Mutations of Two Adjacent Amino Acids Generate Inactive and Constitutively Active Forms of the Human Platelet-activating Factor Receptor*

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We have mutated two residues, Ala230 and Leu231, in the C-terminal portion of the third intracellular loop of the human platelet-activating factor (PAF) receptor into Glu230 and Arg231, respectively. The L231R mutant was able to adopt at least two conformations: (i) a higher affinity state than the corresponding state of the wild-type receptor (WT), dependent on G protein coupling, and (ii) a low affinity state, higher than the one for the uncoupled WT receptor. The Ala230 → Glu230 substitution also resulted in two major modifications: 1) unresponsiveness in terms of phosphatidylinositol hydrolysis in response to PAF and 2) a marked decrease in affinity of the receptor for binding the agonist but not the antagonist. Competition binding studies of transient receptor expression in COS-7 cells and the inability of guanosine 5′-O-(3-thiotriphosphate) to modulate the decrease in affinity of a stable A230E mutant in Chinese hamster ovary cells suggest an inherent low affinity conformation for this mutant. Alternatively, mutation of Ala230 to Gln230 suggested that the residue 230 has a fundamental effect on receptor affinity and its charge is determinant in G protein coupling of the PAF receptor. In this report, we show that substitution of two immediately adjacent residues of the PAF receptor, Ala230 and Leu231, surprisingly leads to an inactive and a constitutively active phenotype, respectively. These results further support the concept of constitutively active G protein-coupled receptors as adopting “active” state conformations similar to those induced by agonist binding to WT receptors.

Platelet-activating factor (PAF), 1 identified as 1-O-alkyl-sn-glycero-3-phosphocholine, is a phospholipid released from stimulated basophils, platelets, macrophages, polymorphonuclear neutrophils, and other cell types (1). PAF is a potent mediator with numerous biological activities related to inflammatory and immune responses, as well as cardiovascular, reproductive, respiratory, and nervous system physiology (1, 2). PAF exerts its action by binding to a specific, high affinity receptor on the target cell surface. This receptor is stereospecific and PAF-dependent cellular responses can be inhibited by a variety of structurally distinct PAF antagonists (2). PAF binding has been found on several cell types and cDNA cloning from various sources revealed that the PAF receptor belongs to the G protein-coupled receptor superfamily (3–7). The PAF receptor couples with various second messenger systems, including phospholipases A, C, and D activation (8–10) and activation of the mitogen-activated protein kinase cascade (9, 11–13).

For the majority of the G protein-coupled receptors studied so far, the ligand-binding pocket appears to be formed by the transmembrane domains of the peptide chain, whereas the coupling to specific G proteins seems to be mediated by the intracellular loops, predominantly by the third intracellular loop, and especially those regions in close proximity to the inner surface of the plasma membrane (Refs. 14–16, and references therein). Recently, it has been reported that site-directed mutagenesis of specific residues in the C-terminal portion of the third intracellular loop of the α1, α2, and β2-adrenergic receptors constitutively activate these receptors (14–16). We were interested in defining a region involved in G protein interaction with the PAF receptor. In addition, we wished to ascertain whether the constitutively active receptor forms induced by mutations in the third intracellular loop of the adrenergic receptor family could also be generated in a G protein-coupled receptor for a lipid ligand. Finally, the aim of this work was to develop a tool for screening inverse agonists for this receptor as potential therapeutic agents in PAF pathophysiology. We report that the Ala230 → Glu230 and Leu231 → Arg231 substitutions result, respectively, in an inactive and a constitutively active PAF receptor.

