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Platelet-activating factor receptor: differential regulation and signaling by agonists and inverse agonists

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Abstract

The receptor for platelet-activating factor (PAFR) is a member of the G-protein-coupled receptor (GPCR) family. Due to the association of platelet-activating factor (PAF) with diverse physiological and pathological processes, considerable efforts have been invested in the development of PAFR antagonists. A large number of these molecules have been shown to specifically interact with the PAFR, but surprisingly, little is known on their impact on receptor activity or conformation. We used three different models of PAFR with high basal activity to study the inverse agonist effects of diverse PAFR antagonists. First, we co-expressed the wild-type (WT) PAFR with the Gαq protein, second, we used a point substitution mutant of PAFR L231R, which is constitutively active, and thirdly, we co-expressed the WT PAFR with another substitution mutant D63N: the co-expression of these two receptors in a 1:3 ratio (WT/D63N) produces a receptor complex with a constitutively active phenotype. Our results indicate that inverse agonists elicit distinct effects depending on the receptor model used. These results may have implications for eventual treatments based on inverse agonists.

Keywords. Platelet-activating factor receptor; G-protein-coupled receptor; Inverse agonist; Dimerization; Signaling

1. Platelet-activating factor and its receptor

Platelet-activating factor (PAF) (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid mediator released from stimulated basophils, platelets, macrophages,

Abbreviations: GPCR, G-protein-coupled receptor; IP, inositol phosphate; PAFR, platelet-activating factor receptor; PKC, Protein kinase C; WT, wild-type.

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polymorphonuclear neutrophils and many other cell types [1]. PAF has been implicated in several pathophysiological conditions including endotoxic shock, dermal inflammation, atherogenesis and allergic asthma among others. The production of platelet-activating factor receptor (PAFR)-transgenic and $PAFR^{-/-}$ mice provided definitive evidence of the involvement of PAF in responses to immunological or inflammatory challenges. For example, PAFR-transgenic mice spontaneously develop melanocyte tumors, respond much more severely to lipopolysaccharide-induced endotoxin shock and demonstrate bronchial hyperresponsiveness, whereas $PAFR^{-/-}$ mice are extremely resistant to antigen-induced systemic anaphylaxis, including circulatory shock and lung edema (reviewed in Ref. [2]).

The cloning of PAFR cDNA, from various sources, revealed that it belongs to the G-protein-coupled receptor (GPCR) family [3–6]. We have shown that PAFR variants co-expressed with the wild-type (WT) receptor result in receptor complexes bearing novel phenotypes [7]. For example, a mutant which is not itself expressed at the cell surface will retain the WT receptor intracellularly, resulting in a low cell surface expression of functional receptors. In addition, co-expression of a mutant (D63N), which is uncoupled from G-proteins but has a higher affinity for PAF than the WT receptor, results in a receptor complex (when expressed at a ratio of 1:1), which has high basal inositol phosphate (IP) production and higher ligand-stimulated activity than the WT, therefore mimicking a constitutively activated receptor. However, when the ratio of expression is changed to 1WT/3D63N, this complex has an extremely high basal IP production, but is no longer responsive to ligand-induced stimulation [7]. This study, among others, shows that GPCR oligomerization has a significant effect on the phenotype of the expressed receptor complexes. A polymorphism within the human PAFR gene was identified in a Japanese population, where an aspartic acid is substituted for an alanine at residue position 224 [8]. The substitution occurs in the putative third cytoplasmic loop and the receptor exhibits impaired interaction with G-proteins. Given the possibilities of oligomerization, this increases the possibilities of phenotypic variants in the population. Functional variants of PAFR may act as predisposing factors for diseases or as modifiers of the disease phenotypes in addition to producing interindividual variations in drug responsiveness.

