Inverse Agonist Activity of Selected Ligands of Platelet-Activating Factor Receptor

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ABSTRACT
The receptor for platelet-activating factor (PAFR) is a member of the G protein-coupled receptor (GPCR) family. According to the allosteric ternary complex model, GPCRs exist in an equilibrium between different conformations. Agonist binding promotes and stabilizes the receptor in an active conformation. On the other hand, ligands that stabilize the inactive conformation are known as inverse agonists. Due to the association of platelet-activating factor (PAF) with diverse physiological and pathological processes, considerable efforts have been invested in the development of antagonists to PAFR. A large number of these molecules has been shown to specifically interact with PAFR but, surprisingly, little is known about their impact on the conformation of the receptor and its activity. By using a constitutively active mutant (L231R) of the human PAFR and by transiently coexpressing the wild-type (WT) receptor with the Gαq subunit of the trimeric G protein, we were able to address this issue with ligands of diverse structures such as phospholipids, benzodiazepines, furans, and others. We demonstrated that some of these molecules are potent inverse agonists. For example, when cells (WT PAFR + Gαq) were exposed to WEB2086, SM10661, or alprazolam, the basal inositol phosphate production was reduced by 53 ± 6, 44 ± 3, and 54 ± 4%, respectively. The decrease in basal inositol phosphate production by WEB2086 was significantly inhibited by a more neutral antagonist BNS2021, confirming the specificity of the reaction. We demonstrate here that WEB2086 and other known ligands previously considered as antagonists can act as inverse agonists on the human PAF receptor.

Platelet-activating factor (PAF) is a potent phospholipid mediator released from many cell types, including stimulated basophils, platelets, macrophages, and polymorphonuclear neutrophils. PAF is involved in a variety of biological activities related to inflammatory and immune responses as well as cardiovascular, respiratory, and nervous system physiology (Braquet and Rola-Pleszczynski, 1987). PAF structural requirements are highly specific for its biological actions, which are mediated through binding and activation of a specific, high-affinity receptor on the target cell surface. cDNA cloning from various sources revealed that the PAF receptor (PAFR) belongs to the G protein-coupled receptor (GPCR) family (Nakamura et al., 1991; Ye et al., 1991; Sugimoto et al., 1992; Kunz et al., 1992; Chase et al., 1993). PAFR signaling is linked to various second messenger systems, through phospholipase A2, C, and D activation (Prescott et al., 1990; Kuruvilla et al., 1993; Liu et al., 1994). This receptor is also known to induce the mitogen-activated protein kinase cascade and the Jak/STAT pathway in some cells (Franklin et al., 1993; Honda et al., 1994; Liu et al., 1994; Franklin et al., 1995; Lukashova et al., 2001). PAF-dependent cell activation can be inhibited by a variety of structurally distinct molecules (Hwang, 1990). The ligands tested in this study were related to different families of molecules such as benzodiazepines, alprazolam (Kornechi et al., 1984) and WEB2086 (Casals-Stenzel et al., 1987); PAF analogs, CV3988 (Terashita et al., 1985) and 1-O-hexadecyl-2-acetyl-sn-glycero-3-phospho-(N,N,N-trimethyl)hexanolamine (hexanolamine) (Grigoriadis and Stewart, 1991); ginkgolides, BN52021 (Nunez et al., 1986); and others such as SM10661 (Komuro et al., 1990), octylxol bromide (Subissi et al., 1989), and FR49175 (Okamoto et al., 1986).