MATERIALS AND METHODS

Construction of Mutant Receptor cDNAs and Expression Vectors—The PAF receptor cDNA derived from Kp132 (a generous gift from Dr. Richard Ye, The Scripps Research Institute, La Jolla, CA) (6) was subcloned into the pRc/CMV expression vector (Invitrogen). Mutated receptors were constructed by polymerase chain reaction (17) using Kp132 as template. To create the Arg substitution for Leu231, we made the oligonucleotide 5′-GCCGGGGGCGGTGATGTTG-3′ and its reverse complement, which changes CTG (Leu) to CCG (Arg). Similarly, to mutate the Ala231 to Glu and Gln, we generated the oligonucleotides 5′-GCCGGGGGAGCTGTGATG-3′ and 5′-GCCGGGGGACCTGTGATG-3′ and their reverse complements, respectively, changing GCG (Ala) to GAG (Glu) and CAG (Gln). Polymerase chain reaction products
were digested Msel-BstEI and the resulting 398-base pair fragment was subcloned into pRC/CMV containing the WT receptor cDNA also digested Msel-BstEI. The region corresponding to the 398-base pair fragment was sequenced on both strands by deoxy sequencing of double-stranded DNA with Sequenase (U. S. Biochemical Corp.).

Cell Culture and Transfections—COS-7 and CHO cells were grown in Dulbecco's modified Eagle's medium (high glucose) and Dulbecco's modified Eagle's medium F-12 (Ham's medium, high glucose), respectively, supplemented with 10% fetal bovine serum. COS-7 cells were plated in 30-mm dishes (1.5 × 10⁴ cells/dish), transiently transfected with constructs encoding the WT and the different mutant receptors using 5 μl of Lipofectamine (Life Technologies, Inc.) and 2 μg of DNA per dish and harvested 48 h after transfection. Stable CHO transfectants were generated in the same way and seeded into one 100-mm dish 24 h after transfection. Clones resistant to Geneticin (1 mg/ml) were isolated and tested for their ability to bind the radiolabeled PAF receptor-specific antagonist [³H]WEB2086 (DuPont NEN). Transient transfection conditions of COS-7 and CHO cells were adjusted to obtain low levels of expression: cells were plated in 100-mm dishes (3 × 10⁴ cells/dish), transfected with constructs encoding the WT and the mutant receptors using 30 μl of Lipofectamine and 4 μg of DNA per dish and harvested 48 h after transfection.

Radioligand Binding Assay—Competition binding curves were done on COS-7 and CHO cell membranes expressing the wild-type and mutated receptor species. Cells were harvested and washed twice in Hepes-Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM d-glucose, 0.49 mM NaH₂PO₄, 25 mM Hepes, pH 7.4) containing 0.1% (w/v) bovine serum albumin (11). The cells were then submitted to three freeze-thaw cycles and homogenized in a Teflon-glass homogenizer. Following centrifugation at 11,000 × g for 10 min, membranes were resuspended in the same buffer at a concentration of 3 mg/ml. Binding reactions were carried out on 30 μg of membranes in a total volume of 0.25 ml in the same buffer with 10 nM [³H]WEB2086 and increasing concentrations of nonradioactive WEB2086 or PAF for 90 min at 25°C. For some experiments, binding reactions were carried out on whole cells using the same conditions with 5 × 10⁴ cells/point. Reactions were stopped by centrifugation. The membrane- or cell-associated radioactivity was measured by liquid scintillation. Binding studies for the experiments involving GTPγS (Sigma) were performed on membranes of CHO transfectants. All points were in duplicate.

Inositol Phosphate Determination—COS-7 cells were transfected as described above with the wild-type and mutant receptors and labeled the following day for 18–24 h with myo-[³H]inositol (Amersham) at 5 μCi/ml in Dulbecco's modified Eagle's medium (high glucose, without inositol) (Life Technologies, Inc.). After labeling, cells were washed once in phosphate-buffered saline and preincubated 5 min in phosphate-buffered saline at 37°C. At the end of this preincubation period, the phosphate-buffered saline was removed and cells were incubated in prewarmed Dulbecco's modified Eagle's medium (high glucose, without inositol) containing 20 μl LICI for 5 min. Cells were then stimulated for 5 min with various concentrations of PAF. Cells were terminated with the addition of perchloric acid followed by a 30-min incubation on ice. In some experiments, cells were treated for 24 h with 100 ng/ml pertussis toxin (Sigma) (13) or with 200 μM of the tyrosine kinase inhibitor Tyrophostin A51 (Calbiochem) for 45 min prior to PAF stimulation. Insoluble phosphates were extracted (18) and separated on Dowex AG1-X8 (Bio-Rad) columns (19). Total labeled inositol phosphates were then counted by liquid scintillation.