Additional phenotype modifications may result from receptor heterodimerization. One report of receptor heterodimerization, among several [9–11], came from the work of Jordan and Devi [12] with the opioid receptor. They found that co-expression of the δ- and κ-opioid receptors in the same cell leads to an almost complete loss of binding to selective δ- and κ-opioid receptor agonists or antagonists, but binding could be restored by the simultaneous addition of the two ligands, indicating that positive cooperativity occurred. However, no positive cooperativity was observed between agonists and antagonists [12].

2. Inverse agonism

Recently, a class of ligands has been described as stabilizing the inactive conformation of receptors. These ligands are known as inverse agonists as they have the opposite effect of agonists. The two-state equilibrium model of receptors can illustrate the inverse agonist

activity of certain ligands. Many GPCRs can be spontaneously active suggesting a two-state conformation, an equilibrium between active and inactive conformational states in the absence of agonist [13–15]. Agonist binding to the receptor alters the equilibrium, favors the active state of the receptor and helps in stabilizing this conformation. In the absence of an agonist, antagonists do not stabilize preferentially either of the two conformations. Compounds displaying inverse agonism should have a higher affinity for the inactive state compared to the active conformation, resulting in a decrease in the proportion of receptors in an active conformation and a reduction in the basal activation of effector mechanisms.

3. PAFR ligands

PAF structural requirements are highly specific for its biological actions (Fig. 1A). The ether-bonded fatty alcohol with C16–18 chain length at the sn-1 position of the glycerol

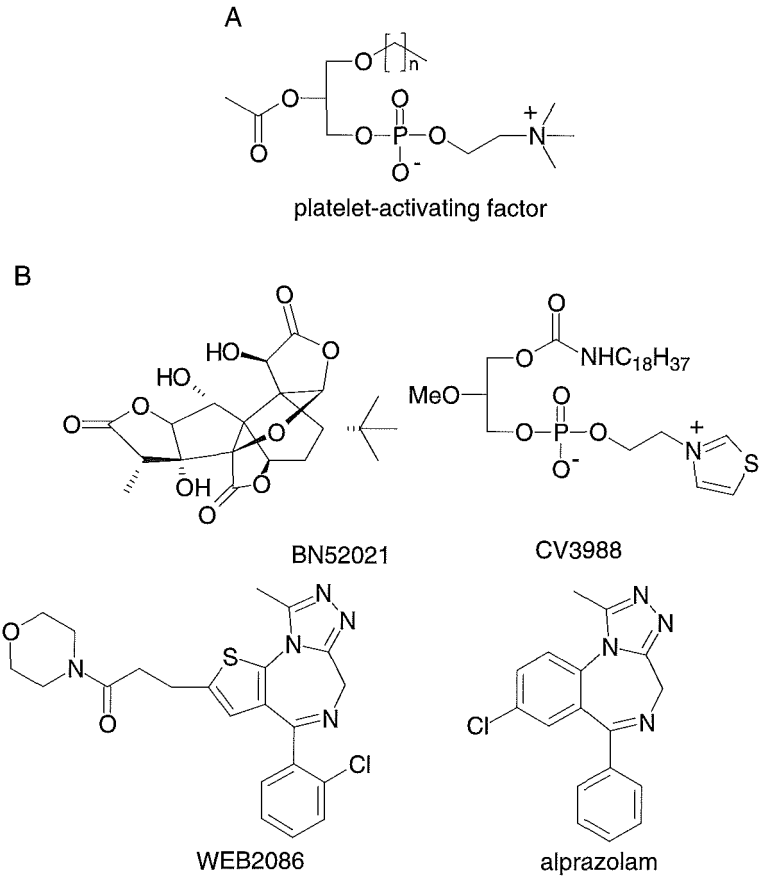

Fig. 1. Schematic representation of PAFR ligands of various structural families.

