., hydrophilic) and *dark brown* indicates lipophilic (i.e., hydrophobic). Representations are rotated 180° about the helical axis to illustrate the membrane-facing (a) and solvent-facing (b) sides of the helix.

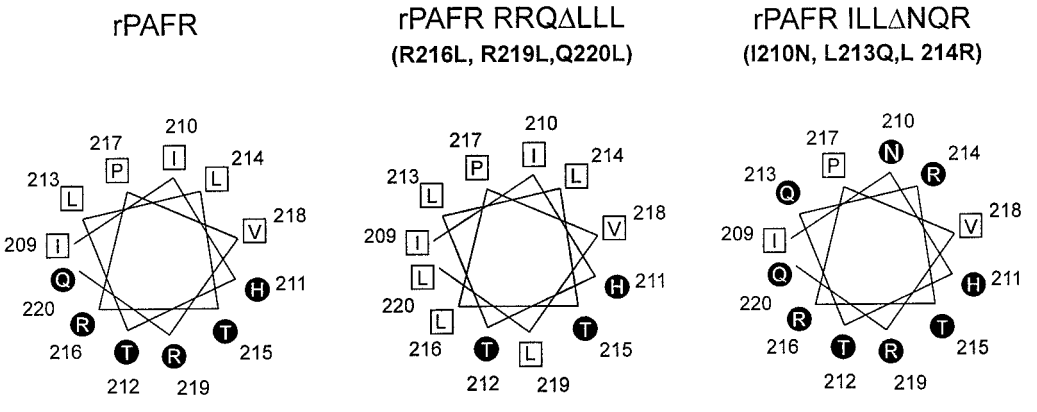


**Fig. 3.** Helical wheel representations showing the location of substitutions in and the disruption of the putative amphipathic helix in 3 i of the rPAFR in rPAFR RRQΔLLL and rPAFR ILLΔNQR mutants. ●, polar amino acids; □, hydrophobic amino acids.

helix and its overall amphipathic character. Fig. 3 shows that these substitutions generate receptors in which the helix is not organized into hydrophobic and hydrophilic faces, a finding supported by the calculated hydrophobic moment/residue of this region in these two mutants (Jones *et al.*, 1992).

Fig. 4 and Table 1 show results of experiments examining the binding of PAF and specific PAF receptor antagonist WEB2086 to membranes derived from cells transiently transfected with rPAFR, rPAFR RRQΔLLL, and rPAFR ILLΔNQR cDNAs. Because of potential effects of substitutions in the putative amphipathic helix in 3 i of the rPAFR on both G protein-coupling and receptor expression, these studies used the antagonist ligand [$^3$H]WEB2086 to label the entire population of receptor sites. Thus, competition binding with PAF was performed in parallel with competition binding with WEB2086 for the [$^3$H]WEB2086-labeled sites. To assess the G protein-coupling status of the expressed receptors, the effects of GTPγS on PAF binding also were assessed. Fig. 4A and Table 1 show that a single class of WEB2086 binding sites with a $K_i$ value of ≈30 nM and a $B_{max}$ value of ≈50 fmol/mg of protein were found in rPAFR, rPAFR RRQΔLLL, and rPAFR ILLΔNQR transfectants. These results show that the performed substitutions within the putative amphipathic helix in 3 i of the rPAFR in the rPAFR RRQΔLLL and rPAFR ILLΔNQR mutants did not alter the expression or antagonist binding activity of these receptors. However, these substitutions produced a dramatic effect on agonist binding to the receptors. Fig. 4B and Table 1 show that rPAFR RRQΔLLL and rPAFR ILLΔNQR exhibit a single class of PAF binding sites with an affinity of 70–80 nM. In contrast, PAF binding to the rPAFR was characterized by a nonlinear Scatchard plot best described by binding of PAF to a high affinity ($K_i$ ≈

