The Third Intracellular Domain of the Platelet-activating Factor Receptor Is a Critical Determinant in Receptor Coupling to Phosphoinositide Phospholipase C-activating G Proteins

STUDIES USING INTRACELLULAR DOMAIN MINIGENES AND RECEPTOR CHIMERAS*

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Platelet activating factor (PAF) is a potent phospholipid mediator which elicits a diverse array of biological actions by interacting with G protein-coupled PAF receptors (PAFR). Binding of PAF to PAFRs leads to activation of G protein(s) that stimulate phosphoinositide phospholipase C and subsequent intracellular signaling responses. To identify the potential role of intracellular domains of the rat PAFR (rPAFR) in signaling, we examined effects of transfecting minigenes encompassing rPAFR intracellular domains 1 (1i), 2 (2i), and 3 (3i) on inositol phosphate (IP) production mediated by the cotransfected rPAFR cDNA. Although transfection of the rPAFR1i and rPAFR2i minigenes had no effects on PAF-stimulated signaling, transfection of the rPAFR3i minigene inhibited PAF-stimulated IP production by approximately 50% compared to controls. The rPAFR3i domain did not inhibit IP production mediated by the multifunctional rat pituitary adenylate cyclase-activating polypeptide receptor (rPACAPR), demonstrating the specificity of the competition by the rPAFR3i domain. In further experiments, the rPAFR3i domain was engineered onto the homologous domain of a monofunctional transmembrane variant of the rPACAPR (rPACAPRr) that activates only adenyl cyclase. The rPACAPRr/rPAFR3i chimera responded to PACAP with increases in IP production which were attenuated nearly completely in cells cotransfected with the rPAFR3i domain. In contrast, PACAP had no effects on IP production in a receptor chimera expressing a mutated form of the rPAFR3i domain (rPACAPRr/rPAFR3i_mut). These results demonstrate the ability of the rPAFR3i domain to confer a phospholipase C-signaling phenotype to a receptor deficient in this activity and show that this activity is specific for the engineered rPAFR3i domain. These results suggest that the third intracellular loop of the rPAFR is a primary determinant in its coupling to phosphoinositide phospholipase C-activating G proteins, providing the first insight into the molecular basis of interaction of PAFRs with signal-transducing G proteins.

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# The abbreviations used are: PAF, platelet-activating factor; G protein, guanine nucleotide-binding protein; IP, inositol phosphate; PAFR, platelet-activating factor receptor; rPAFR, rat PAFR; rPACAPR, rat PACAP receptor; rPACAPRr, transmembrane 2 variant of the rPACAPR; rPACAPRr/rPAFR3i, chimera of rPACAPRr and rPAFR3i domain; PAFR, platelet-activating factor receptor; rPAFR, rat PAFR; PCR, polymerase chain reaction; rPAFR1i, first intracellular domain of the rPAFR; rPAFR2i, second intracellular domain of the rPAFR; rPAFR3i, third intracellular domain of the rPAFR; 3i, first intracellular domain; 2i, second intracellular domain; BHK, baby hamster kidney.
clear that PAFR signaling is a consequence of receptor coupling to G proteins. Recent studies indicate that coupling of recombinant PAFRs to phospholipase C is mediated by pertussis toxin-insensitive G<sub>α11</sub> proteins (16, 18).

Despite the wealth of information implicating PAF in a multitude of physiological and pathophysiological cascades and the cloning of its receptor cDNA from several species (17, 19, 20), the molecular basis of its interaction with G proteins, and hence signaling, is unknown. The present study was initiated to ascertain which intracellular domain(s) of the rPAFR mediates its coupling to phospholipase C-activating G protein(s). We used a combination of minigene expression of intracellular domains of the rPAFR to antagonize PAFR-mediated signaling and a novel gain of function approach using insertional receptor chimeraigens to demonstrate that the rPAFR<sub>3i</sub> plays a crucial role in PAFR signaling. These results provide the first insight into the structural determinants of the PAFR that are involved in its coupling to G proteins.

**EXPERIMENTAL PROCEDURES**

**Materials—**Full-length cDNAs encoding the rPAFR, rPACAPR, and a novel<sup>2</sup> transmembrane domain 2 variant of the rPACAPR (rPACAPR<sub>2</sub>) were isolated in our laboratory using a PCR-based strategy essentially as we described (21). Double-stranded sequencing of the cloned rPACAPR, rPAFR, and rPACAPR<sub>2</sub>cDNAs verified their identity to cDNAs isolated by hybridization screening (20, 22). Oligonucleotide primers used for sequencing and PCR were synthesized by the University of Iowa DNA Core Facility.

PAF (1-O-hexadecyl-2-acyetyl-sn-glycero-3-phosphocholine) and PACAP-27 were purchased from Bachem. [H]<sup>3</sup>PAF (1-O-[hexadecyl-1,2-<sup>3</sup>H]-sn-glycero-1-phosphocholine) (16.5 Ci/mmol) was from Amersham. LipofectAMINE, Opti-MEM, and inositol-free Dulbecco's modified Eagle's medium were obtained from Life Technologies, Inc. AmpliTaq was from Perkin Elmer, F<sub>60</sub> polymerase was from Stratagene, pCRIII vector was obtained from Invitrogen, and Perfect Plasmid was from Promega. Cell culture medium and serum were obtained from the Diabetes Endocrinology Research Center, the University of Iowa. Other molecular biological reagents were obtained from the University of Iowa DNA Core. BHK-21 cells (ATCC) were a gift from Dr. Jeffrey Pessin, the University of Iowa.

