The role of the third intracellular loop of the neutrophil *N*-formyl peptide receptor in G protein coupling

Eric R. PROSSNITZ, Oswald QUEHENBERGER, Charles G. COCHRANE and Richard D. YE*

Department of Immunology, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037, U.S.A.

The G-protein-coupled *N*-formyl peptide receptor (FPR) contains one of the smallest known third intracellular loops of this class of receptors, consisting of only 15 amino acids. To study the role of this region of the receptor in G protein coupling and signal transduction, we generated a deletion mutant (D3i) in which 10 amino acids of the loop were removed, as well as a series of site-directed mutants containing substitutions of the charged and polar amino acids of this loop. The D3i mutant, expressed at normal levels on the cell surface, displayed a K_D for labelled *N*-formyl-Met-Leu-Phe ($[^3H]$ FMLP) of 165 nM. This value compares with a K_D for the wild-type FPR of 1.0 nM, or 20 nM in the presence of guanosine 5'-[γ -thio]triphosphate, which uncouples G proteins from the receptor. These results indicate that D3i contains significant structural defects, beyond the disruption of G protein coupling, that affect ligand binding properties. Ten site-directed mutants generated in the third

intracellular loop (T²²⁶A, K²²⁷E, H²²⁸A, K²³⁰Q, K²³⁵Q, S²³⁶A, S²³⁶A/S²³⁷G, R²³⁸G, R²⁴¹E and S²⁴⁴A) displayed K_D values between 0.5 and 1.0 nM, with expression levels between 22 % (K²²⁷E) and 111 % (H²²⁸A) of that of wild type receptor. The capacity of the mutants for signal transductions was determined by measuring intracellular Ca²⁺ mobilization. Eight of the ten mutants displayed EC₅₀ values for FMLP of between 0.07 and 0.9 nM, as compared with 0.12 nM for the wild-type receptor. The two mutants K²²⁷E and R²³⁸G had EC₅₀ values of 2.7 and 2.9 nM respectively. The increase in EC₅₀ could be accounted for partially by the low levels of receptor expression. All ten mutants gave maximum levels of Ca²⁺ mobilization similar to that produced by the wild-type FPR. These results contradict the conclusions reached with other G-protein-coupled receptors and indicate that the third intracellular loop of the FPR does not have a critical role in the functional coupling of G proteins.

INTRODUCTION

The *N*-formyl peptide receptor (FPR) belongs to the class of G-protein-coupled receptors containing seven transmembrane regions. The number of cloned receptors in this class is greater than 100 and continues to grow rapidly (for a review of the sequences of known G protein coupled receptors, see [1]). The first such receptor to have its amino acid sequence determined was rhodopsin [2,3]. Numerous adrenergic, muscarinic and 5-hydroxytryptamine receptors have been subsequently cloned (e.g. [4–6]). In contrast to rhodopsin, these receptors, though members of the same class, are considerably larger. Whereas rhodopsin has 348 amino acids, the adrenergic, muscarinic and 5-hydroxytryptamine receptors consist of between 402 and 590 amino acids. These size differences are found almost exclusively in the third intracellular loop and the C-terminus. The third intracellular loop of rhodopsin is predicted to consist of 22 amino acids; this same loop in the adrenergic receptors varies between 54 (β_2) and 180 (α_2C_2) amino acids, and in the muscarinic receptors it varies between 157 (m_1) and 240 (m_3) amino acids. Within this same group of receptors the C-terminus varies between about 20 and 164 amino acids. In contrast to the pattern represented by the adrenergic receptors, a large number of receptors have been cloned in the last 3 years that contain third intracellular loops with ~16–30 amino acids and C-termini with ~16–50 amino acids. This group includes receptors for *N*-formyl peptides, C5a, thrombin, interleukin-8, platelet-activating factor, bradykinin, vasoactive intestinal peptide, substance K and a collection of odourant receptors.

Deletion mutagenesis has revealed that multiple segments of the third intracellular loop of the adrenergic receptors are critical for G protein coupling [7,8]. The analysis of chimaeric receptors formed from the β_2 and α_2 adrenergic receptors (which couple to G_s and G_i respectively) further implicates the third intracellular loop as being important in G protein specificity [9]. Additionally, chimaeric receptors in which almost the entire third intracellular loop of the phospholipase-stimulating α_1 adrenergic receptor replaced that same region of the β_2 adrenergic receptor were capable of stimulating phosphatidylinositol hydrolysis in response to β agonists [10]. In other experiments, peptides corresponding to the C-terminal portion of the third intracellular loop were able to disrupt α_2 adrenergic receptor–G protein interactions [11]. These observations indicate an important role for the third intracellular loops of G-protein-coupled receptors in signal transduction.