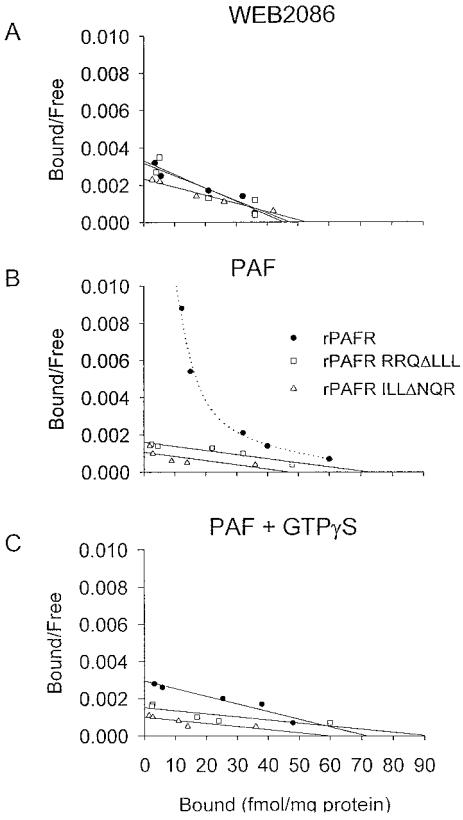


**Fig. 4.** Binding of PAF and specific PAFR antagonist WEB2086 to membranes prepared from rPAFR, rPAFR RRQΔLLL, and rPAFR ILLΔNQR transfectants. Competition of [$^3$H]WEB2086 binding by WEB2086 and PAF was examined in membranes derived from rPAFR, rPAFR RRQΔLLL, and rPAFR ILLΔNQR transfectants. Binding data were transformed and are shown as Scatchard plots for [$^3$H]WEB2086 binding in the presence of (A) WEB2086, (B) PAF, and (C) PAF plus 10 $\mu$M GTPγS. *Solid lines*, linear regression analysis of the binding data. Binding of PAF to the rPAFR in the absence of GTPγS (B) did not conform to a one-site fit as did the other data. *Dashed line*, best fit of PAF binding assuming a two-site model in which PAF binds to two sites with different affinities. Data are from a single experiment performed in duplicate and are representative of three experiments that provided essentially identical results. Transfections and [$^3$H]WEB2086 binding assays were performed as described in the text.

TABLE 1

Ligand binding parameters of WEB2086 and PAF in wild-type and mutant PAFRs

Competition of [$^3$H]WEB2086 binding by WEB2086 and PAF was examined in membranes derived from rPAFR, rPAFR RRQΔLLL, and rPAFR ILLΔNQR transfectants as shown in legend to Fig. 4. PAF binding was performed in the absence and presence of 10 $\mu$M GTPγS. $K_i$ and $B_{max}$ values were determined by Scatchard transformation of the binding data. The $K_i$ value shown for PAF in the absence of GTPγS (10 $\mu$M) represents the high affinity binding component. Values represent mean ± standard error from three experiments, each performed in duplicate.

| | WEB2086 $B_{max}$ | WEB2086 $K_i$ | PAF $K_i$ | PAF + GTPγS $K_i$ |
|---|---|---|---|---|
| | *fmol/mg of protein* | | *nM* | |
| rPAFR | 50 ± 4 | 29 ± 2 | 1.0 ± 0.3 | 84 ± 13[b] |
| rPAFR RRQΔLLL | 41 ± 3 | 28 ± 6 | 83 ± 11[a] | 86 ± 6 |
| rPAFR ILLΔNQR | 50 ± 9 | 27 ± 9 | 71 ± 8[a] | 102 ± 6[b] |

[a] $p < 0.05$ compared with value in rPAFR transfectants. [b] $p < 0.05$ compared with value in the absence of GTPγS.