**Construction of rPAFR Minigenes—**A set of minigenes containing cDNA fragments encoding the rPAFR<sub>1i</sub>, rPAFR<sub>2i</sub>, and rPAFR<sub>3i</sub> domains was derived from the rPAFR<sub>3i</sub>cDNA. The oligonucleotide primers used for PCR amplification of these sequences included the sequence 5′-GCCGCGACATTGGGA-3′ representing a consensus ribosomal binding site sequence followed by Met/Gly codons, at the 5′ end of the forward primers and a stop codon at the 3′ end of the reverse primers. The resulting minigene PCR products were cloned following ligation into the eukaryotic expression vector pCRIII. Plasmids with constructs in the proper orientation were reamplified and purified using the Qiagen MaxiPrep Kit. Sequences of both strands of minigenes were confirmed by automated fluorescent deoxyxynucleotide sequencing by the University of Iowa DNA Core Facility.

**Construction of a Chimeric rPACAPR/rPAFR<sub>3i</sub> cDNA—**A chimeric cDNA, in which the rPAFR<sub>3i</sub> domain was integrated into the existing homologous domain of the rPACAPR<sub>2</sub>, was constructed by reverse primer encompassing the start codon of the rPACAPR<sub>2</sub> and stop codon, respectively, were used. The resulting rPACAPR<sub>2</sub>/rPAFR<sub>3i</sub>cDNA was cloned into pCRII. The sequence of both strands of the rPACAPR<sub>2</sub>/rPAFR<sub>3i</sub>cDNA was confirmed by automated fluorescent deoxyxynucleotide sequencing by the University of Iowa DNA Core Facility.

**Construction of a Chimeric rPACAPR/rPAFR<sub>3i</sub>mut cDNA—**A chimeric cDNA, in which a mutated version of the rPAFR<sub>3i</sub> domain (rPAFR<sub>3i</sub>mut) was engineered into the existing homologous domain of the rPACAPR<sub>2</sub>, was constructed by reverse primer encompassing the stop codon of the rPACAPR<sub>2</sub> and a mutagenic reverse primer encompassing the stop codon of the rPACAPR<sub>2</sub>. The mutagenic forward 36-mer (5′-GGGCACACCGGCAGTG-3′) encodes for substitution of 8 amino acids within rPAFR<sub>3i</sub>. Similarly, the 3′ end of the rPACAPR<sub>2</sub>/rPAFR<sub>3i</sub>mut cDNA was amplified using a mutagenic forward primer and a reverse primer encompassing the stop codon of the rPACAPR<sub>2</sub>. The mutagenic forward 36-mer (5′-GGGCACACCGGCAGTG-3′) encodes for the same rPAFR<sub>3i</sub> substitutions encoded by the mutagenic reverse 36-mer. These two overlapping rPACAPR<sub>2</sub>/rPAFR<sub>3i</sub>mut receptor chimera fragments were purified using the Wizard PCR Prep Kit and then used in recombinant PCR. In this reaction, the two overlapping fragments served as templates and reverse primer encompassing the rPACAPR<sub>2</sub> start codon and stop codon, respectively, were used. The resulting rPACAPR<sub>2</sub>/rPAFR<sub>3i</sub>cDNA was cloned into pCRII. The sequence of both strands of the rPACAPR<sub>2</sub>/rPAFR<sub>3i</sub>cDNA was confirmed by automated fluorescent deoxyxynucleotide sequencing by the University of Iowa DNA Core Facility.

**Inositol Phosphate Production—**For measurement of inositol phosphates (IPs), transfected BHK cells were allowed to recover for 30 h following terminations of transfections then labeled with 16 h with [H]inositol (2–8 Ci/mmol) in inositol-free Dulbecco's modified Eagle’s medium containing 10% dialyzed fetal bovine serum (Sigma). 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°C and stimulated with vehicle, PAF, or PACAP-27 for 20 min. Incubations were terminated by removing the medium and adding 1.0 mL of methanol. Total IPs were extracted following addition of chloroform (1.0 mL) and water (0.5 mL) and then separated on Dowex AG1-X5 columns as described by Brown et al. (<sup>23</sup>) except that 15 mL of 1 M ammonium formate, 0.1 M formic acid. IP accumulation is expressed as disintegrations/min of IPs/10<sup>6</sup> dpm in the lipid fraction.

**PAF Binding—**Binding studies were performed to evaluate possible effects of the rPAFR<sub>3i</sub> minigene on rPAFR expression. These experiments were performed in 12-well dishes using the 2-h transfection protocol used for transient transfection of rPAFR minigene cDNAs,

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adjusting the amount of DNA and lipofectamine for twice as many cells. Binding studies were performed 48 h after transfection. Cells were washed three times with ice-cold Ca\(^{2+}\)- and Mg\(^{2+}\)-free Dulbecco’s phosphate-buffered saline and then resuspended in 0.4 ml of this same buffer containing 10 nM \[^3H\]PAF alone or with various concentrations of unlabeled PAF. Cells were incubated for 2 h at 0–4 °C prior to termination of the binding assay by removing the medium, quickly washing the cells three times with ice-cold Ca\(^{2+}\)- and Mg\(^{2+}\)-free Dulbecco’s phosphate-buffered saline and finally adding 1 ml of 1 M NaOH for 30 min to solubilize the cells. Bound radioactivity was determined by scintillation counting of the cell lysates following neutralization with 1 ml of 1 M HCl. The resulting competition binding data was transformed to a Scatchard plot using a least square regression analysis to determine the number of \[^3H\]PAF binding sites in rPAFR transfectants. B\(_{max}\) values were normalized on the basis of cell number by counting the number of cells in three individual wells (for each transfection condition) following their trypsinization.