backbone, an acetyl residue at sn-2, and phosphocholine at sn-3 are all required for optimal PAF activity [16]. However, PAF-dependent cell activation can be inhibited by a variety of structurally distinct molecules (Fig. 1B). These molecules are classified in three structural classes of PAF antagonists: natural products (BN52021 or ginkgolide B derived from the leaves of *Ginkgo biloba*), synthetic structural analogs of PAF (CV3988 and others) and other synthetic compounds such as alprazolam and WEB2086, also referred to as Apafant. Some of these compounds are already used in clinical settings and we have shown that some of them exhibited interesting inverse agonism activities. For example, WEB2086, a benzodiazepine, which cannot bind to benzodiazepine receptors, has already been tested in studies for treatment of asthma [17,18], but a better knowledge of how this molecule acts on the PAF receptor signaling may lead to an improvement in its therapeutic potential.

4. PAFR and inverse agonism

Constitutively activated mutant receptors have been a valuable tool to demonstrate that certain ligands stabilize inactive conformations. It has been established that many GPCRs can exist in a spontaneously active form in the absence of agonist [19–22]. This agonist-independent activity has been observed, in the majority of cases, in cell lines or transgenic mice in which receptors were overexpressed or mutated [21,23–26].

We analyzed the inverse agonist activity of structurally different PAF antagonists and showed that many can exhibit inverse agonism at different levels [27]. Three models were used to verify the inverse agonism properties of ligands. As in the majority of G-protein-coupled receptors, substitution of a leucine of the third intracellular loop to an arginine (L231R) close to the sixth transmembrane domain stabilizes the active form and leads, in COS 7 cells, to a significant increase in the basal production of inositol phosphates [28]. A previous study using the muscarinic receptor showed that co-expression of a Gαq protein shifted the equilibrium towards the active form of the receptor [29]. We found that co-expression of the Gαq protein with PAFR led to an increase in the basal production of inositol phosphates which was two-fold higher than the level of PAFR expressed alone and stimulated with PAF 10^{-6} M [27]. In the last few years, dimerization of GPCRs has been demonstrated by different methods. It now appears that not only can receptors exist as dimers, but that dimerization may also play important roles in signaling. In a previous study, we reported that COS 7 cells co-expressing the WT PAFR and a G-protein uncoupled mutant (D63N, a substitution of aspartate 63 to an asparagine) exhibited higher basal levels of inositol phosphates than WT or D63N expressed alone (Table 1) [7]. Unlike

Table 1
Inositol phosphate production by different receptor complexes

	IPs produced by indicated receptor complexes/IP produced by WT+PAF stimulation
WT+PAF 10^{-6} M	1
WT+Gαq	2.15
WT+3 D63N	2.21
L231R	3.43

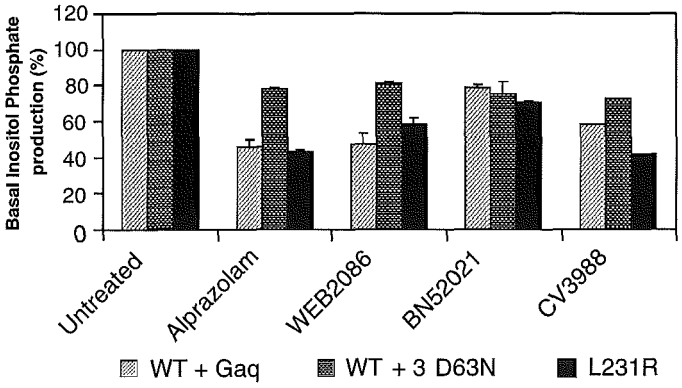

Fig. 2. Effects of different PAF antagonists on inositol phosphate production by wild-type receptors co-expressed with $G\alpha_q$ protein and mutants of the PAF receptor: L231R (constitutively active) or the complex of 1 WT+3D63N receptors (constitutively active). COS 7 cells were incubated for 45 min with the indicated compounds; inositol phosphate accumulation was then measured. Different families of PAF antagonists: BN52021 (7×10^{-6} M), alprazolam (4.2×10^{-5} M), WEB2086 (1×10^{-5} M) and CV3988 (1.6×10^{-5} M) were compared. The results are expressed as the means ± S.E. of three independent experiments, each done in duplicate.