0.7 nM) and a low affinity ($K_i \approx 50$ nM) binding site. The ability of GTPγS to abolish high affinity PAF binding to the rPAFR (Fig. 4C, Table 1) demonstrates that this high affinity binding component represents binding to the G protein-coupled form of the rPAFR. In contrast to its effects on PAF binding to the rPAFR, GTPγS had little or no effect on PAF binding to rPAFR RRQΔLLL and rPAFR ILLΔNQR (Table 1). In fact, the affinity of rPAFR RRQΔLLL and rPAFR ILLΔNQR for PAF, in assays performed in either the presence or absence of GTPγS, was the same as that of the G protein-uncoupled form of the rPAFR. The lack of high affinity PAF binding and the insensitivity of PAF binding to the G protein-uncoupling effects of GTPγS in rPAFR RRQΔLLL and rPAFR ILLΔNQR mutants indicate that these receptors are impaired in their ability to undergo coupling to G proteins. These results show that substitutions in either the hydrophobic or hydrophilic face of the putative amphipathic helix in 3 i of the rPAFR that disrupt the amphipathic nature of this helix abolish the ability of the receptor to form a high affinity agonist/receptor/G protein ternary complex. In contrast, we found that a rPAFR mutant with substitutions in 3 i of the rPAFR that were outside of the predicted amphipathic helical domain retained the ability to undergo coupling to G proteins (not shown). This rPAFR mutant, rPAFR ERRΔALL, had substitutions comparable to those of the amphipathic helix mutant rPAFR RRQΔALL. Although the expression of rPAFR ERRΔALL was poor relative to that of the rPAFR or amphipathic helix rPAFR mutants, its affinity for PAF was reduced significantly by treatment with GTPγS. Thus, mutations in 3 i of the rPAFR comparable to those of the amphipathic helix mutants of the PAFR do not prevent receptor coupling to G proteins.

To further test the hypothesis that the putative amphipathic helix in 3 i of the rPAFR plays a role in receptor coupling to G proteins, we compared the receptor signaling activity of rPAFR, rPAFR RRQΔLLL, and rPAFR ILLΔNQR. In most PAF-responsive cells, the biological effects of PAF can be attributed to activation of PI PLC. Therefore, we assessed the ability of PAF to stimulate PI PLC in cells transiently transfected with each of the receptor cDNAs. Previous studies in our laboratory have demonstrated that PAF stimulates PI PLC by a pertussis toxin-insensitive mechanism in rPAFR transfectants, suggesting the involvement of G proteins in the $G_q$ family in this response. Fig. 5 shows that PAF stimulated dose-dependent increases in IP

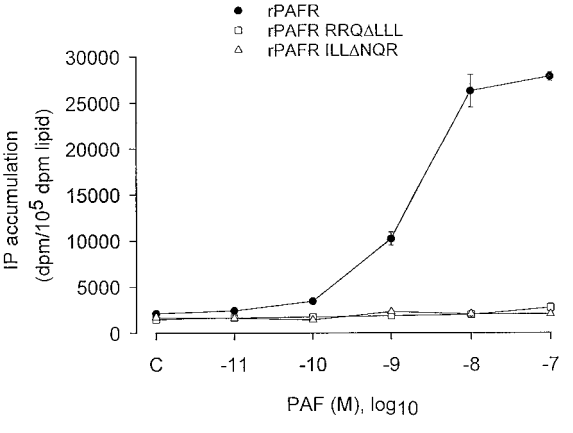


**Fig. 5.** Dose response of PAF-stimulated IP accumulation in rPAFR, rPAFR RRQΔLLL, and rPAFR ILLΔNQR transfectants. BHK cells were transiently transfected with cDNAs encoding rPAFR, rPAFR RRQΔLLL, or rPAFR ILLΔNQR in parallel with the binding studies shown in Fig. 4 and summarized in Table 1. Transfected cells were challenged with vehicle (*C*) or the indicate concentrations of PAF, and accumulation of IPs was measured as described in the text. Values represent mean ± standard error of three separate transfections.

accumulation in rPAFR transfectants but had no effects on IP accumulation in rPAFR RRQΔLLL or rPAFR ILLΔNQR transfectants. These results show that disruption of either the hydrophobic or hydrophilic face of a putative amphipathic helix in 3 i of the rPAFR abolishes the ability of the receptor to activate PI PLC-activating G proteins.