It has been established that many GPCRs can exist in a spontaneously active form in the absence of agonist (Costa et al., 1992; Lefkowitz et al., 1993; Chidiac et al., 1994). This agonist-independent activity has mostly been observed in cell lines or transgenic mice in which receptors were overexpressed or mutated (Adie and Milligan, 1994; Barker et al., 1994; Chidiac et al., 1994; Bond et al., 1995; Newman-Tancredi et al., 1997). In the PAFR, a substitution of leucine 231 of the third intracellular loop to an arginine (L231R) stabilizes the active form and leads, in COS 7 cells, to a significant increase in the basal production of inositol phosphates (IP) (Parent et al., 1996a). In a previous study, we also reported...
that COS 7 cells coexpressing the wild-type (WT) PAFR and a G protein-uncoupled mutant D63N (a substitution of aspartate 63 to an asparagine) at a ratio of three D63N mutants to one WT receptor exhibited higher basal levels of inositol phosphates than WT or D63N expressed alone (Le Gouill et al., 1999). Unlike the constitutively active mutant L231R, however, the 1WT + 3D63N complex did not respond to PAF in terms of increased IP production.

Constitutively active receptor mutants have been a valuable tool to demonstrate that certain ligands stabilize inactive conformations. These ligands are known as inverse agonists, because they have the opposite effect of agonists. Until now, most of the PAFR ligands were loosely classified as antagonists, thus as molecules that interfere with the agonist (PAF) activation of the receptor. In this study, we characterize the effects of these molecules on the activated state of the PAFR to define which ones have inverse agonist, partial agonist, or neutral antagonist (no effect on activation) properties.

By either expressing the combination WT/Goαq, WT/D63N (1:3 ratio), or the L231R mutant of PAFR, we also studied whether the properties of the ligands would vary depending on the structural conditions leading to the constitutive activation of PAFR.

**Experimental Procedures**

**Materials.** Alprazolam, BN52021, (±)-trans-2,5-bis(3,4,5-trimethoxyphenyl)-1,3-dioxolane (dioxolane), enantio-PAF, FR49175, hexanolamine, octylonium bromide, SM10661, and PAF were from BIOMOL (Plymouth Meeting, PA). WEB2086 was from Boehringer Ingelheim GmbH (Ingelheim, Germany). CV3985 was from Takeda Chemical Industries, Osaka, Japan.

**Cell Culture and Transfections.** COS 7 cells were grown in Dulbecco’s modified Eagle’s medium high glucose (Invitrogen Canada, Inc., Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Bio Media Canada, Drummondville QC, Canada) and transfected using FuGENE-6 (Roche, Mississauga, ON, Canada). Cells were plated at a density of 3 × 10^4 cells/well in six-well plates and the following day, transfected exactly as instructed in Roche’s protocol, by using 2 μl of FuGENE-6 and 1 μg of DNA. Experiments were carried out 2 days after transfection.

**Radioligand Binding Assay.** [3H]WEB2086 (PerkinElmer Life Sciences Products, Boston, MA) binding reactions were performed, as previously described by Parent et al. (1996a), on COS 7 cells transfected with DNA encoding a c-myc epitope-tagged (N terminus) WT PAFR in pcDNA3 (Invitrogen, Carlsbad, CA). Briefly, cells were harvested, washed twice with phosphate-buffered saline (PBS) and resuspended in HEPES-Tyrode’s buffer (140 mM NaCl, 2.7 mM KCl, 1 mM CaCl_2, 12 mM NaHCO_3, 5.6 mM p-glucose, 0.49 mM MgCl_2, 0.37 mM NaH_2PO_4, 25 mM HEPES, pH 7.4) containing 0.1% bovine serum albumin. The binding assays were done on 5 × 10^5 cells in a total volume of 0.25 ml of the same buffer, containing 10 nM [3H]WEB2086, at room temperature for 90 min. In some experiments, different antagonists were also included in the mix to compete with the radioligand binding on the receptor. Binding reactions were stopped by centrifugation. The cell-associated radioactivity was measured by liquid scintillation.