the constitutively active mutant receptor L231R, the 1WT+3D63N complex did not respond to PAF in terms of increased IP production. In addition, the 1:3 complex exhibited a different phenotype from the WT and D63N expressed in a 1:1 ratio, since this complex showed significantly higher IP accumulation in response to agonist than the WT receptor expressed alone. We hypothesized that the 1WT/3D63N complex had acquired some

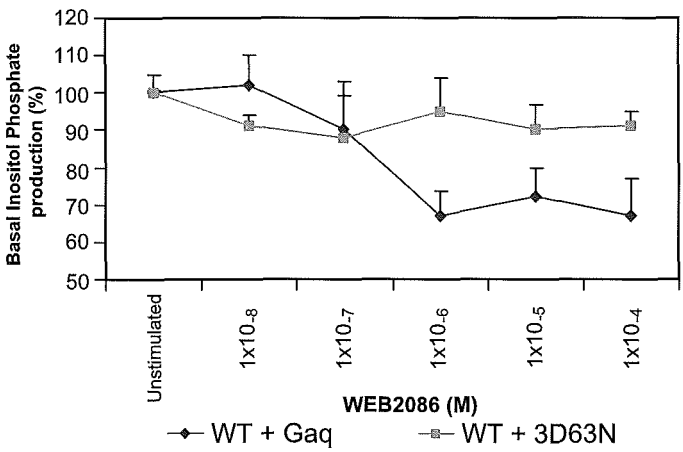

Fig. 3. Effects of graded concentrations of WEB2086 on inositol phosphate production by wild-type receptors co-expressed with $G\alpha_q$ protein and the complex of 1 WT+3D63N receptors (constitutively active). Comparison of the concentration–response curves of inhibition of IP production by WEB2086 with two different constitutively active complexes. The results are the means of three independent experiments, each done in duplicate.

rigidity and the agonist could no longer induce the appropriate modification of the conformational state needed for ligand-induced activation of effector mechanisms.

This resistance to conformational change could also be what resulted in the lack of response of this constitutively active receptor complex to the inverse agonists, which were active in decreasing basal IP production by the WT + Gαq and L231R receptors (Fig. 2). The majority of the inverse agonists used, except BN52021, which was a very weak inverse agonist, decreased the activity of the WT + Gαq and L231R receptors significantly more than the reduction in the activity of the 1WT/3D63N complex. Interestingly, although there was some reduction in activity of the latter with WEB2086, this reduction was not concentration-dependent, unlike the effect of WEB2086 on the WT + Gαq complex (Fig. 3). Similarly, a H2 receptor mutant which had a limited response to histamine also failed to respond to an inverse agonist in the same manner as the WT receptor [30]. These results indicate that different inverse agonists will not have the same effect on all types of receptor configurations and it will therefore be very important to study all possible homo- or heterodimerization capacities of target receptors in potential therapeutic interventions.

5. Inverse agonism, signaling and receptor maturation

PAF-dependent signaling is linked to various second messenger systems, through phospholipase A_2, C and D activation [31–33], such as IP turnover and calcium mobilization. The activation of the mitogen-activated protein kinase cascade is both pertussis toxin sensitive and insensitive, depending on the cell type studied [32,34,35]. Several tyrosine kinase pathways are also activated, including the Jak/STAT pathway [36]. Inverse agonist signal transduction has not yet been studied. Our preliminary data, however, indicate that WEB2086 induces PAFR receptor phosphorylation, which is approximately 40% of that induced by PAF. This phosphorylation is completely inhibited by inhibitors of protein kinase C (PKC), unlike the PAF-induced one which is only partially inhibited. Interestingly, the use of partially selective PKC isozyme inhibitors or constitutively active isozyme mutants indicates that PAF and WEB2086 stimulate different PKC isoforms. In addition, although none of the studied inverse agonists modulated receptor internalization, preliminary results suggest that WEB2086 increases PAFR downregulation whereas PAF upregulates its expression [37]. We are presently studying whether the differential activation of PKC isoforms results in the opposite effects the two ligands have on PAFR expression. Further investigations will be needed to understand the possible effects of different inverse agonists on receptor trafficking.