In this paper, we present the results of studies with mutations created in the third intracellular loop of the FPR. The FPR is of interest because it has one of the smallest third intracellular loops known to date, predicted to consist of only 15 amino acids, and little is currently known about the structure–function relationships of receptors in this class. We predicted that if the third intracellular loop of G-protein-coupled receptors is involved in signal transduction, then the sequence of this loop of the FPR would constitute a core structure contributing to the activation of G proteins. To address this, we created a deletion mutant and systematically mutated the charged and polar amino acids of the loop and analysed the resulting receptors for their ability to couple to G proteins. Our present results and others that we have

Abbreviations used: FPR, *N*-formyl peptide receptor; FMLP, *N*-formyl-methionine-leucine-phenylalanine; FLPEP, *N*-formyl-norleucine-leucine-phenylalanine-norleucine-tyrosine-lysine-fluorescein; GTP[S], guanosine 5'-[γ -thio]triphosphate

* To whom correspondence and reprint requests should be addressed.

obtained [12] indicate that the third intracellular loop of the FPR is not crucially involved in the signal transduction process.

MATERIALS AND METHODS

Materials

The mouse L cell fibroblast line 2071 was obtained from American Type Culture Collection, Bethesda, MD, U.S.A. Reagents for DNA mutagenesis were purchased from Amersham, Arlington Heights, IL, U.S.A. Oligonucleotides were synthesized by Operon Technologies, Alameda, CA, U.S.A. *N*-Formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein (FLPEP) was obtained from Molecular Probes, Eugene, OR, U.S.A., and ³H-labelled *N*-formyl-Met-Leu-Phe ([³H]FMLP) was from New England Nuclear, Cambridge, MA, U.S.A. Restriction enzymes were purchased from Gibco BRL, Gaithersburg, MD, U.S.A.; and from New England Biolabs, Beverly, MA, U.S.A.

Mutagenesis and expression of the human FPR cDNA

Oligonucleotide primers were synthesized based on the published FPR cDNA sequence [13,14]: 5'-GCGAATCCCCAGCCATG GAGACAAATTCCTCTCTC-3' (corresponding to the 5' FPR cDNA coding sequence with the initiation codon underlined and containing an upstream translation initiation sequence [15] and an *Eco*RI restriction site) and 5'-ACGAATTCATTACTT-TGCCTGTAATC-3' [corresponding to the 3' FPR cDNA coding sequence and containing two stop codons (underlined) and an *Eco*RI restriction site]. These primers were used in the PCR to amplify the FPR coding sequence from a 1.6 kb FPR cDNA originally isolated from an HL-60 cell cDNA library. The PCR product was digested with *Eco*RI and the 1.1 kb fragment was subcloned into the same site in the polylinker of phage M13mp18. Single-stranded M13 DNA, in conjunction with 20–29mer oligonucleotides (containing one or two altered nucleotides or containing a gap in the sequence to generate deletions), was used in the Amersham oligonucleotide-directed mutagenesis system to introduce the desired mutations into the FPR gene. Plaques were amplified and the mutations were confirmed by dideoxy sequencing. For expression, the mutated FPR genes were subcloned into the *Eco*RI site of the vector SFFV.neo, which contains the selectable marker aminoglycoside transferase [16]. Mouse L cell fibroblasts 2071 (CCL 1.1; American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 5% newborn bovine serum and 2 mM L-glutamine. Approx. 8×10^5 cells were plated out in a 25 cm² flask 20 h prior to transfection with 10 µg of linearized vectors by the calcium phosphate precipitation method [17]. Transfectants were selected by their resistance to G418 sulphate (Gibco BRL) at an active drug concentration of 0.35 mg/ml. For each mutant, approx. 20–50 individual colonies from transfected cells were pooled for analysis.