**Inositol Phosphate Determination.** COS 7 cells were cotransfected, as indicated in Figs. 1 and 2, with DNA encoding the α_q subunit of the human trimeric Gq protein and either a c-myc epitope tagged (N terminus) WT or a mutant receptor (L231R or D63N). The following day, cells were labeled for 18 to 24 h with 5 μCi/ml of [3H]myoinositol (Amersham Pharmacia Biotech, Piscataway, NJ) in Dulbecco’s modified Eagle’s medium (high glucose, without inositol) (Invitrogen). After labeling, they were washed once with PBS and preincubated for 45 min at room temperature in presence of different PAF antagonists. LiCl was then added at a final concentration of 10 mM and cells incubated in presence or absence of 10 nM PAF, at 37°C. The reaction was terminated after 30 min by the addition of perchloric acid. After an incubation of 30 min on ice, inositol phosphates were extracted and separated on Dowex AG1-X8 columns (Bio-Rad, Hercules, CA) as previously described (Parent et al., 1996b). [3H]-Labeled inositol phosphate levels were then evaluated by liquid scintillation.

**Receptor Sequestration.** The evaluation of receptor sequestration was done on COS 7 cells transiently expressing a c-myc epitope-tagged (N terminus) WT receptor. Cells were exposed or not to different PAFR ligands at 37°C for 30 min in HEPES-Tyrode’s buffer. They were then harvested, washed with PBS, and incubated at 16°C with or without anti-c-myc antibody (clone 9E10; American Type Culture Collection, Manassas, VA). After 90 min, cells were washed with PBS and incubated for an additional hour with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson Immunoresearch, West Grove, PA). Receptor expression on the cell surface was then evaluated using a FACScan flow cytometer (BD Biosciences, Oakville, ON, Canada).

Statistical significance was established with Student’s t test.

**Results**

To further amplify the effect of the activated state of PAFR on inositol phosphate levels, COS 7 cells were also transfected with the cDNA of the human Goαq. Other members of the α_q family were tested (Go11, Go14, Go16) but their effect on basal inositol phosphate production was lower than with
Goq. With this increase in basal activity, we were able to use the WT receptor to test both antagonists’ and inverse agonists’ properties of various ligands.

Efficiency of PAF Antagonists. COS 7 cells expressing WT PAFR and Goq stimulated with 10⁻⁷ M PAF were used to confirm the antagonist activity of indicated compounds (Fig. 1). The concentrations chosen for these experiments were approximately 30-fold their reported IC₅₀ for diverse biological responses to PAF. Although these compounds are all PAFR ligands, because they interfered with [³H]WEB2086 binding (Fig. 2) or [³H]PAF (data not shown), some of them, FR49175 (2.6 × 10⁻⁵ M) and dioxolane (1 × 10⁻⁵ M), did not antagonize PAF-induced inositol phosphate production in COS 7 cells. On the other hand, CV3988 (1.6 × 10⁻⁴ M), octylamine bromide (2.4 × 10⁻⁴ M), hexanolamine (1.1 × 10⁻⁵ M), WEB2086 (1 × 10⁻⁴ M), and enantio-PAF (1 × 10⁻⁵ M) were all efficient antagonists, reducing the level of PAF-induced inositol phosphate production to 4 ± 6, 12 ± 16, 11 ± 12, 17 ± 8, and 20 ± 21% of maximal response, respectively. Others, such as BN 52021 (7 × 10⁻⁵ M), alprazolam (4.2 × 10⁻⁴ M), and SM10661 (1.2 × 10⁻⁴ M) showed weak antagonist properties by only reducing response to PAF to levels of 54 ± 8, 66 ± 10, and 70 ± 11%, respectively.

Basal Inositol Phosphate Production. COS 7 cells expressing WT + Goq, L231R, or 1WT+ 3D63N were used for the following experiments. The increased basal level of inositol phosphate production in these cells was essential to examine a wide range of PAF antagonists for their potential inverse agonist activity. Table 1 shows levels of basal inositol phosphate production by constitutively active mutants compared with levels induced by stimulation of the WT receptor with 10⁻⁶ M PAF.