Data shown in figures represent means ± S.E. from three transfections assayed in one experiment. Each experiment shown is representative of at least two experiments that provided essentially identical results. The data were analyzed by ANOVA followed by Fisher’s post-hoc analysis when multiple comparisons were made. When comparisons involved only two parameters, statistical significance was determined by an independent t test.

**RESULTS**

Minigene constructs encoding the rPAFR1i, rPAFR2i, and rPAFR3i domains were prepared to determine effects of their transient expression on signaling by the rPAFR. Fig. 1A shows the putative membrane topology of the rPAFR identifying these three intracellular domains and their primary sequences. A minigene encoding the carboxyl-terminal cytoplasmic domain of the rPAFR was not prepared in view of recent studies by Takano *et al.* (24) demonstrating that a truncated PAFR lacking this domain was not impaired in its ability to stimulate phosphoinositide phospholipase C or intracellular Ca\(^{2+}\) mobilization. The minigenes encoding these three intracellular domains of the rPAFR were amplified by PCR so that a consensus ribosomal binding site and Met/Gly codons were engineered onto the 5' cDNA end and a stop codon at the 3' cDNA end. The resulting constructs were cloned into the eukaryotic expression vector pCRIII for expression of the minigene sequence (Fig. 1B). Similar minigene expression systems have been used previously to express intracellular domains of the \(\alpha\)-adrenergic, D1 dopamine, and muscarinic receptors (25, 26) as well as for expression of the COOH-terminal domain of \(\beta\)-adrenergic receptor kinase (27) and pleckstrin homology domains of several proteins (28).

To determine potential role(s) of the three intracellular loops of the rPAFR on rPAFR-mediated intracellular signaling, we cotransfected BHK cells with pCRIII containing cDNAs encoding the rPAFR and the three intracellular domains of the rPAFR. Preliminary experiments showed that untransfected BHK cells do not express endogenous PAF receptors or respond to PAF with phosphoinositide hydrolysis and that the recombinant rPAFR exhibits the characteristic activation of phosphoinositide phospholipase C in response to PAF stimulation.

Fig. 2 summarizes effects of PAF on IP accumulation in cells cotransfected with cDNAs encoding the rPAFR and rPAFR1i, rPAFR2i, or rPAFR3i domain peptides, alone and in combina-
tion. Cells were transfected with 0.8 μg of rPAFR cDNA and 0.67, 1, or 2 μg of the intracellular domain cDNA. Transfection of cells with cDNAs encoding the intracellular domains of the rPAFR had no effects on the basal rate of IP accumulation. However, as shown, rPAFR-mediated IP production in response to a maximally effective concentration of PAF (100 nM) was reduced significantly in cells transfected with the rPAFR3i domain minigene but not in cells transfected with cDNAs encoding rPAFR1i or -2i domains. The rPAFR3i domain minigene inhibited rPAFR activity approximately 50% when cotransfected (1.0 or 2.0 μg) individually with the rPAFR cDNA. Transfection of the rPAFR3i domain minigene simultaneously with the rPAFR1i or 2i domain minigenes (1.0 μg each) did not produce more inhibition of rPAFR activity than seen with the rPAFR3i domain alone and cotransfection of the rPAFR1i and -2i domains did not inhibit significantly rPAFR activity. In addition, when a submaximally active amount of the rPAFR3i domain minigene (0.67 μg) was transfected simultaneously with both the rPAFR1i and -2i domains, no significant inhibition of rPAFR activity was seen, as observed with this amount of the rPAFR3i alone. This evidence for inhibition of rPAFR activity by the rPAFR3i domain minigene suggests that this region of the rPAFR is involved in receptor/G protein coupling. In addition, these data do not support a role of the rPAFR1i or -2i in receptor/G protein coupling or for interactions of these domains with the rPAFR3i domain in this coupling.

The extent of rPAFR3i-mediated inhibition of rPAFR activity was dependent on the amount of rPAFR3i minigene transfected. Fig. 3 shows that cotransfection of the rPAFR3i minigene with the rPAFR cDNA (0.8 μg) produced dose-dependent reductions in rPAFR-mediated IP production with maximal effects apparent at 1.0 μg of rPAFR3i domain minigene. As shown similarly in Fig. 2, this amount of rPAFR3i minigene produced more than 50% inhibition of rPAFR activity with no further inhibition observed using 2.0 μg of the minigene. The observed attenuation in rPAFR activity by cotransfection of the rPAFR3i minigene could result from a nonspecific effect producing a reduction in the expression of the rPAFR. This seems unlikely, in part, because similar effects were not observed during cotransfection of the rPAFR1i or -2i minigenes. However, to address this possibility, we performed [3H]PAF binding assays in cells cotransfected with rPAFR cDNA and either the rPAFR3i minigene or empty pCRIII vector. Simultaneous transfections were performed in these studies for parallel measurements of rPAFR-mediated IP production to document the inhibitory effect of the rPAFR3i domain on rPAFR activity.