Ligand binding

Cells were harvested using trypsin-free dissociation buffer (Gibco BRL), washed once with PBS, and resuspended to 10^6 cells/ml in RPMI-1640 containing 0.5% BSA (binding buffer). Equilibrium binding was carried out in 0.5 ml of the binding buffer with 5×10^5 cells and FLPEP at the desired concentrations, in the absence or presence of 5 µM unlabelled *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys. Following incubation for 60 min on ice, the cells

were analysed on a FACSCAN flow cytometer (Becton Dickinson) for fluorescence intensity. Mean fluorescent intensity was determined as a function of ligand concentration. Binding data were analysed by the method of Scatchard to obtain the K_D and the relative number of binding sites per cell (B_{max}). Using [³H]FMLP, the absolute B_{max} for cells expressing the wild-type FPR was determined to be about 1 pmol/mg of protein. Binding of the ligand [³H]FMLP was determined as described [18]. Briefly, membranes of transfected cells were prepared by nitrogen cavitation and isolated by centrifugation [19]. Membranes (20–30 µg) were incubated with various concentrations of [³H]FMLP, in the presence or absence of excess unlabelled FMLP (100-fold). Where indicated, guanosine 5'-[γ-thio]-triphosphate (GTP[S]) was added to 100 µM. After 60 min at room temperature, the binding was stopped by filtration through Whatman GF/C filters. Retained radioactivity was counted by scintillation spectrometry. Binding data were analysed by the Scatchard method with the aid of the LIGAND computer program [20].

Measurement of Ca²⁺ mobilization

Cells were harvested as described for ligand binding, washed once with PBS and resuspended to 5×10^5 /ml in RPMI-1640 medium containing 10% fetal bovine serum. The cells were incubated with 5 µM indo-1/AM (Molecular Probes) at 37 °C for 25 min. Following a brief wash with the same medium, cells were resuspended to 1×10^6 /ml in RPMI-1640 (lacking Phenol Red) containing 10% fetal bovine serum and maintained at room temperature until use. Continuous measurements of calcium-bound and free indo-1 fluorescence were made using an SLM 8000 photon counting spectrofluorometer (SLM-Aminco) detecting at 400 nm and 490 nm respectively, with an excitation wavelength of 340 nm. For each determination, the response to agonist was taken as the peak fluorescence. For high concentrations of ligand, this occurred within 5 s after the addition of ligand, whereas for low concentrations of ligand, it occurred between 20 and 30 s from the ligand addition. Dose-response data were analysed using a least-squares non-linear regression program of Sigmaplot (Jandel Scientific) with the equation $y = a + \{(b - a) / [1 + (10^c / 10^d)^4]\}$, where a and b represent the bottom and top plateaus of the curve respectively, x is the log(ligand concentration), c is the logEC₅₀ and d is the slope factor (here taken as 1).

Immunofluorescence

Cells were grown on coverslips and fixed with 2% formaldehyde. The cells were permeabilized with 0.1% saponin in PBS. The same solution containing 5% BSA was used in all subsequent steps. The cells were incubated with the anti-FPR antiserum αC12 [18] at a 1:500 dilution for 10 min. After three washes, goat anti-rabbit antibody conjugated with rhodamine B was added at a 1:200 dilution for 10 min. After three final washes, the specimens were mounted on glass slides with 60% glycerol and 2.5% 1,4-diazabicyclo[2,2,2]-octane (Sigma). Cells were examined with an epifluorescence microscope (Carl Zeiss, Inc.), using a filter set selective for rhodamine. Photographs were taken on 400 ASA Ektachrome film.

RESULTS

The third intracellular loop of G-protein-coupled receptors has been implicated in G protein activation (reviewed in [21]). Because the entire third intracellular loop of the FPR (Figure 1) is smaller

Figure 1 Schematic representation of the third intracellular loop of the FPR

The third intracellular loop is depicted as protruding from the membrane (stippled region) into the cytoplasm. The amino acids (one-letter abbreviations) that are the focus of this work are numbered, with mutated amino acids indicated by the arrows.