When used at concentrations corresponding to 3-fold their reported IC₅₀, many of the ligands tested had a weak inverse agonist activity (Fig. 3). However, some such as dioxolane, SM10661, alprazolam, and WEB2086 had a much stronger effect and were able to lower the basal levels of inositol phosphates to 65 ± 19, 56 ± 3, 46 ± 4, and 47 ± 6%, respectively, in cells cotransfected with the WT receptor and Goq (Fig. 3, A and B). With the constitutively active mutant receptor L231R, the basal levels of inositol phosphates were reduced by 51 ± 4, 50 ± 3, 57 ± 4, and 42 ± 4%, with the same compounds, respectively. However, these ligands had a much weaker inverse agonist effect on cells expressing the 1WT + 3D63N receptor combination. The basal level of inositol phosphate production was inhibited by 17 ± 1, 32 ± 7, 22 ± 1, and 19 ± 1% for dioxolane, SM10661, alprazolam, and WEB2086, respectively. Hexanolamine has been reported to act as a partial agonist (Grigoriadis and Stewart, 1991) when tested in rabbit platelet aggregation or O₂ production in guinea pig peritoneal macrophages. Our results showed a partial agonist activity of hexanolamine and enantio-PAF, which led to an increase in inositol phosphate production of 54 ± 54 and 581 ± 88%, respectively, in cells overexpressing the WT receptor and the Goq protein (Fig. 3C). A much smaller increase was seen in cells expressing the constitutively active receptors. Extended concentration-response curves for WEB2086, alprazolam, and CV3988, which exhibited efficient inverse agonist activity, were compared in cells coexpressing WT + Goq and showed that WEB2086 is a more potent inverse agonist than either CV3988 or alprazolam (Fig. 3D).

In comparison with stimulation by 10⁻⁷ M PAF, hexanolamine, and enantio-PAF demonstrated a slightly lower inositol phosphate production corresponding to 80 and 94% respectively, of PAF stimulation in cells overexpressing WT + Goq (data not shown). In presence of the L231R constitutively active mutant, hexanolamine and enantio-PAF induced IP production corresponding to 49 and 39% of PAF stimulation. In cells expressing 1WT + 3D63N, 10⁻⁷ M PAF, hexanolamine, and enantio-PAF all demonstrated a very modest increase of IP production corresponding to 167, 186, and 154% of basal level of inositol phosphate production (data not shown).

To determine whether the relative unresponsiveness of the 1WT + 3D63N receptor complex was concentration-dependent, we treated cells expressing this complex or coexpressing WT + Goq with graded concentrations of selected ligands (Fig. 4). WEB2086, SM10661, and dioxolane induced a concentration-dependent reduction of basal inositol phosphate levels in cells transfected with the WT receptor and Goq. However, in cells with the 1WT + 3D63N receptor combination, the inositol phosphate levels remained relatively unaffected by increasing concentrations of these ligands.

The action of an inverse agonist should be inhibited by a neutral antagonist, in the same way as a full or partial agonist would be blocked by an antagonist. We treated COS

**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor Combinations</th>
<th>IP Produced by Indicated Receptor Combinations</th>
<th>IP Produced by WT + PAF Stimulation</th>
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<tbody>
<tr>
<td>WT + PAF 10⁻⁶ M</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>WT + Goq</td>
<td>2.15</td>
<td></td>
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<tr>
<td>WT + 3D63N</td>
<td>2.21</td>
<td></td>
</tr>
<tr>
<td>L231R</td>
<td>3.43</td>
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Fig. 2. Effect of PAF antagonists on [³H]WEB2086 binding to wild-type PAF receptors. Cells were transfected with overexpressed WT receptor and the binding reaction was performed in presence of different antagonists and [³H]WEB2086 as described under Experimental Procedures. The results are shown here as one experiment representative of two others.
A different family of PAF antagonists, BN52021 (7 \times 10^{-6} M), dioxolane (1 \times 10^{-6} M), octylammonium bromide (2.4 \times 10^{-5} M), SM10661 (1.2 \times 10^{-5} M), and FR49175 (2.6 \times 10^{-5} M) were used. B, drugs of the benzodiazepines family, alprazolam (4.2 \times 10^{-5} M) and WEB2086 (1 \times 10^{-5} M), were compared. C, PAF analogs, hexanolamine (1.1 \times 10^{-6} M), enantio-PAF (1 \times 10^{-6} M), and CV3988 (1.6 \times 10^{-5} M), were compared. The results are expressed as the means ± S.E. of three independent experiments, each done in duplicate. D, comparison of the concentration-response curves of inhibition of IP production by WEB2086, alprazolam, and CV3988. The results are the means of three independent experiments, each done in duplicate.
creased the WEB2086-induced reduction of inositol phosphate production.