RESULTS

We were interested in defining a region in the third intracellular loop of the PAF receptor that was involved in G protein interaction or whose modification might lead to the constitutive activation of the receptor. Substitutions of amino acids were made with residues which had a high probability of causing a change in the structure-function of the PAF receptor, based on the work of Kjelsberg and co-workers (15). This group reported the constitutive activation of the ε₂β-adrenergic receptor by all amino acid substitutions at the Ala²³⁰ position (the residues with the most pronounced effects being Arg, Lys, and Glu) which is positioned similarly to Ala²³⁰ and Leu²³⁵ of the PAF receptor with respect to the inner surface of the plasma membrane. Fig. 1 shows a representation of the putative seven membrane-spanning domain topography of the PAF receptor and indicates the two amino acids which were replaced in the mutant receptors.

We first examined the agonist-independent IP production and agonist-specific increase of binding affinity in these mutants. For these initial experiments, COS-7 cells were chosen for their high expression levels, which maximized basal activity, and for determining ligand binding properties independent of G protein coupling (16).

Inositol Phosphate Accumulation in the Mutant Receptor—transfected Cells—Expression levels in the COS-7 cells ranged from 1 to 1.4 pmol/mg of membrane protein for the WT and the mutant receptors (Table I). IP accumulation was insensitive to pertussis toxin and to the tyrosine kinase inhibitor Tyrophostin A51 (data not shown). Thus, in our system, the PAF receptor seems to stimulate IP production by a pertussis toxin-insensitive G protein and not to involve a tyrosine kinase signaling pathway for PI metabolism. Basal levels of IP accumulation were measured in COS-7 cells transiently transfected either with the expression vector alone or with the vector containing the WT or the mutant receptors. As shown in Fig. 2A, basal IP levels were higher in cells transfected with the WT PAF receptor than in those transfected with the vector alone. Expression of the L231R mutant receptor resulted in basal levels of IP accumulation which were about 10 times higher than in cells expressing the WT receptor. The substitution of the immediate adjacent residue, in the A230E mutant, resulted in basal IP production barely above that of the cells transfected with the vector alone and about one-fourth of the WT basal level in terms of total IPs. Cells transfected with the different constructions were then stimulated with 10⁻⁷ M PAF and the resulting IP accumulation was measured (Fig. 2B). The stimulation of the L231R mutant induced a 3-fold higher accumulation of IPs than the WT receptor. Unexpectedly, the A230E mutant showed no response at all in terms of IP production following PAF stimulation. Given the dramatic effects on IP production of the A230E mutation, we were interested in further characterizing this position. To verify if it was the position 230 of the PAF receptor which was critical or if the effects observed were due to the charge of the Glu²⁴⁰ substituting residue, the Ala²³⁰ was mutated to the Glu isostere, Gln²³⁰. Fig. 2A shows that the A230Q mutation had no significant effect on the basal IP accumulation in COS-7 cells. Following a PAF 10⁻⁷ M stimulation, the A230Q mutant receptor produced only one-fifth of the WT total IP production (Fig. 2B). The response of the mutant receptors was further studied by stimulation with PAF over a wide range of concentrations. Fig. 3 illustrates the concentration-response curves of IP accumulation for the wild-type and the three mutant receptors in response to PAF concentrations from 0 to 10⁻⁶ M. PAF concentrations higher than 10⁻⁶ M were not used as PAF has been shown to have nonreceptor mediated
effects at these concentrations (20), making the interpretation of results difficult. IP production for the WT receptor reached a plateau at $10^{-2} \text{M}$ agonist. The IP accumulation produced by the L231R mutant was from two to three times more than that of the WT as reported above, but did not reach a plateau: IP accumulation continued to increase up to $10^{-6} \text{M}$ PAF.