## Discussion

Despite the broad range and well described pathophysiological actions of PAF, there is little understanding of how the receptor for this potent lipid mediator activates G proteins to initiate its biological responses. Recently, we presented evidence that 3 i of the rPAFR is a primary determinant in its coupling to PI PLC-activating G proteins (Carlson *et al*., 1996a). These studies showed that rPAFR-stimulated IP accumulation was inhibited by up to 75% by cellular transfection of minigenes encoding 3 i of the rPAFR. The presumed competitive inhibitory effect of the transfected intracellular domain on coupling of the rPAFR to G proteins was not observed in cells transfected with minigenes encoding 1 i or 2 i of the rPAFR. Our ability to confer a PI PLC-activating phenotype to a pituitary adenylate cyclase-activating polypeptide receptor variant by inserting the rPAFR 3 i into its existing homologous domain demonstrated clearly that 3 i of the rPAFR contains sequence determinants required for G protein coupling. The current study extends these findings by providing the first evidence for a site of G protein coupling within this domain. Our results suggest that an amphipathic helix located in the amino-terminal region of the rPAFR is required for coupling to and activation of G proteins.

It is important to consider the current results in relation to previous studies examining structural functional aspects of the PAFR. Parent *et al*. (1996) reported the effects of mutation of two adjacent amino acids located in the carboxyl-terminal region of 3 i of the human PAFR on receptor affinity and activity. Mutation of A230 to glutamate in the human PAFR inactivated the receptor, whereas mutation of the adjacent L231 to arginine constitutively activated the receptor. Both mutations produced alterations in the affinity of the receptors for PAF that could not be accounted for by their state of G protein coupling. Thus, these authors suggested a role for these amino acids in a receptor isomerization process (R to R*) that precedes and is required for receptor coupling to G proteins, by analogy to the revised ternary complex model proposed by Samama *et al*. (1993). The current results show that mutations within the putative amphipathic helix in the amino-terminal region of 3 i of the rPAFR inactivate the receptor in a way that can be ascribed entirely to uncoupling of the receptors from G proteins. Both rPAFR RRQΔLLL and rPAFR ILLΔNQR exhibited agonist binding affinities that were equivalent to that of the G protein-uncoupled form of the rPAFR and insensitive to the G protein-uncoupling effects of GTPγS. Our modeling of the 3 i of the human PAFR (not shown) shows that an amphipathic helix can be accommodated in the same region as that of the rPAFR described here, although the polar face of this helix is less charged than that of the rPAFR. In view of the current findings, it is possible that the suggested role of A230 and L2321 in isomerization of the human PAFR may involve interactions with such an amphipathic helix leading to its stabilization or formation. However, our recent finding that A230E substitution in the rPAFR has no effects on receptor activity (Carlson *et al*., 1996b) suggests that this residue does not play an equivalent role in the rPAFR. No further studies have evaluated structural functional aspects of the 3 i of PAFRs.