Receptor Sequestration. After ligand binding to the receptor, a cascade of events often leads to sequestration of the receptor from cell surface. Because lower numbers of cell-surface receptors could produce the same phenomenon of decreased basal activity as inverse agonists, we examined whether indicated molecules induced an accelerated internalization of the receptor. Receptor expression on the cell surface was assessed using flow cytometry. As shown in Fig. 6, only hexanolamine, which has partial agonist activity, was able to induce PAFR internalization at a level (85 ± 42%) comparable with that induced by the agonist PAF (10^{-7} M). The other compounds induced a much lower level of receptor internalization.

Discussion

It has been shown in the context of several GPCRs that certain molecules could act as inverse agonists and reduce
the levels of spontaneous receptor activity and functional cellular responses (Chidiac et al., 1994; Samama et al., 1994; Shryock et al., 1998; Spadoni et al., 1998). Here, we report that molecules known to antagonize diverse responses to PAF can also inhibit spontaneous activity of PAFR. The antagonists characterized in this study included families of molecules of unrelated structure such as benzodiazepines (alprazolam and WEB2086), PAF analogs (CV3988 and hexanolamine), ginkgolides (BN52021), and other families. Until now, none of these antagonists had been examined for their inverse agonist properties. The structural diversity of these molecules suggested a possible variety of properties, and we therefore decided to characterize one or two members of each family.

Benzodiazepines were more efficient at reducing the levels of constitutive activity of PAF WT and mutant receptors. SM10661 and dioxolane, members of the diaryl tetrahydrofurane family, also reduced constitutively active receptor signaling, demonstrating a decrease of approximately 50% of inositol phosphate production. Among the other antagonists assayed, octylonium bromide, a very good antagonist, did not act as an inverse agonist. Others, such as FR49175 or dioxolane, were less capable of competing with PAF for a binding site on the receptor but demonstrated inverse agonist activity. These molecules may be interacting less tightly with the receptor, which would facilitate their displacement by PAF, or may not bind to the receptor at the same site as PAF, which would make them less effective antagonists but maintain their capacity for inverse agonist activity.

A 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 (Leff, 1995; Milligan et al., 1995; Leurs et al., 1998). 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, neutral antagonists do not stabilize preferentially either of the two conformations. Compounds displaying inverse agonism should have a higher affinity for the inactive state compared with 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. This has been suggested for the β2-adrenoceptor, that inverse agonists would have a preferential affinity for the R form of the receptor over the R* state (Samama et al., 1994). On the other hand, in the case of opiate receptors, it has been proposed that the compounds with inverse agonist activity have a preferential affinity for the free receptor over the receptor-G protein complex and thus suppress agonist-independent activity (Costa et al., 1992). Our results indicate that a molecule described as having an inverse agonist activity for a certain receptor conformation will not necessarily demonstrate the same activity for another type of receptor structure, as shown here with the L231R mutant receptor and the 1WT + 3D63N constitutively active complex, where ligands with inverse agonist activity on the L231R did not decrease the IP production by the 1WT + 3D63N complex.

Fig. 5. Blocking the inverse agonist activity of WEB2086 by BN52021. Cells overexpressing wild-type PAF receptor and Gαs were unstimulated, treated with BN52021, and then stimulated with WEB2086, or stimulated only with WEB2086 or BN52021. Inositol phosphate accumulation was then measured as described under Experimental Procedures. The results are expressed as the means ± S.E. of three independent experiments. *p < 0.05.