No IP was produced by the A230E mutant over the entire range of PAF concentrations. Surprisingly, the A230Q mutant accumulated approximately half of the WT maximal IP level after a stimulation with $10^{-6} \text{M}$ agonist. The A230Q concentration-response curve seemed to be shifted to the right by 2 orders of magnitude relative to the curve of the WT receptor. These results were not caused by differences in receptor expression levels as binding studies using $^3\text{H}$-WEB2086 were simultaneously carried out on whole cells and revealed that the WT and mutant receptors cell surface expression levels were very similar (between $1.6 \times 10^6$ and $2.0 \times 10^6$ receptors/cell for the three experiments), thus unlikely to influence the degree of responsiveness of the receptors to PAF. Since the A230E mutant did not respond and the IP accumulation of the L231R and the A230Q mutants did not reach a plateau within the “acceptable” PAF concentrations, EC50 values could not be obtained and compared between mutants and WT receptors.

Characterization of the Binding Affinities of the Mutant Receptors—Competition binding experiments indicated that the affinity of the specific PAF receptor antagonist WEB2086 for the three mutant receptors was unaltered as compared to the WT receptor (Fig. 4A). As it is well known, PAF being a phospholipid, binding experiments using $^3\text{H}$-PAF comprise a large nonspecific component which renders reproducibility and interpretation of the results difficult (21). Since the affinity of the antagonist was the same for all the receptors in this study ($K_i = 20 \text{nM}$, Table I), we used competition of $^3\text{H}$-WEB2086 binding by cold PAF to assess the PAF affinity for the different receptors and obtained high reproducibility between experiments.

![Table I](https://example.com/TableI.png)

**Table I.** Ligand binding parameters of WEB2086 and PAF to the wild-type and mutant receptors. $^3\text{H}$-WEB2086 binding was determined as described under “Materials and Methods” on membranes derived from transfected COS-7 or CHO cells. Receptor densities ($B_{max}$) are indicated in pmol/mg protein. Dissociation constants are expressed in nanomolar. $K_i$ represents the dissociation constant for the high affinity state of the receptor. $K_i^{(G)}$ corresponds to the affinity of receptors when uncoupled from G proteins with $10 \mu\text{M} \text{GTP}\gamma\text{S}$. The results are the mean ± S.E. of three independent determinations.
Ki showed a very significant decrease in affinity for PAF (Kᵢ = 671 nM and 733 nM, respectively) relative to the WT receptor (Kᵢ = 66 nM). The affinity of the L231R mutant for PAF is increased (Kᵢ = 19 nM) in comparison with the WT receptor. The changes in affinity displayed by the A230E, the A230Q, and the L231R mutant receptors would seem to be an intrinsic property of the mutant molecules independent of G protein coupling as inferred by the high expression level in COS-7 cells (Table I) (16).

Binding Characteristics in CHO Cells Expressing Low Levels of Receptors—We next wished to investigate the role of G protein coupling in the ligand binding properties of the mutant PAF receptors. The study of the interaction between a receptor and a G protein can be performed by the analysis of biphasic competition binding isotherms (22). We thus decided to establish the binding characteristics of the mutants on CHO cell membranes stably expressing low levels of receptors (16). Expression levels for the different constructions are indicated in Table I, and are comparable between transfectants.

We examined the ligand binding properties of the WT and mutant receptors in membranes derived from transfected CHO cells in the presence or absence of 10 μM GTPγS. Binding isotherms revealed that WT and mutated receptors bound the antagonist [³H]WEB2086 with the same affinity (Table I) which is not modulated by the presence of GTPγS (data not shown). In contrast with the situation described above for the COS-7 cells overexpressing the receptors, competition of [³H]WEB2086 binding by PAF in membranes of CHO stable transfectants resulted in curves that could be resolved into high and low affinity components, except for the A230E mutant (Fig. 5).