Fig. 2. Effects of transfecting minigenes encoding intracellular domains of the rPAFR on rPAFR-mediated IP production. BHK cells were transiently cotransfected with rPAFR cDNA (0.8 μg/well) plus the indicated amounts of pCRIII containing rPAFR1i, -2i, or -3i cDNAs alone and in the indicated combinations (2.0 μg of DNA/well, i.e. using pCRIII vector DNA to maintain a constant amount of plasmid DNA per transfection). PAF (100 nM)-stimulated IP accumulations in cells transfected with vector alone or minigene constructs are expressed relative to vehicle controls (i.e. basal values). Vehicle-stimulated IP levels ranged from 528 ± 67 to 758 ± 120 dpm/10^6 dpm lipid and did not differ statistically among the various transfecants. *, p < 0.05 compared to control (vector-cotransfected cells), †, p > 0.05 (i.e. statistically insignificant) compared to cells cotransfected with 1.0 μg of DNA encoding rPAFR3i alone.

Fig. 3. Effect of increasing amounts of rPAFR3i cDNA on rPAFR-mediated IP production. BHK cells were transiently cotransfected with rPAFR cDNA (0.8 μg/well) and various amounts of the rPAFR3i minigene. The total amount of plasmid DNA per transfection was kept constant (2.8 μg/well) by the addition of pCRIII vector DNA. PAF (100 nM)-stimulated increases in IP accumulation in transfectants is expressed relative to the vehicle control (i.e. basal values). Vehicle-stimulated IP levels were 589 ± 61 dpm/10^6 dpm lipid. Transfections and measurements of IPs were performed as described under "Experimental Procedures." *, p < 0.05 compared to vector-cotransfected cells (i.e. 0 μg of rPAFR3i domain minigene).

Fig. 4. Schematic representation of the structural features of the third intracellular domains of the rPACAPR2, rPAFR, and chimeric rPACAPR/rPAFR3i. The fifth and sixth transmembrane domains and the third intracellular loop of the various receptors are shown with the circles indicating individual amino acids. Amino acid sequences of the third intracellular loops of the rPACAPR2 and rPAFR are shown along with the sequence of rPAFR3i mut. The arrow shown in the rPACAPR2 sequence indicates the site at which the rPAFR3i or rPAFR3i mut domains were inserted into the homologous domain of the rPACAPR2 to create the rPACAPR2/rPAFR3i and rPACAPR2/rPAFR3i mut chimeras, respectively. The third intracellular loop of the chimera illustrates the insertion of the rPAFR3i or rPAFR3i mut domains into the existing rPACAPR2 domain.
under the same experimental conditions used to measure expression of the rPAFR. In two separate experiments, cells co-transfected with the rPAFR cDNA and pCRIII vector expressed 210,000 and 220,000 PAF receptors/cell and cells cotransfected with the rPAFR cDNA and the rPAFR3i minigene expressed 200,000 and 230,000 PAF receptors/cell. In contrast to the absence of an effect of the rPAFR3i minigene on rPAFR expression, parallel experiments showed that cells transfected with the rPAFR3i minigene exhibited reductions in PAF-stimulated IP production of 56% (i.e. 672 ± 67% to 293 ± 51%, n = 3) and 49% (509 ± 47% to 258 ± 38%, n = 3) compared to cells transfected with pCRIII alone. These results show that the attenuation of rPAFR activity in cells transfected with the rPAFR3i minigene is not due to alterations in rPAFR expression.

To investigate further the role of the rPAFR3i domain in signaling by the rPAFR, we genetically engineered this domain onto a variant of the rPACAPR to create a chimera of this receptor and the rPAFR3i domain. This variant of the rPACAPR, recently identified in our laboratory,2 has been designated rPACAPR2 because it differs from the rPACAPR in discrete sequences located in transmembrane domain 2. However, unlike the multifunctional rPACAPR which activates both adenylyl cyclase and phosphoinositide phospholipase C (22), rPACAPR2 activates adenylyl cyclase but has no effects on phospholipase C.2 The approach we used to create a chimera of rPACAPR2 with the rPAFR3i domain is unique in that we engineered the rPAFR3i domain onto this receptor while retaining its homologous domain (Fig. 4). We reasoned that this approach was feasible because the rPACAPR mRNA can be alternatively spliced in the region encoding the 3i domain to yield forms of the rPACAPR with additional 28 amino acid sequences (i.e. PACAPR-hip or -hop) (22). Surprisingly, the rPAFR3i domain is identical in length to the 28-amino acid inserts present in the hip- and hop-cassette forms of the rPACAPR. Because the insertion of the hip- and hop-cassette sequences are obviously tolerated in these two spliced forms of the rPACAPR, we engineered the rPAFR3i domain into this site of the rPACAPR2.