Fig. 6. Sequestration of PAF receptors. Sequestration of WT PAF receptor was measured following a stimulation with 10⁻⁷ M PAF and PAF antagonists at concentrations equivalent to 3-fold the IC₅₀ described in literature BN52021 (7 × 10⁻⁶ M), dioxolane (1 × 10⁻⁶ M), octylonium bromide (2.4 × 10⁻⁵ M), SM10661 (1.2 × 10⁻⁶ M), FR49175 (2.6 × 10⁻⁵ M), hexanolamine (1.1 × 10⁻⁶ M), enantio-PAF (1 × 10⁻⁶ M), CV3988 (1.6 × 10⁻⁵ M), alprazolam (4.2 × 10⁻⁶ M), and WEB2086 (1 × 10⁻⁵ M) at 37°C for 30 min. The cells were then incubated with anti-ε-nect antibody and followed by a fluorescein isothiocyanate-conjugated goat anti-mouse antibody. Cell-surface receptor expression was determined on a FACScan flow cytometer. The results are the means ± S.E. of three independent experiments.
The overexpression of WT + Goq or the constitutively active receptor (L231R) both conserve their potential to respond to agonist activation, whereas the combination of WT and D63N produces a receptor complex that has a different phenotype from both the WT and D63N expressed alone (Le Gouill et al., 1999). We have shown that when the WT and D63N are expressed at a 1:1 ratio, this complex has higher basal IP production and has a potentiated response to PAF in comparison with WT alone. Although the complex composed of 1:3 ratio has high basal IP production, it no longer responds adequately to PAF, indicating that this complex has acquired some rigidity and the agonist can no longer induce a conformational change needed for the appropriate activation of effector mechanisms. This resistance to conformational change could also result 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 + Goq and L231R receptors. This could explain the major differences in activity displayed by the two models of constitutively active receptors. Similarly, a mutant of the H2 receptor, which had a limited response to histamine, also failed to respond to an inverse agonist in the same manner as the WT receptor (Smit et al., 1996).

We also studied whether the decrease in signaling by inverse agonists could be attributed to an increased sequestration of the receptors from the cell surface. It had been shown that inverse agonists could induce the internalization of the receptors from the cell surface. It had been shown that inverse agonists could induce the internalization of the cholecystokininA receptor (Roettger et al., 1997), although in that inverse agonists could induce the internalization of the receptors from the cell surface. It had been shown that inverse agonists could be attributed to an increased sequestration of the WT receptor (Smit et al., 1996). We failed to respond to an inverse agonist in the same manner as the WT receptor (Smit et al., 1996).

However, we demonstrated that dioxolane, WEB2086, alprazolam, and SM10661 also induced low levels of receptor sequestration, comparable with less potent ones. The only compounds that induced an increase in PAFR sequestration were the partial agonist hexanolamine and the agonist PAF, which had been shown previously to induce PAFR sequestration (Le Gouill et al., 1997). These results indicate that the decrease in basal activity induced by selected ligands is not due to an accelerated rate of receptor internalization.

In this work, we demonstrated that dioxolane, WEB2086, alprazolam, and SM10661 have potent inverse agonist activity on constitutively active receptors overexpressed in COS 7 cells. These inverse agonists all induce a decrease in inositol phosphate production, without changing the receptor sequestration rate. Our results also demonstrate that the inverse agonist effect of WEB2086 can be significantly inhibited by a more neutral antagonist such as BNS2021. By comparing WT coexpressed with Goq, D363N coexpressed with 1WT, and L231R mutant PAF receptors, we demonstrated that inverse agonist activity can differ from one constitutively active receptor to another. This could be explained by conformational differences in the receptor binding sites and may indicate that a molecule known for its inverse agonist activity will not necessarily have the same activity on a mutant receptor. Studies of the relationship between ligand structure and their properties may make it possible to predict secondary effects of some of these molecules in therapy and to develop new molecules with the desired characteristics, such as ligands that can induce short- or long-term desensitization without inducing an effector cascade.

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agonist, spiperone but not the neutral antagonist, WAY 100,635. Br J Pharmacol 120:737–739.


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