Similar results were obtained in COS-7 cells transiently transfected using conditions to allow for low levels of receptor expression (data not shown). Binding properties of the CHO transfectants are summarized in Table I. As shown by the competition binding curves, the high affinity state of the L231R mutant (Kᵢ(h) = 0.2 nM) is much higher than the WT receptor (Kᵢ(h) = 6.7 nM). According to Fig. 5A, around 60% of total [³H]WEB2086 binding loss by competition with PAF is associated with high affinity receptors for the L231R mutant compared to 40% for the WT receptors, respectively. These data suggest that there could be a higher percentage of receptors coupled to a G protein for the L231R mutant compared with the WT receptor. GTPγS mediated the conversion of both WT and L231R mutated receptors from their high affinity states into their respective low affinity states. In agreement with our observations in COS-7 cells, these experiments indicated that the low affinity state of the mutant receptor has higher affinity for PAF than the corresponding state of the WT receptor (Fig. 4B).

The inactive A230E mutant shows no high affinity component in its competition binding curve (Fig. 5B). In fact, the curve obtained is monophasic and the PAF binding properties for the A230E mutant are unaltered by GTPγS. This mutant binds the agonist with at least 10 times less affinity than the uncoupled WT receptor. Again, these results are in agreement with the experiments carried out in COS-7 cells (Fig. 4B).

The A230Q mutant displayed a biphasic binding isotherm with a high affinity state almost 100 times lower than the corresponding state of the WT receptor (Fig. 5C). The percentage of [³H]WEB2086 binding loss associated with high affinity PAF receptors was essentially the same between the A230Q and the WT receptors. The uncoupled A230Q receptor had an affinity for PAF approximately 10 times lower than the uncoupled WT receptor. Together, these results suggest that the A230Q mutation brings a severe loss of affinity of the PAF receptor for its agonist and a decreased responsiveness of the PAF receptor to agonist stimulation in terms of IP accumulation without apparently altering the G protein interaction with the receptor.

**DISCUSSION**

The PAF receptor activates multiple signaling pathways in response to its agonist, which has numerous biological activities (11). The present study was undertaken to determine a region involved in G protein coupling of the PAF receptor. Constitutively active receptors of the G protein-coupled receptor family have been reported by several groups to be involved in certain human diseases. These constitutively active receptors have residues that have been substituted in various regions of their seven membrane-spanning structure (reviewed in Ref. 23). The in vitro mutation of residues in the C terminus of the third intracellular loop of the adrenergic receptor family resulted in constitutively active receptors (14–16). That region has been shown to be important in receptor coupling to its G protein (24) and some of its sites to be essential in keeping the receptor in a constrained, inactive conformation in absence of agonist (14–16).

We have mutated Ala[^230] and Leu[^231] of the C terminus of the

**FIG. 4.** Competition binding isotherms of [³H]WEB2086 by WEB2086 (A) or PAF (B) in COS-7 cells. [³H]WEB2086 binding was measured as indicated under "Materials and Methods" on membranes derived from COS-7 cells transiently expressing the WT, A230E, A230Q, or the L231R mutant receptors. The results are representative of three independent experiments the mean values of which are reported in Table I.
third intracellular loop of the human PAF receptor into Glu\textsuperscript{230} and Arg\textsuperscript{231}, respectively. These non-conservative mutations were chosen to maximize chances of obtaining a constitutive mutant receptor (15). The major findings of the present study are that the substitutions of two immediately adjacent residues, Ala\textsuperscript{230} and Leu\textsuperscript{231}, surprisingly led to completely opposite phenotypes; the A230E mutant receptor is totally unresponsive and has a decreased affinity for PAF; and the L231R mutated receptor shows constitutive activity and displays a higher affinity for PAF than the WT receptor.