The current findings are the first to support a role for an amphipathic helix in G protein coupling of a receptor other than muscarinic or adrenergic receptors. In addition to being a receptor for a lipid mediator rather than a biogenic amine, the rPAFR is structurally unrelated to these other receptors. Of particular note is the lack of conservation in size and sequence of the 3 i of the rPAFR compared with these receptors in which amphipathic helices within 3 i mediate their coupling to G proteins. The rPAFR 3 i is approximately one sixth as large as the 3 i of the $G_q$-coupled m3 and m5 muscarinic and the $G_i$-coupled m2 muscarinic receptors and one half as large as the $G_q$-coupled $\alpha_{1B}$-adrenergic and $G_s$-coupled $\beta_2$-adrenergic receptors. This dissimilarity in size and sequence within 3 i of receptors in which this domain plays a role in their coupling to G proteins that often are the same is consistent with G protein coupling being mediated by a higher order structure like an amphipathic helix. The amphipathic helices are located conspicuously at the amino region (rPAFR, $\alpha_{1B}$- and $\beta_2$-adrenergic; Strader *et al*., 1987; Cotecchia *et al*., 1990), carboxyl region ($m_2$ muscarinic; Liu *et al*., 1995), or amino and carboxyl regions ($m_3$ muscarinic; Blin *et al*., 1995) of 3 i near or encompassing ($m_2$ and $m_3$ muscarinic; Bluml *et al*., 1994; Blin *et al*., 1995; Liu *et al*., 1995) the adjacent transmembrane domains of these receptors. Thus, it seems likely that these amphipathic domains represent a conserved switch that responds to agonist-mediated conformational changes in the receptor regardless of their location within 3 i or adjacent transmembrane domains. Obviously, other receptor determinants must be involved in determining the specificity of receptor coupling in view of the varied G proteins to which these receptors couple. Indeed, coupling of the $m_3$ receptor to $G_{q11}$ involves interactions between sequences within 2 i and the two amphipathic helices present in 3 i (Blin *et al*., 1995).

It is unclear whether G protein coupling by receptor amphipathic helices represents a universal mechanism for interaction of receptors with G proteins. This is due in part to the relatively small number of receptors in which the role of amphipathical helical domains in G protein coupling has been evaluated. Amphipathic helices are not present in G protein-coupling domains of all receptors, and there is evidence for the lack of a role of such domains in G protein coupling. Mutational analysis of a putative amphipathic helix in 3 i of the LHCG receptor showed that neither the amphipathic helix nor the basic amino acids in this region are required for coupling to $G_s$ (Wang *et al*., 1993). Voss *et al*. (1993) reported that peptides derived from putative G protein-coupling regions of $\alpha_1$-, $\alpha_2$-, and $\beta_1$-adrenergic receptors lacked amphipathic character and did not activate G proteins directly or interfere with native receptor coupling to G proteins. Moreover, G protein coupling seems to be mediated or regulated by intracellular domains other than 3 i in some receptors, including those for calcitonin (1 i; Nussenzveig *et al*., 1994), vasopressin (2 i; Liu and Wess, 1996), and prostaglandin $E_2$ (carboxyl-terminal tail; Namba *et al*., 1993). Whether amphipathic helices exist in these domains and

associated transmembrane regions or mediate coupling of such receptors to G proteins or whether these receptor domains interact with amphipathic helices in other receptor regions to regulate G protein coupling remains to be determined. However, our finding that an amphipathic helix in 3 i of the rPAFR is required for receptor coupling to G proteins suggests a conserved role of 3 i amphipathic helices in G protein coupling of receptors ranging from those for lipid mediators to biogenic amines.

The precise mechanism by which agonist occupancy of the rPAFR leads to its coupling to G proteins via an amphipathic helix in 3 i of the receptor cannot be determined at present. At least two mechanisms seem possible. Agonist binding to the rPAFR could induce a conformational change in the receptor, leading to exposure of the existing amphipathic helix in 3 i. Alternatively, agonist binding to the receptor could stabilize or induce the formation of this amphipathic helix. Both possibilities conform to the revised ternary complex model of G protein-coupled receptors (Samama *et al*., 1993) in which agonist binding to receptors induces conformational changes in the receptor (R to R*) that are required before it undergoes coupling to G proteins. It is interesting to speculate that the proline located near the carboxyl end of the amphipathic helix in the rPAFR could normally destabilize the amphipathic helix and respond to the conformational changes in the receptor on agonist binding to allow helix formation in accordance with the second mechanism. In this regard, it is noteworthy that prolines can exist within amphipathic helices (Lee *et al*., 1992; Cox *et al*., 1993; Henderson *et al*., 1993) and confer stability to $\alpha$-helices of integral membrane proteins (Li *et al*., 1996). Moreover, crystallographic studies showed that T4 lysozyme can adapt to the potentially destabilizing effects of prolines on $\alpha$-helix integrity (Sauer *et al*., 1992). Alternatively, this proline may hydrogen bond to other regions of the receptor that respond to conformational changes in the receptor to regulate exposure of the G protein-coupling amphipathic helix in accordance with the first mechanism.