Thus, our experimental approach was to determine whether inclusion of the rPAFR3i domain into PACAPR2 could restore the transmembrane alteration-induced loss of rPACAPR-mediated activation of phosphoinositide phospholipase C. Fig. 5a summarizes effects of PACAP-27 (100 nM) on IP accumulation in cells transfected with pCRIII vector alone or pCRIII containing rPACAPR, rPACAPR2, or rPACAPR/rPAFR3i cDNAs (0.5 µg/well). Transfected cells were challenged with vehicle or PACAP-27 (100 nM) and accumulation of IPs was measured as described under “Experimental Procedures.” *, p < 0.05 compared to response in rPACAPR2 transfectants. b, dose-response of PACAP-stimulated IP accumulation in cells transfected with rPACAPR2 or rPACAPR/rPAFR3i cDNA. BHK cells were transiently transfected with pCRIII containing cDNAs encoding the rPACAPR, rPACAPR2, and rPACAPR/rPAFR3i. As expected, agonist-stimulated increases in IP accumulation were observed in rPACAPR2 transfectants and not in cells transfected with pCRIII alone or rPACAPR2 cDNAs. In contrast to its effects in rPACAPR2 transfectants, PACAP-27 (100 nM) provoked increases in IP accumulation of approximately 600% in cells transfected with the rPACAPR2/rPAFR3i chimera. The chimeric receptor retained the phenotype of the rPACAPR2 to stimulate increases in cAMP in response to PACAP (data not shown). Fig. 5b demonstrates that rPACAPR and rPACAPR/rPAFR3i have identical EC50 values (0.5 nM PACAP) for activating phospholipase C while rPACAPR has a greater maximal response for phospholipase C activation. These results suggest that inclusion of the rPAFR3i domain in the homologous region of the rPACAPR2 conferred to the receptor an ability to couple to phospholipase C-activating G

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**Fig. 5.** a, PACAP-induced IP accumulation in cells transfected with the rPACAPR/rPAFR3i chimera cDNA. BHK cells were transiently transfected with pCRIII vector alone or pCRIII containing rPACAPR, rPACAPR2, or rPACAPR/rPAFR3i cDNAs (0.5 µg/well). Transfected cells were challenged with vehicle or PACAP-27 (100 nM) and accumulation of IPs was measured as described under “Experimental Procedures.” *, p < 0.05 compared to response in rPACAPR2 transfectants. b, dose-response of PACAP-stimulated IP accumulation in cells transfected with rPACAPR2 or rPACAPR/rPAFR3i cDNA. BHK cells were transiently transfected with pCRIII containing rPACAPR, rPACAPR2, or rPACAPR/rPAFR3i cDNAs (0.5 µg/well). Transfected cells were challenged with various doses of PACAP-27 and IP accumulation was measured as described under “Experimental Procedures.” c, absence of PACAP-stimulated IP accumulation in cells transfected with rPACAPR2/rPAFR3imut cDNA. BHK cells were transfected with pCRIII containing rPACAPR2, rPACAPR2/rPAFR3i, or rPACAPR2/rPAFR3imut cDNAs (0.5 µg/well). Transfected cells were challenged with vehicle or PACAP-27 (100 nM) and IP accumulation was measured as described under “Experimental Procedures.” *, p < 0.05 compared to response in rPACAPR2 transfectants.
but not in cells transfected with rPACAPR2/rPAFR3imut or rPACAPR2/rPAFR3imut domain of rPACAPR2. Fig. 4 demonstrates the location of the 8 amino acids within rPACAPR2 that were mutated in the cDNA encoding rPACAPR2/rPAFR3imut. Then, these same cells were subjected to a second transfection with pCRII vector, pCRII containing rPACAPR2 cDNA, or rPAFR3CDNA (1.25 μg/well) as described under “Experimental Procedures.” Transfected cells were challenged with vehicle and either 100 nm PACAP (rPAFR transfectants) or 100 nm PACAP-27 (rPACAPR and rPACAPR/rPAFR3i transfectants) and accumulation of IP was measured as described under “Experimental Procedures.” Agonist-stimulated increases in IP accumulation in transfectants are expressed relative to the vehicle control (i.e., basal values). Vehicle-stimulated IP levels ranged from 1150 ± 26 to 1452 ± 188 dpm/10^5 dpm lipid and did not differ statistically among the various transfectants. *, p < 0.05 versus cells transfected with vector in the second transfection.

Fig. 6. Specificity of rPAFR3i domain-mediated inhibition of receptor-stimulated IP accumulation. BHK cells first were transiently transfected with cDNA encoding the rPAFR, rPACAPR2/rPAFR3i, rPACAPR, or pCRII vector (0.5 μg/well). Transfected cells were challenged with PACAP-27 (10 μM) and cAMP in cells expressing rPACAPR2/rPAFR3i, rPACAPR, or pCRII vector (0.5 μg/well) as described under “Experimental Procedures.” Transfected cells were challenged with vehicle and either 100 nm PACAP, (rPAFR transfectants) or 100 nm PACAP-27 (rPACAPR and rPACAPR/rPAFR3i transfectants) and accumulation of IP was measured as described under “Experimental Procedures.” Agonist-stimulated increases in IP accumulation in transfectants are expressed relative to the vehicle control (i.e., basal values). Vehicle-stimulated IP levels ranged from 1150 ± 26 to 1452 ± 188 dpm/10^5 dpm lipid and did not differ statistically among the various transfectants. *, p < 0.05 versus cells transfected with vector in the second transfection.

To define the specificity of the rPAFR3i domain in conferring phospholipase C signaling to rPACAPR2, we engineered a mutated version of rPAFR3i (rPAFR3imut) into the homologous domain of rPACAPR2. Fig. 4 demonstrates the location of the 8 amino acids within rPAFR3i domain that were mutated in the cDNA encoding rPACAPR2/rPAFR3i mut. As shown, PACAP stimulated IP accumulation in cells transfected with rPACAPR2, rPAFR3i, rPACAPR/rPAFR3i mut, or rPAFR3i. However, PACAP produced significant increases in cAMP in cells expressing rPACAPR2/rPAFR3i, rPACAPR/rPAFR3i mut, or rPAFR3i (data not shown). These results demonstrate the sequence specificity of the rPAFR3i domain in conferring the phospholipase C-signaling phenotype to the rPACAPR2/rPAFR3i chimera.