According to the extended ternary complex model, G protein-coupled receptors exist in an equilibrium between "inactive" and "active" conformational states: R $\leftrightarrow$ R*, respectively (16). The agonist is thought to stabilize the active conformation by virtue of a preferentially higher affinity for R*, thereby shifting the equilibrium to the right and resulting in productive receptor-G protein coupling (15).

The lack of response of the A230E mutant may be coming from an incapacity to interact productively with a G protein suggested by the absence of IP accumulation at high agonist concentrations and by the competition binding curves that are monophasic in the CHO expression system and unaffected by guanyl nucleotides. To ascertain whether the A230E mutant properties were conferred by the negative charge of the Glu residue brought into the basic microenvironment of the C terminus of the third intracellular loop of the PAF receptor, the Ala\textsuperscript{230} residue was also mutated to the Glu isoster, Gln. The A230Q mutant was a 100-fold less sensitive to PAF in IP production than the WT receptor. In terms of agonist binding, the A230Q mutant displayed a $\sim$10- and $\sim$100-fold decreased affinity for PAF when compared to the WT receptor for the low and high affinity states, respectively. The A230Q mutant was still able, however, to interact with G proteins as shown by the IP production, by its biphasic binding isotherm and by the percentage of G protein-coupled receptors which was similar to the WT receptors. The decreased response following PAF stimulation of this receptor mutant seems to be due to its decreased affinity for the agonist.

It is interesting to note that both the A230E and the A230Q mutants had lower intrinsic affinities for PAF than the WT receptor but that only the A230E mutant seemed unable to interact with G proteins. It appears that the Ala\textsuperscript{230} position is critical in regulating the PAF receptor conformation, as judged by the agonist binding affinities of the mutants at this position. Moreover, our results show that the change of the residue found at this position is determinant in the PAF receptor ability to interact with G proteins. The change of the Glu\textsuperscript{230} residue of the A230E mutant could prevent the R $\leftrightarrow$ R* isomerization in this receptor, as supported by the very low basal IP accumulation, and/or by interfering in the interaction between the receptor and the G protein.

Mutations conferring a constitutively active phenotype make the R to R* transition agonist-independent and should bestow a higher affinity for agonists (15). The L231R mutated receptor showed a basal activity approximately 10 times higher than the WT receptor. Similar results were obtained in a study where a constitutively active $\beta_2$-adrenergic receptor accumulated CAMP about 10 times more than the WT receptor when the levels of expression were considered (16). Constitutive mutants of the $\alpha_2$-adrenergic receptor also resulted in basal activity approximately 10 times higher when the Thr\textsuperscript{348} residue was substituted by a Cys or a Lys residue (14). Unlike the L231R mutant, the constitutively active $\alpha_2$- and $\beta_2$-adrenergic receptors had basal activities that were similar to the stimulated WT receptors (14, 16). When stimulated with $10^{-7}$ M PAF, the L231R mutant accumulated 3-fold higher levels of IPs than the WT receptor.
receptor. If the stimulated IPs levels are considered as relative increases above the elevated basal levels, the WT receptor accumulates 52 times more IPs when stimulated as compared to basal activity whereas the stimulated L231R mutant has a relative increase of 16. Such differences may be explained by a higher fraction of the L231R mutant receptors already engaged in coupling to a G protein (basal activity) that cannot be further stimulated, resulting in a lower relative increase. Nonetheless, at any given agonist concentration, the L231R mutant accumulated more total IPs than the WT receptor and this accumulation did not reach a plateau within the agonist concentrations used. Hypersensitivity of biological responses has also been reported for other receptors such as the α1-adrenergic (25) and the yeast a-factor (26) receptors.