than the sum of the regions within this same loop implicated to be critical for G protein coupling in other receptors, such as the β -adrenergic receptor, we hypothesized that within the FPR this region may represent a core sequence necessary for G protein activation. In order to study the role of the third intracellular loop in signal transduction, we constructed a deletion mutant, D3i (a deletion of K²³⁰–P²³⁹; see Figure 1), in which 10 residues of this loop were removed. Using previously established methods [16], we transfected the mutant as well as the wild-type FPR into mouse L cell fibroblasts, which are devoid of any endogenous receptors capable of responding to FMLP or related ligands. Flow cytometry was used to examine the expression characteristics and homogeneity of the cell population. Using FLPEP we observed that greater than 90 % of the wild-type-FPR-transfected cells expressed ligand binding sites (results not shown). This demonstrated that the transfected cells represented a homogeneous cell population capable of binding the ligand. Using the same procedure, we were unable to detect binding of the fluoresceinated ligand to D3i-transfected cells. This indicated either that the mutated receptor was not expressed or that the binding affinity was lower than the detection limit of flow cytometry using the described procedure (≤ 50 nM; see [22]). To examine whether the D3i mutant was expressed we used immunofluorescence microscopy with an antibody directed against the C-terminal 12 amino acids of the FPR. Like the cells transfected with wild-type FPR, D3i-transfected cells showed binding of the antibody at the periphery of the cells (Figure 2). This suggests that the D3i mutant receptor is expressed at the surface of the transfected cells. Using [³H]FMLP, which can be employed at concentrations higher than those for FLPEP, we observed specific binding but with characteristics significantly different from those of the wild-type receptor (Table 1). The dissociation constant of D3i (K_D 165 nM) is higher than that of the wild-type FPR measured in the presence of GTP[S] (K_D 20 nM), which uncouples the high-affinity receptor–G protein complex [23]. Similarly, no FMLP-induced calcium mobilization was observed in cells expressing the D3i mutant (results not shown). These findings suggest that, in addition to any possible effects due to a disrupted

Figure 2 Expression of the D3i deletion mutant

Immunofluorescence staining of mock-transfected L cells (a), wild-type FPR-transfected L cells (b) and D3i-transfected L cells (c). Staining was carried out as described in the Materials and methods section using an antiserum directed against the C-terminal 12 amino acids of the FPR.

receptor–G protein interaction, deletion of 10 of the 15 amino acids in the third intracellular loop of the FPR altered global receptor structure in such a way as to reduce ligand binding affinity.

Because the results obtained with the D3i mutant do not allow a conclusion to be drawn regarding the direct involvement of the third intracellular loop of the FPR in G protein coupling and signal transduction, we constructed point mutations of the charged and polar amino acids within the third intracellular loop that are likely to interact with G proteins because of their probable solvent accessibility. Substitution of one or two amino acids does not alter the size of the relatively short third

Table 1 Ligand binding properties of wild-type and D3i deletion mutant FPRs

K_d and B_{max} values for FMLP [3 H]P binding were determined from equilibrium binding experiments as described in the Materials and methods section. Where one K_d value is given, there was no statistical improvement with a two-site model. Where two K_d values are provided, a two-site model produced a superior fit to the experimental data. Values are representative of duplicate experiments.

Receptor	K_d (nM)	B_{max} (fmol/mg)
Wild-type FPR	1.0	1200
Wild-type FPR + GTP[S]	0.5	80
	20	400
D3i mutant	165	1100

Table 2 Ligand binding and agonist-mediated Ca^{2+} mobilization by wild-type and mutant FPRs

Ligand binding parameters were determined using FLPEP as described in the Materials and methods section. Maximum Ca^{2+} mobilization is expressed as a percentage of that induced by ATP.

Receptor	Relative B_{max}	K_d (nM)	Ca^{2+} mobilization EC_{50} (nM)	Maximum Ca^{2+} mobilization (%)
Wild-type FPR	100 $\pm$ 5	1.4 $\pm$ 0.2	0.12 $\pm$ 0.04	75 $\pm$ 8
T ²²⁶ A	67 $\pm$ 3	0.6 $\pm$ 0.2	0.07 $\pm$ 0.02	74 $\pm$ 10
K ²²⁷ E	22 $\pm$ 4	0.7 $\pm$ 0.3	2.7 $\pm$ 0.4	87 $\pm$ 7
H ²²⁹ A	111 $\pm$ 9	0.8 $\pm$ 0.2	0.5 $\pm$ 0.3	76 $\pm$ 3
K ²³⁰ Q	58 $\pm$ 10	0.8 $\pm$ 0.1	0.5 $\pm$ 0.2	94 $\pm$ 9
K ²³⁵ Q	27 $\pm$ 4	0.9 $\pm$ 0.2	0.9 $\pm$ 0.2	89 $\pm$ 3
S ²³⁶ A	56 $\pm$ 2	1.0 $\pm$ 0.2	0.8 $\pm$ 0.3	80 $\pm$ 3
S ²³⁶ A/S ²³⁷ G	45 $\pm$ 4	0.9 $\pm$ 0.2	0.2 $\pm$ 0.1	84 $\pm$ 10
R ²³⁸ G	62 $\pm$ 3	0.7 $\pm$ 0.1	2.9 $\pm$ 0.9	84 $\pm$ 12
R ²⁴¹ E	38 $\pm$ 2	0.6 $\pm$ 0.1	0.3 $\pm$ 0.2	93 $\pm$ 6
S ²⁴⁴ A	69 $\pm$ 3	0.5 $\pm$ 0.2	0.2 $\pm$ 0.1	92 $\pm$ 7