To define further the specificity of the rPAFR3i domain in rPACAPR2/rPAFR3i-mediated IP production as well as in antagonism of rPAFR-mediated IP production, we assessed the effects of cotransfection of the rPAFR3i minigene on agonist-stimulated IP accumulation in cells transfected with cDNAs encoding the rPAFR, rPACAPR, rPAFR3i, and rPAFR. The rPACAPR served as a negative control in these experiments because it stimulates phosphoinositide phospholipase C but has a 23-amino acid 3i domain that is completely different from the 28-amino acid rPAFR3i. The results of these studies are shown in Fig. 6. As shown, cotransfection of the rPAFR3i had no effects on rPACAPR-mediated IP production but inhibited rPAFR3i-mediated IP production by approximately 50%. Also, cotransfection of the rPAFR3i domain inhibited rPACAPR2/rPAFR3i-mediated IP production by approximately 80%.

the inhibition of receptor-mediated IP production was limited to the receptors expressing this domain. These results demonstrate the specificity of the antagonism of receptor signaling by the rPAFR3i domain and its role in receptor/G protein coupling by the rPACAPR2/rPAFR3i chimera.

DISCUSSION

These results provide the first insight into the structural determinants of the PAFR that are involved in its coupling to G proteins. In this study, we transfected minigenes encoding the three intracellular loops of the rPAFR to antagonize rPAFR-mediated IP production to identify potential intracellular regions of the receptor required for coupling to phospholipase C-activating G proteins. This approach, first described by Luttrell et al. (25), relies upon disruption of receptor/G protein coupling by competition of intracellular domain peptides with the ligand-receptor complex at the receptor/G protein interface. These studies were complemented by a gain-of-function strategy in which one of the intracellular loops of the rPAFR was engineered onto a receptor deficient in signaling via phospholipase C in an attempt to confer the phospholipase C-signaling phenotype. This combined approach enabled us to ascertain that the third intracellular loop of the rPAFR is a primary, if not absolute, epitope for coupling the receptor to phospholipase C-activating G proteins.

Minigenes encoding the three intracellular domains of the rPAFR first were employed to identify potential G protein-coupling domains of the rPAFR. These studies showed that PACAP-stimulated IP production was inhibited in a dose-dependent manner by transfection of increasing amounts of the rPAFR3i minigene. This effect was not due to nonspecific effects resulting in a reduction in the expression of the rPAFR. In contrast, transfection of minigenes encoding the rPAFR1i or -2i domains had no significant effects on PACAP-stimulated IP production. These findings implicated the rPAFR3i domain in coupling rPAFRs to phospholipase C-activating G protein(s) and suggested that the rPAFR1i and -2i domains do not contribute individually to rPAFR coupling in the same manner as does the rPAFR3i domain. Additional experiments designed to investigate possible synergistic effects among the three intracellular loops of the rPAFR failed to show any such interactions. It is possible, however, that the rPAFR1i and -2i domains could play small but important roles in coupling of rPAFRs to phospholipase C-activating G protein(s) that are not detectable by the methods used in this study. Poor expression of the rPAFR1i or -2i domains could preclude detection of their involvement in rPAFR signaling, although this possibility seems unlikely for several reasons. First, the three intracellular domains of the rPAFR are of similar size and hydrophobicity and the corresponding minigenes for these domains were constructed in the same expression vector. Second, in the two previous studies investigating inhibition of receptor signaling by intracellular domain minigenes (25, 26), minipeptides from two different intracellular receptor domains, and from four different receptors were expressed at comparable levels although these domains shared no similarities in sequence or in size (i.e., 71–181 amino acids). Unfortunately, the small size of the rPAFR domains (i.e. 16–28 amino acids) precluded analysis of their expression by immunoblotting. Third, such differences in expression would have to be fairly large because transfection of 1 μg of the rPAFR3i minigene maximally inhibited rPAFR activity whereas no inhibition of rPAFR activity was apparent in cells transfected with twice as much rPAFR1i or -2i minigene DNA. Finally, the extent of inhibition of rPAFR signaling by the rPAFR3i domain observed in this study (i.e., 50% or more) is comparable to that produced by the α1B-adrenergic 3i domain on phospholipase C activation mediated by the α1B-
adrenergic receptor, a receptor in which a primary role of the 3i domain in receptor/G protein coupling has been well established (29).

Interestingly, transfection of the rPAFR3i domain minigene did not attenuate phospholipase C activation mediated by the rPACAPR. These results can be interpreted as evidence for specificity in the inhibitory effect of the rPAFR3i domain on receptor signaling because the rPACAPR 3i domain exhibits no sequence similarity to that of the rPAFR. However, Hawes et al. (26) demonstrated that 3i domain-induced inhibition of signaling by receptors was receptor-specific for receptors coupling to different G proteins but was generalized for receptors coupling to the same G protein. The generalized inhibition of receptor signaling by a nonhomologous receptor domain may reflect competition of intracellular domains of different receptors for the same region on the Gα subunit. In light of these findings, our results are consistent with the notion that the rPAFR and rPACAPR interact with different G proteins to stimulate phospholipase C or that they interact at different sites of a single G protein. The first of these two possibilities is supported by recent studies in our laboratory. We found that sequestration of βγ subunits by coexpression of minigenes encoding the β-adrenergic receptor kinase carboxy-terminal domain attenuated rPACAPR, but not rPAFR-mediated IP production, suggesting that these two receptors activate phospholipase C by coupling to different G proteins. Thus, the lack of inhibitory effects of the rPAFR3i domain on rPACAPR-mediated IP production likely reflects the specificity of this domain for coupling to a G protein unique from that which couples to the rPACAPR.