The constitutive L231R mutant has a higher affinity for PAF than the WT receptor both in the coupled and the uncoupled states. The lower affinity which is considerably higher than the corresponding state of the WT receptor is an intrinsic property of the mutant molecule as shown by binding experiments in COS-7 cells expressing receptors at high levels (16) and by using GTP·γ-S in CHO cells. Such intrinsic higher affinities have been described for constitutive mutants of the adrenergic receptor family (14–16). The intrinsic high affinity property of a constitutively activated β2-adrenergic receptor was shown not to reflect an altered interaction with a G protein, since when receptors were solubilized and purified, the mutant receptor retained its higher affinity than the WT (16). The increase in affinity of the “high affinity state” of the L231R mutant compared to the WT receptor is remarkable and appears to be G protein coupling-dependent. In fact, we see with the competition binding studies in CHO cells that the binding curve for the L231R mutant is biphasic, indicating that there is an equilibrium between the two states of the L231R receptor, R*+R; that is, the constitutive receptor is not uniquely in a high affinity state and it needs to couple to a G protein to be so. The “low affinity state” of the L231R mutant is intermediate between the “high” and the low affinity states of the WT receptor. This low affinity state, therefore, does not correspond to the coupled WT receptor. Moreover, we have shown that this high intrinsic affinity of the low affinity state appears to be the uncoupled form of the L231R receptor. Thereby, the elevated basal activity of the L231R mutant would not result from its high intrinsic affinity, but could result from a bigger tendency of the L231R mutant to isomerize to R*, favoring productive interaction with G proteins. This tendency seems to be reflected in our data by what could be a higher percentage of receptors coupled to a G protein for the L231R mutant relative to the WT receptor. The L231R mutation does not confer the “relaxed” conformation to the receptors but rather allows them to achieve more easily the R* state in a dynamic process; otherwise monophasic high affinity competition binding curves unaffected by guanyl nucleotides would be obtained. Supporting our data, computer simulations implied that constitutive mutant receptors isomerize to an active state more readily than do WT receptors (16). At present, we cannot exclude the possibility that the primary effect of the alterations is to increase coupling to the G protein that would result in higher activity of the L231R mutant. But the L231R mutation must influence the PAF receptor molecule in other ways because of the differences between the high affinity states of this mutant and of the WT receptor that cannot be explained only by the intrinsic higher affinity or by increased G protein coupling. Perhaps the combination of those factors can lead to a conformational change resulting in this remarkable higher affinity.

There is considerable evidence that receptor interaction with G proteins is driven by two factors, conformation and electrostatic interactions (27). The introduction of the negatively charged Glu at position 230 led to total loss of G protein coupling, while substitution of Gin for Ala230 brought back the coupling of the receptor. We might speculate that the L231R mutation is bringing one more positively charged residue in an already basic microenvironment (KRRARL) that could favor the attachment of the G protein. Further experiments will be necessary to delineate the mechanisms involved in the higher affinity of the L231R mutant, as well as in its basal and stimulated activities. It must be noted that the present studies are necessarily performed in a transfected system, in which the PAF receptor preferentially couples to the α1 class of G proteins. The implication of the residues described herein in leukocytes in which α1 subunits are also involved, remains to be evaluated.

In summary, both residues at position 230 and 231 are important in the regulation of the PAF receptor conformation. Mutation of Leu231 to Arg231 led to higher agonist affinity and higher agonist-independent and -dependent activity of the PAF receptor. Mutation of Leu231 into other residues will reveal whether only the specificity of the WT sequence at this position is uniquely constraining the activity of the receptor in the unliganded state. The residue 230 has profound effects on agonist affinity and its charge is determinant in G protein coupling. Substitutions by other residues will enlighten the relationship between amino acid side chains at this position and the structure-function of the PAF receptor. We have defined two adjacent residues that when mutated lead to completely opposite phenotypes. Work is presently underway in our laboratory to determine if a “hinge” region has been identified, by substituting neighboring residues.

This system extends the notion that the C terminus of the third intracellular loop of the G protein-coupled receptors plays a determinant role in the overall conformation of the receptor molecule and the L231R mutant receptor should lend itself to screening for inverse agonists as potential therapeutic tools in PAF pathophysiology.

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