6. Conclusion

Our recent studies on inverse agonism of PAFR ligands have shown that these ligands cannot decrease the activity of all constitutively active mutant receptors. Some receptor complexes appear to be impervious to both agonist and inverse agonist activation while others maintain the capacity to be further activated, resulting in a differential regulation of

certain types of receptors by inverse agonists. The effects of inverse agonists on G-protein-coupled receptors may also be affected by the capacity of the complex to form a homo- or heterodimer, resulting in modulation of the pharmacological properties of the ligands.

Further characterization of the mechanisms of action of inverse agonists would yield important elements for our understanding of GPCR signaling and eventual therapeutic tools for a wide range of diseases.

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Discussion 15

P. Strange

How do you see the inverse agonist actually signalling this way? What's your picture of what's going on here?

J. Staňková

I think you get a different conformation of the receptor from the agonist, but you still get a change in conformation with the antagonist and therefore you get the possibility of displaying a different part of the receptor and permitting the phosphorylation.

G. Milligan

You obviously did some quite interesting observations there, but one of the things that seemed a little bit odd was the PKC effect, given that the WEB compound is inhibiting IP production. Did you actually make any direct measures of the activation state of the different PKC isoforms? Or indeed, even test which of the PKC isoforms are present in the cell systems that you're working with?

J. Staňková

Most of these PKC isoforms are present in the cells that we work with. We haven't done any more work than I've shown you with the PKCs. These are very preliminary results that we have obtained. However, these particular mutants have been used by a colleague of mine, and they seem to have equivalent activity.

G. Milligan

It's just that obviously, you'd think if the WEB compound was inhibiting IP production, you wouldn't think it terribly obvious that PKC was going to be activated, unless there is a very distinct signalling pathway to generate that. And that is clearly possible, because you're saying for the PAF receptor, you can clearly go through the Jak/STAT pathway and some others as well. But it just seemed a little bit unusual.

J. Staňková

I agree with you that it is a little strange, but so far, this is the only work we have really done on it, and I am not sure what to think about it yet.

T. Schwartz

I was just wondering whether you could clarify a little to me, because you said that PAF was actually activating the cells at femtomolar concentrations.

J. Staňková

In real cells, such as macrophages and smooth muscle cells, it can, but not in transfected cells. And I think maybe I now understand a little more when I saw the nice bell-shaped curves that you showed, because when we do, for example, look at the production of cytokines from macrophages, we have a very nice bell-shaped curve. But when we were looking at IP production in transfected cells, the curve is definitely not bell-shaped, we

can't even saturate because we can't put in enough of the lipid agonist, since 1 μM is the maximal concentration we can use before non-specific membrane effects. So I have a feeling that it's just a question of the receptor coupling to the $G\alpha_i$ or the $G\alpha_q$ proteins.

T. Schwartz

But you say normally PAF will do things at femtomolar concentrations, but what happens here at quite a few orders of magnitude away, so does the Jak/STAT activation start down at low concentrations?

J. Staňková

No, I think it's more the different usage of G proteins, because in COS cells, for example, we can't find any coupling to $G\alpha_i$. But in other cells it couples to $G\alpha_i$. For example, PAF is a chemoattractant, and the movement can be completely inhibited by pertussis toxin, so I think that the responses to femtomolar concentrations are either the PAFR coupling to the $G\alpha_i$ or other proteins or it's a different receptor. However, knock-out studies in rodents indicate there is only one receptor and at this time there is no molecular evidence of another receptor protein in humans.