Direct evidence that the rPAFR3i domain represents a structural determinant for coupling to phospholipase C-activating G proteins was obtained using a chimera of a transmembrane domain variant of the rPACAPR and the rPAFR3i domain. Genetically engineered chimeras of G protein-coupled receptors have been used previously to identify domains conferring G protein-coupling specificity (29–38). In these previous studies, however, receptor chimeras were created by replacing an intracellular domain(s) of one receptor with the homologous domain of another receptor derived from the same group of G protein-coupled receptors (i.e. groups I, II, and III; Refs. 39 and 40). The present study is the first to describe signaling by a receptor chimera created from structurally unrelated receptors and in which the intracellular domain of one receptor was engineered onto the existing domain of the recipient receptor. Our results show that engineering the rPAFR3i domain onto the rPACAPR2 conferred to the receptor the ability to activate phospholipase C. However, engineering a substituted version of rPAFR3i into rPACAPR2 did not confer to the receptor the ability to activate phospholipase C. Activation of phospholipase C by the rPACAPR2/rPAFR3i chimera was not due to constitutive activation of the receptor, as produced by mutation of specific regions of the 3i domain of G protein-coupled receptors (41–43), because rPACAPR2/rPAFR3i-mediated IP production was entirely ligand-dependent. Importantly, we were able to attenuate nearly completely the ability of this chimeric receptor to stimulate phospholipase C by cotransfection of the rPAFR3i minigene. These results demonstrate the ability of the rPAFR3i domain to confer a phospholipase C-signaling phenotype to a receptor deficient in this activity and show that this activity is due to the engineered rPAFR3i domain rather than to some nonspecific effect involving conformational alterations in the receptor or constitutive receptor activation. These results suggest that the third intracellular domain of the rPAFR3i remarkably can undergo the appropriate ligand-induced conformational changes that occur in its native environ-

ment when placed into an unrelated receptor with a peptide ligand rather than the natural ether phospholipid ligand.

While much evidence has implicated the 3i domain of G protein-coupled receptors in signaling specificity (44, 45), other studies have ascribed the specificity of G protein-coupling to sequences in 1i, 2i, and the carboxyl-terminal domain of G protein-coupled receptors. Indeed, Hawes et al. (26) found that minigene expression of homologous 3i domains inhibited IP production mediated by α11-adrenergic, M1 muscarinic cholinergic, and D1 dopamine receptors and that α11-adrenergic receptor signaling was attenuated similarly by expression of 4i but not 2i of the α11-adrenergic receptor. In contrast, studies with M2 and M3 muscarinic receptor chimeras suggest that regions in 2i and 3i of the M3 muscarinic receptor contribute equally to its coupling to Gq11 (34). Other studies utilizing muscarinic cholinergic:β-adrenergic receptor chimeras suggest that interactions between 2i and 3i are required to fully determine G protein-coupling specificity (30, 46). G protein-coupling appears to be determined by intracellular domains other than 3i in several receptors including receptors for FMLP (1i, 31; alternatively 2i and carboxyl-terminal tail, Ref. 47), calcitonin (1i, Ref. 37), and prostaglandin E2 (carboxyl-terminal tail, Ref. 48). Evidence for the involvement of the carboxyl-terminal tail in this latter receptor was derived from observations that alternative splicing of the carboxyl-terminal tail of the EP3 receptor produced receptors that stimulated different second messenger systems. In contrast, the carboxyl-terminal tail of the human PAFR was not required for signaling by a truncated mutant of this receptor (24). Unfortunately, these latter two studies implicating a role, or lack thereof, of the carboxyl-terminal cytoplasmic domain of receptors for prostaglandin E2 and PAF comprise our understanding of the structural basis of signaling by receptors for lipid mediators.

In conclusion, this study provides the first insight into the molecular basis of interaction of PAFRs with signal-transducing G proteins. Our results demonstrate that the 3i domain of the rPAFR plays a critical role in its coupling to phospholipase C-activating G proteins, a primary step underlying short- and long-term pathophysiologic effects of PAF. Thus, binding of the phospholipid mediator PAF to its receptor produces the same type of receptor-G protein interactions as occur during binding of biogenic amines, peptides, and other substances to their cognate receptors (44, 45). Moreover, our studies with an insertional chimera of the rPAFR3i domain and the rPACAPR2 lead to the suggestion that ligand interactions with G protein-coupled receptors promote receptor activation and coupling to G proteins by an almost universal alteration in 3i regardless of the biochemical nature of the ligand or the subfamily (and hence surrounding structural features) to which the receptor belongs. This is surprising in view of the diversity of this domain among different receptors and the apparently distinct evolutionary origins of these receptors.