intracellular loop (schematically shown in Figure 1), and should have minimal impact on the conformation of this loop and on the global structure of the FPR. For most of the mutants, we attempted merely to remove or replace the predominant structural property of the side chain (e.g. K $\rightarrow$ Q or T $\rightarrow$ A). However, for the two charged amino acids at the transmembrane boundaries (K²²⁷ and R²⁴¹), we elected to maintain charged amino acids at these positions with the aim of maintaining receptor topology and structure. We therefore substituted acidic residues for these basic ones, since acidic residues are present at other transmembrane boundaries within the receptor. The presence of two serines juxtaposed at positions 236 and 237 led us to speculate that either one alone might be sufficient to enable receptor function. For this reason, in addition to the mutant S²³⁶A, i.e. Ser²³⁶ replaced with alanine, we created the double mutant S²³⁶A/S²³⁷G instead of the two individual single mutations.

Analysis of the binding properties of the mutated forms of the FPR was carried out by flow cytometry. The FPR in neutrophils displays a K_D in the range 0.5–1.0 nM [22,24,25]. The recombinant wild-type FPR expressed in L cells displayed a K_D of 1.4 nM (Table 2). With this method, we estimated transfected fibroblasts to express between 70 000 and 80 000 wild-type FPRs

per cell, a number similar to the neutrophil FPR expression level [22]. Mutant FPR expression levels varied by 5-fold, from 22 % to 111 % of the wild type. This variation is typical for the transfected cells and does not significantly affect the efficacy of ligand-induced signal transduction mediated by the receptor (see text below and Table 3). Mutations at the presumed transmembrane boundaries, i.e. K²²⁷E and R²⁴¹E (along with K²³⁵Q, in the middle of the loop), resulted in the lowest FPR expression. The remaining mutants fell within a 2-fold range of expression compared with the wild-type receptor. Only one receptor mutant, H²²⁹A, was expressed at slightly higher levels than the wild-type receptor. The dissociation constants determined for the mutant receptors ranged from 0.5 to 1.0 nM; the dissociation constant for the wild-type receptor was 1.4 nM (Table 2). This is consistent with the idea that mutations on the cytoplasmic face of the receptor should not have direct effects on the ligand binding pocket, which might be expected with mutations in the extracellular or intramembranous regions. The lack of observable effects of the mutations on the ligand binding properties indicates that these mutations do not indirectly alter the interaction of the receptor with ligand.

The ability of these mutant receptors to transduce binding signals was determined by assaying ligand-stimulated Ca^{2+} mobilization. In neutrophils and FPR-transfected L cells, binding of ligand to the receptor results in the activation of the pertussis toxin-sensitive G protein, which then activates a phospholipase C. The $InsP_3$ thus generated causes the release of Ca^{2+} from intracellular stores (for a review, see [26]). Thus the ligand concentration required for a half-maximal response (EC_{50}) can be used as a measure of the coupling of the mutant receptors to G proteins. The wild-type FPR expressed in L cells mobilized Ca^{2+} with an EC_{50} of 0.1–0.2 nM FMLP, whereas L cells not expressing the receptor exhibited no Ca^{2+} response at ligand concentration up to 100 μ M. Most of the mutants displayed EC_{50} values in the range 0.07–0.50 nM FMLP (Table 2), not substantially different from that of the wild-type receptor. Two mutants, K²³⁵Q and S²³⁶A, displayed EC_{50} values of 0.9 and 0.8 nM respectively. Of these, K²³⁵Q expressed low levels of receptor, which could result in the observed increase in EC_{50} . This is also true for K²²⁷E (EC_{50} 2.7 nM), which carries the mutation at the transmembrane boundary (Figure 1). Reversal of the charge at this position could affect functional coupling indirectly through deleterious changes in conformation. In addition to the S²³⁶A mutation, we also assayed a double mutation of S²³⁶A and S²³⁷G. This double mutant was expressed at levels comparable with those of the single mutant S²³⁶A and displayed an EC_{50} very similar to that of the wild-type FPR. This would suggest that either the presence or absence of both serine residues is required for optimal receptor function, but that the lack of only one serine results in a small impairment of receptor function. The final mutation to have a significant effect on transmembrane signalling was R²³⁸G. This mutant had an expression level and K_D values similar to those of the wild-type receptor, but it displayed an EC_{50} of 2.9 nM, a 20-fold increase as compared with the wild-type receptor.