It is possible that the putative amphipathic helix located in 3 i of the rPAFR constitutes only a necessary part of the G protein-coupling domain of the receptor. Although the rPAFR lacks the dual interacting amphipathic helix structure present in 3 i of the m3 muscarinic receptor (Blin *et al*., 1995), we cannot preclude the possibility that the amphipathic helix of the rPAFR interacts with polar or hydrophobic regions of 3 i or other regions of the rPAFR to form the G protein-coupling site. However, several observations argue against interactions of this domain with 1 i, 2 i, or the carboxyl-terminal tail of the PAFR to form a G protein-coupling site. First, the ability of guinea pig PAFRs truncated in their carboxyl-terminal tail to exhibit signaling responses to PAF (Takano *et al*., 1994) suggests that this domain is dispensable for G protein coupling. Second, our inability to attenuate rPAFR-mediated signaling by cellular transfection of minigenes encoding 1 i or 2 i of the rPAFR, alone or in combination with rPAFR 3 i minigenes (Carlson *et al*., 1996a), does not support a role for interactions of the 3 i amphipathic helix with these domains of the rPAFR. We acknowledge, however, that the 1 i and 2 i peptides encoded by the minigenes may not assume an appropriate conformation to compete for interactions of the authentic domain with the amphipathic helix in 3 i of the rPAFR. Further studies with rPAFRs in which the 1 i or 2 i is deleted or substituted with homologous domains from a related receptor will be required to address this issue. Third, insertional chimerogenesis of 3 i of the rPAFR into a receptor with structurally unrelated 1 i and 2 i (pituitary adenylate cyclase-activating polypeptide receptor 2 variant) was sufficient to confer coupling of the chimeric receptor to PI PLC-activating G proteins (Carlson *et al*., 1996a). Finally, mastoparan, a peptide present in wasp venom, directly activates G proteins as a result of its ability to form an amphipathic helix (Sukumar and Higashijima, 1992). This finding indicates that amphipathic helical peptides alone can interact with and activate G proteins, although clearly the specificity and efficiency of this process may be regulated by other structural elements in the context of a receptor. Thus, it seems reasonable to consider the possibility that the amphipathic helix in 3 i of the rPAFR may mediate coupling to G proteins independent of intramolecular interactions with these other receptor domains.

The ability of the PAFR to undergo coupling to G proteins underlies the profound pathophysiological sequelae in response to PAF during acute anaphylactic and allergic situations (Braquet *et al*., 1987), in children with deficiencies in the PAF-degrading enzyme PAF acetylhydrolase (Miwa *et al*., 1988; Hattori *et al*., 1994), and in transgenic mice in which PAFRs are overexpressed (Ishii *et al*., 1997). Here, we provide the first insight into the structural element of the PAFR required for its coupling to G proteins. Our ability to completely prevent G protein coupling of the rPAFR by rational mutations in the hydrophobic and hydrophilic faces of a predicted amphipathic helix located in the amino-terminal region of 3 i of the receptor indicates a required role of this structure in interaction with and activation of G proteins. Our results also suggest that a common mechanism of interaction of receptors with G proteins has been retained in receptors ranging from the lipid mediator PAFR to the structurally dissimilar receptors for biogenic amines, raising interesting questions about the design and evolution of these receptors.



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