REFERENCES
1. Benveniste, J., Henson, P. M., and Cochrane, C. G. (1972) J. Exp. Med. 136, 1356–1377
2. Siriganian, R. P., and Oster, A. G. (1971) J. Immunol. 106, 1244–1251
3. Snyder, F. (1990) Annu. Rev. Pharmacol. 30, 697–719
4. Braquet, P., Touqui, L., Shen, T. Y., and Vargahtig, B. B. (1987) Pharmacol. Rev. 39, 97–145
5. Hanahan, D. J. (1986) Annu. Rev. Biochem. 55, 483–509
6. Kolhai, M., Hosford, D., Guinot, P., Esau, A., and Braquet, P. (1991) Drugs 42, 9–29
7. Kolhai, M., Hosford, D., Guinot, P., Esau, A., and Braquet, P. (1991) Drugs 42, 274–294
8. Del Cerro, A., Arni, S., and Lynch, G. (1990) Behav. Neural Biol. 54, 213–217
9. Hwang, S-B. (1990) J. Lipid Mediators 2, 123–158
10. Chao, W., and Olson, M. S. (1993) Biochem. J. 292, 617–629
11. Shimizu, T., Hnda, Z., Nakamura, M., Bito, H., and Izumi, T. (1992) Biochem. Pharmacol. 44, 1001–1008
12. Haslam, R. J., and Vanderwel, M. (1982) J. Biol. Chem. 257, 6879–6885
13. Balsinde, J., and Mollinedo, F. (1991) J. Biol. Chem. 266, 18726–18730
14. Nakashima, S., Suganuma, A., Sato, M., Tohmatsu, T., and Nozawa, Y. (1989) J. Immunol. 143, 1295–1302
15. Dhar, A., and Shukla, S. D. (1991) J. Biol. Chem. 266, 18797–18801
16. Honda, Z., Takano, T., Gotoh, Y., Nishida, E., Ito, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 20707–20715
17. Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okude, H., Toh, H., Ito, K., Miyamoto, T., and Shimizu, T. (1991) Nature 349, 342–346
18. Amatruda, T. T., III, Gerard, N. P., Gerard, C., and Simon, M. I. (1993) J. Biol. Chem. 268, 10139–10144
19. Nakamura, M., Honda, Z., Izumi, T., Skanaka, C., Mutoh, H., Minami, M., Bito, H., Seyama, Y., Matsumoto, T., Noma, M., and Shimizu, T. (1991) J. Biol. Chem. 266, 20400–20405
20. Bito, H., Honda, Z., Nakamura, M., and Shimizu, T. (1994) Eur. J. Biochem. 221, 211–218
21. Chatterjee, T. K., Sarkar, G., Bolander, M., and Fisher, R. A. (1995) Methods in Neuroscience, Academic Press, New York
22. Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., and Journot, L. (1993) Nature 365, 170–175
23. Brown, E., Kendall, D., and Nahorski, S. (1984) J. Neurochem. 42, 1379–1387
24. Takano, T., Honda, Z., Sakanaka, C., Izumi, T., Kameyama, K., Haga, K., Haga, T., Kurokawa, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 22453–22458
25. Luttrell, L. M., Ostrowski, J., Kjelsberg, M. A., Caron, M. G., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 1430–1433
26. Samama, P., Cotechia, S., Costa, T., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 4625–4636
27. Koch, W. J., Hawes, B. E., Inglesie, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 15776–15785
28. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. F. (1994) Annu. Rev. Biochem. 63, 101–132
29. Liggett, S. B., Caron, M. G., Lefkowitz, R. J., and Hnatowich, M. (1991) J. Biol. Chem. 266, 4816–4821
30. Wang, Z.-F., and Ross, E. M. (1994) J. Biol. Chem. 269, 18968–18976
31. Schreiber, R. E., Prossnitz, E. R., Ye, R. D., Cochrane, C. G., and Bokoch, G. M. (1994) J. Biol. Chem. 269, 326–331
32. Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993) Nature 365, 166–170
33. Wang, C., Jayadev, S., and Escobedo, J. A. (1995) J. Biol. Chem. 270, 16677–16682
34. Nunnally, John, D., and Gershengorn, M. C. (1994) J. Biol. Chem. 269, 28123–28129
35. Takagi, Y., Ninomiya, H., Sakamoto, A., Miwa, S., and Masaki, T. (1995) J. Biol. Chem. 270, 10072–10078
36. Wang, Z., Wang, H., and Ascoli, M. (1993) Mol. Endocrinol. 7, 85–93
37. Strader, C. D., Fong, T. M., Graziano, M. P., and Tota, M. R. (1995) FASEB J. 9, 745–754
38. Kjelsberg, M. A., Cotechia, S., Ostrowski, J., Caron, M. G., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 1430–1433
39. Samama, P., Cotechia, S., Costa, T., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 16483–16487
40. Ren, Q., Kurose, H., Lefkowitz, R. J., and Cotechia, S. (1993) J. Biol. Chem. 268, 16483–16487
41. Ostrowski, J., Kjelsberg, M. A., Caron, M. C., and Lefkowitz, R. J. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 167–183
42. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. F. (1994) Annu. Rev. Biochem. 63, 101–132
43. Wang, S. K.-F., and Ross, E. M. (1994) J. Biol. Chem. 269, 18968–18976
44. Schreiber, R. E., Prossnitz, E. R., Ye, R. D., Cochrane, C. G., and Bokoch, G. M. (1994) J. Biol. Chem. 269, 326–331
45. Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993) Nature 365, 166–170
46. Schreiber, R. E., Prossnitz, E. R., Ye, R. D., Cochrane, C. G., and Bokoch, G. M. (1994) J. Biol. Chem. 269, 326–331
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