Receptor expression levels might be expected to influence the EC_{50} in the following manner if the only determining factor in the transmission of the signal were receptor occupancy. Cells expressing fewer receptors would require higher ligand concentrations to achieve a given level of receptor occupancy compared with cells expressing high levels of receptor. This has previously been investigated by isolating individual clones which inherently express varying amounts of stably transfected receptor [21], by transiently transfecting COS-7 cells with varying amounts of DNA [27], or by expression in *Xenopus* oocytes [8]. We

Table 3 Effect of FPR expression level on FMLP stimulated Ca^{2+} mobilization

The FPR expression level is given as a percentage of that in the unsorted cell population. Maximum Ca^{2+} mobilization is expressed as a percentage of that induced by ATP.

Wild-type FPR expression level (%)	Ca^{2+} mobilization EC_{50} (nM)	Maximum Ca^{2+} mobilization (%)
25 ± 2	0.5 ± 0.1	71 ± 8
100 ± 5	0.16 ± 0.06	73 ± 7
170 ± 10	0.17 ± 0.07	74 ± 8

isolated two additional populations of cells expressing the wild-type FPR by sorting a cell population with a flow cytometer. In this way, we obtained cells expressing an average of 25 % and 170 % of the receptors of the unsorted population. The lower expression level was comparable with the lowest expression levels of the site-directed mutants (K^{227}E and K^{235}Q). The cell population with lower expression level displayed an EC_{50} 2–3-fold greater than that of the unsorted cells (Table 3). However, EC_{50} values for the highly expressing population (170 %) and the unsorted cells (100 %) were similar. This suggests that for the mutants such as R^{241}E (38 % receptor expression) and K^{235}Q (27 % receptor expression) the small increases in EC_{50} can largely be accounted for by the differences in receptor expression levels. However, for the mutant K^{227}E (22 % receptor expression) only a portion of the increase in EC_{50} can be attributed to expression levels. Furthermore, for the mutant R^{238}G the expression level (62 %) is similar to that of mutants with EC_{50} values almost identical to that of the wild type receptor (e.g. T^{226}A , 67 % receptor expression, EC_{50} 0.07 nM; S^{244}A , 69 % receptor expression, EC_{50} 0.2 nM). Thus mutants K^{227}E (EC_{50} 2.7 nM) and R^{238}G (EC_{50} 2.9 nM) do exhibit alterations in their signal transduction properties that are unrelated to receptor expression levels. Receptor efficacy, as measured by the maximum level of Ca^{2+} mobilization at saturating concentrations of ligand, reflects the ultimate potential of the receptor to activate G proteins. Maximum Ca^{2+} mobilization was determined relative to that due to an unrelated receptor, the endogenous purinergic receptor, which mobilizes Ca^{2+} but through a pertussis toxin-insensitive G protein. The response of this receptor to its ligand, ATP, should be independent of the presence of the wild-type or mutated forms of the FPR and thus serves as a standard against which the maximum responses of the FPR can be compared. The wild-type FPR displayed a maximum Ca^{2+} response which was 75 % of the response of the same cells to ATP. The mutant receptors displayed maximum responses in the range 74–94 %, equal to or slightly greater than that of the wild-type receptor. This indicates that, although in some cases higher concentrations of ligand may be required, the potential of the mutant receptors to activate G proteins has not been affected.

DISCUSSION

A large body of evidence indicates that the third intracellular loop of many G-protein-coupled receptors is required for G protein activation and is able to confer selectivity among the second messenger systems. The receptors used for such studies, primarily the α and β adrenergic and muscarinic receptors, have third intracellular loops that range in size from 54 to 240 amino

acids. In the past 3 years, a large number of novel G-protein-coupled receptors have been cloned and the majority of these have small third intracellular loops, in the range 15–30 amino acids. Thus it becomes necessary to study the function of these smaller third intracellular loops in G protein coupling.

In this study we have examined the role of the third intracellular loop of the FPR in G protein coupling. We have systematically substituted charged and polar residues with uncharged or oppositely charged ones, and analysed the resulting mutants with ligand binding and Ca^{2+} mobilization assays. Based on the results presented in this paper, we conclude that the process of G protein activation by the FPR is probably not dependent on any electrostatic or polar interaction between the third intracellular loop of the FPR and its G protein. Of the mutations we studied, only two (K^{227}E and R^{238}G) were significantly altered in their dose-response properties, having EC_{50} values in the 2–3 nM range. This represents a 10–20-fold shift to the right compared with the wild-type FPR (EC_{50} 0.1–0.2 nM). Of these, mutant K^{227}E is located on the transmembrane boundary and was determined to express a low number of binding sites on the surface of the cell. Mutations at the transmembrane boundary have previously been shown to have deleterious effects on the expression of receptors, possibly due to alterations in receptor structure [28]. The lack of a decrease in efficacy of Ca^{2+} mobilization for the FPR mutant K^{227}E suggests that such alterations would be minimal in this case. Furthermore, the mutant R^{241}E at the transmembrane boundary at the opposite end of the third intracellular loop behaved normally, suggesting that this effect is not universal. The FPR mutant R^{238}G , located towards the C-terminal end of the third intracellular loop, has an EC_{50} of 2.9 nM, but the fact that maximal efficacy seen with this mutant is the same as that for the wild-type FPR indicates that the mutation does not result in a critical impairment of receptor function.

We compared our results with those from studies of rhodopsin, which contains a third intracellular loop of 22 amino acids. Conflicting reports regarding the involvement of this region have been reported. König et al. [29] found that peptides corresponding to the second and third intracellular loops and the N-terminal portion of the C-terminal tail were able to compete with meta-rhodopsin II for the formation of a complex with G_t , the G protein that couples to rhodopsin. On the contrary, Weiss et al. [30] demonstrated that G_t was not able to prevent the binding of an antibody directed against the third intracellular loop of rhodopsin, leading to the conclusion that a significant portion of the third intracellular loop does not interact directly with G_t . Recent mutagenesis studies have demonstrated that deletions of between 20 and 90 % of the third intracellular loop result in a rhodopsin able to bind the ligand retinal but unable to activate G_t [31]. Interestingly, a deletion of 13 amino acids did not prevent G_t from binding to rhodopsin [32,33]. Extensive point mutagenesis showed that only substitution of the two serine residues and a threonine residue adversely affected G protein coupling [31]. These mutagenesis results substantiate our conclusion that the third intracellular loop plays a limited role in signal transduction.

A common feature of the small third intracellular loops is the prevalence of basic residues within the loop. This is reminiscent of mastoparan, a wasp venom peptide (INLKALAALAKKIL) capable of directly stimulating G proteins [34,35]. However, mastoparan contains only two sites of basic residues and is otherwise very hydrophobic, providing it with the capability of disrupting membrane structures [36]. The ability of mastoparan to bind to and stimulate G proteins, thus mimicking receptor function, may be a result of its structural similarity to non-

Figure 3 Size distribution of the third intracellular loop versus the C-terminal tail of G-protein-coupled receptors

The receptors are coded as follows: m_1 – m_5 , human m_1 – m_5 muscarinic acetylcholine; oct, *Drosophila* octopamine; D_1 – D_5 , human dopamine D_1 – D_5 ; α_1 , α_{1B} , α_2 (includes C2, C4, C10 and R20 subtypes), α -adrenergic of various sources; β_1 – β_3 , human β -adrenergic; S_{1a} , S_{1c} , S_{1d} and S_2 , human and rat 5-hydroxytryptamine (serotonin); Olf, 10 odourant; Op, human rhodopsin and red, blue and green opsins; Tb, human thromboxane A_2 ; Nt, rat neurotensin; A_1 and A_2 , dog adenosine subtypes; Trh, mouse thyrotropin-releasing hormone; Nk, human neurokinin A; P, rat substance P; K, rat neuromedin K; cAMP, *Dictyostellium* cyclic AMP. The oval includes the following 16 receptors: human FPR, C5a, thrombin, interleukin-8, vasoactive intestinal peptide, *mas* oncogene (angiotensin), lutropin–choriogonadotropin, thyrotropin and follicle-stimulating hormone; rat bradykinin, neuromedin B-preferring bombesin and non-isopeptide-selective endothelin; bovine adrenal angiotensin II type I and endothelin I; mouse bombesin/gastrin-releasing peptide; and guinea-pig (and human) platelet-activating factor. For complete sequences and references, see Probst et al. [1].

contiguous regions of G-protein-coupled receptors, which contain basic residues throughout their intracellular surfaces. However, numerous G-protein-coupled receptors contain an almost equal distribution of basic and acidic amino acids within their third intracellular loops (e.g. the opsins and the substance P receptor), indicating that a basic third intracellular loop cannot be essential for G protein interactions.

It has recently been shown that the insulin-like growth factor II/mannose 6-phosphate receptor can couple to G_i proteins, despite the fact that the receptor is a single membrane-spanning protein [37,38]. A sequence of 14 amino acids within the cytoplasmic domain of this receptor was found to have the ability to activate G_i protein [39]. The activity of peptides corresponding to this region depended upon the presence of two basic amino acids at the N-terminal side and the sequence BBXB or BBXXB (where B is a basic residue and X is not) at the C-terminal end. When a search for this sequence pattern was carried out, it was found to be present in the second or third intracellular loop or C-terminal portion of the muscarinic, α_2 -adrenergic and dopaminergic receptors [40]. Furthermore some, but not all, peptides meeting these criteria were capable of directly stimulating G protein activity. When receptors with small third intracellular loops, including the FPR, are searched for this sequence pattern, it is almost non-existent within the intracellular loops, suggesting these receptors utilize different sequence motifs to activate G proteins.

The muscarinic and α_2 -adrenergic receptors studied above have third intracellular loops in excess of 150 amino acids. It is therefore not unanticipated that receptors with third intracellular loops of less than 30 amino acids may utilize different sequences or rely more on sequences of other intracellular loops and the C-

terminus for G protein recognition and activation. In addition to the third intracellular loop, the C-termini of G-protein-coupled receptors also vary greatly in size, from 14 amino acids (D_2 and D_3 dopamine receptors) to 164 amino acids (α_{1B} -adrenergic receptor). The best studied receptors (adrenergic and muscarinic) have large third intracellular loops despite the fact that a majority of G-protein-coupled receptors possess third intracellular loops with less than about 30 amino acids. The distribution of amino acids between the third intracellular loop and the C-terminus of a large number of known G-protein-coupled receptors is shown in Figure 3. Of interest is the observation that receptors with large third intracellular loops do not have large C-termini, and conversely those receptor with large C-termini do not have large third intracellular loops. It should be noted here that the first and second intracellular loops of all these receptors do not vary substantially in size. This lack of receptors with both large third intracellular loops and C-termini may suggest that within the three-dimensional structure of G-protein-coupled receptors these two regions occupy the same space, and therefore if one is large the other must be small so as to allow the proper structure and function of the receptor. The reason for the existence of large third intracellular loops or C-termini is not clear, especially given the fact that most receptors lack both. It can be speculated that the function of these large domains may involve receptor regulation or desensitization. Whatever the function of these large domains, the presence of such regions in either the third intracellular loop or the C-terminus may also suggest that the position of this region within the linear sequence is not critical. Extrapolation of this idea would suggest that the location of regions involved in G protein coupling may vary between different receptors.

Recently we obtained results which confirm those reported above [12]. Using synthetic peptides corresponding to the intracellular loops and portions of the C-terminus and a fusion protein containing the C-terminal 47 amino acids of the FPR, we investigated the physical association of FPR subdomains with G proteins. The results indicate that the second intracellular loop and the N-terminal portion of the C-terminus, but not the third intracellular loop, are involved in G protein binding. Further studies will determine whether these results may be extended to other receptors with small third intracellular loops. An analysis of the sequences involved in G protein coupling by these receptors may define a novel mode of G protein coupling.

We thank Stacey Cavanagh for her expert technical assistance. This work was supported in part by National Institutes of Health Grant GM46572. E.R.P. is the recipient of an International Human Frontiers Science Program fellowship. O.Q. is supported by an American Heart Association Fellowship, California Chapter. This is publication # 7624-IMM from the Department of Immunology, the Scripps Research Institute.

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