0026-895X/91/060788-10$03.00/0

MOLECULAR PHARMACOLOGY, 39:788-797

Wheat Germ Agglutinin Potentiates Specific Binding of Platelet-Activating Factor to Human Platelet Membranes and Induces Platelet-Activating Factor Synthesis in Intact Platelets

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Received September 24, 1990; Accepted February 19, 1991

SUMMARY

Specific binding of tritium-labeled platelet-activating factor (PAF) and a nonmetabolizable bioactive analog of PAF, 1-*O*-alkyl-2-*N*-methylcarbamyl-*sn*-glyceryl-3-phosphorylcholine, to human platelet membranes was found to be potentiated by wheat germ agglutinin (WGA) and erythroagglutinin. As demonstrated in Scatchard plots, the potentiation effect is due to an increase in the maximal number of receptor sites, with no alteration in the equilibrium dissociation constant. The WGA-potentiated specific binding can be specifically inhibited by *N*-acetylglucosamine, shows identical affinity for PAF agonists and a receptor antagonist, L-659,989, and has an identical Na^+ inhibition pattern to non-treated membranes in the absence of WGA. The WGA-induced potentiation is preferential in the plasma membrane-enriched fraction. The maximal number of receptor sites increases in membranes pretreated with neuraminidase and β-*N*-acetylglucosaminidase. Therefore, WGA may bind to an endogenous PAF receptor modulator, which then either dissociates from or associates with the PAF receptor and regulates the receptor conformation. The membrane fraction enriched with intracellular membranes is also enriched with PAF receptors. WGA was also found to increase the maximal aggregation of rabbit and human platelets induced by PAF and to induce the synthesis of PAF, which preceded aggregation in human platelets. An intracellular PAF receptor may also exist, and it could modulate the function of PAF retained inside of the stimulated cells.

PAF is a potent phospholipid mediator generated in inflammatory and allergic reactions (1). The structure of this mediator has been established as 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (2, 3). PAF was originally shown to be released from antigen-stimulated IgE-sensitized rabbit basophils (4). Other cell types, including macrophages, neutrophils, platelets, basophils, eosinophils, and endothelial cells, also possess the capacity to synthesize PAF when stimulated *in vitro* (5). However, considerable quantities of PAF produced intracellularly are retained within cells (6, 7). Therefore, it was speculated that PAF may act as both an intracellular and an extracellular mediator (8).

PAF has potent biological effects on a variety of cell types, and its mechanism of action appears to be receptor mediated. Specific PAF receptors have been identified in several cell types, including rabbit (9) and human platelets (10, 11), human polymorphonuclear leukocytes (12, 13), human lung tissue (14), and rat liver tissue (15). However, the role of PAF as an intracellular mediator is not yet clearly defined.

GTP, as well as monovalent and divalent cations, modulates the conformation of PAF-specific receptors (13, 16, 17). The existence of endogenous PAF inhibitors has also been reported in liver (18) and in uterus (19). Although the function of PAF receptor could be modulated by second messengers or endogenous PAF inhibitors, the physiological significance of these reported endogenous lipid inhibitors of PAF responses is still poorly understood. We will report here the possible existence of an additional type of endogenous inhibitor, which modulates the binding characteristics of the PAF receptor, and also the possible existence of an intracellular receptor specific for PAF.

Experimental Procedures

Materials

The tritium-labeled PAF agonists 1-*O*-[alkyl-1′,2′-^{3}H]-2-*N*-methylcarbamyl-*sn*-glyceryl-3-phosphorylcholine ([^{3}H]*N*-methylcarbamyl-

ABBREVIATIONS: PAF, platelet-activating factor; C_{16}-PAF, 1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine; C_{18}-PAF, 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine; *N*-methylcarbamyl-PAF, 1-*O*-alkyl-2-*N*-methylcarbamyl-*sn*-glyceryl-3-phosphorylcholine; enantio-C_{16}-PAF, 3-*O*-hexadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine; WGA, wheat germ agglutinin; L-659,989, (±)-(*trans*)-2-(3-methoxy-5-methylsulfonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)tetrahydrofuran; L-648,611, 3-(*N*-palmitoylamino)propylphosphocholine; CV-6209, 2-[*N*-acetyl-*N*-(2-methoxy-3-octadecylcarbomyloxypypropoxycarbonyl)amino methyl]-1-ethylpyridinium chloride; PHA-E, erythroagglutinin from *Phaseolus vulgaris*; Conc A, concanavalin A from *Canavalia ensiformis*, type IV; PAN, lectin from *Arachis hypogaea*; Gorse, lectin from *Ulex europaeus*; BS-1, lectin from *Bandeiraea simplicifolia*; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; PMN, polymorphonuclear leukocyte lyso-PAF, 1-0-hexadecyl-sn-glyceryl-3-phosphorylcholine.

PAF) (specific activity, 54.6 Ci/mmol) and 1-*O*-[octadecyl-9,10-^{3}H]-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine ([^{3}H]C_{18}-PAF) (specific activity, 120 Ci/mmol), [^{3}H]acetyl coenzyme A (specific activity, 200 mCi/mmol), and [^{3}H]acetic acid, sodium salt (specific activity, 3.3 Ci/mmol) were obtained from NEN-Du Pont (Boston, MA). The purity of tritium-labeled *N*-methylcarbamyl-PAF and C_{18}-PAF was higher than 98% (NEN, technical information). The purity remained more than 95% even after 3 months, as checked with high performance liquid chromatography. The labeled compounds were used within 3 months. Unlabeled C_{16}-PAF and enantio-C_{16}-PAF were purchased from BACHEM (Terrance, CA). NMR and mass spectroscopic data indicated that the purity of both C_{16}-PAF and enantio-C_{16}-PAF was greater than 99% (20). Enantio-C_{16}-PAF was about 10,000-fold less potent than C_{16}-PAF either in inhibiting [^{3}H]PAF binding to its receptor on isolated rabbit platelet membranes or in stimulating the aggregation of gel-filtered rabbit platelets (21). C_{16}-PAF and enantio-C_{16}-PAF were, therefore, used without further purification. WGA from *Triticum vulgaris*, PHA-E, Conc A, PAN, Gorse, and BS-1 were purchased from Sigma Chemical Co. (St. Louis, MO). L-659,989 (17, 22) and L-648,611 (23, 24) were synthesized at Merck, with purity greater than 99%. CV-6209 (25) was kindly provided by Dr. Y. Oka (Takeda Chemical Industries, Ltd.) (Fig. 1). *N*-Acetyl-β-D-glucosamine, *N*-acetyl-β-D-galactosamine, D-glucosamine, and L-fucose were obtained from Sigma. All other chemicals were reagent grade and were commercially available.

Methods

Preparation of human platelet membranes. Human platelets were prepared from freshly drawn blood, following the same procedure as described for rabbit platelets (9). The isolated human platelets were finally suspended in 5 mM $MgCl_2$, 10 mM Tris, 2 mM EDTA, pH 7.0, and the cells were lysed by following the freezing and thawing procedure (9). The lysed membranes were then further fractionated, on a discontinuous sucrose density gradient, into membrane fractions A and B (11). The isolated membranes were stored at −80° and thawed before use. The membrane protein content was determined with the method of Lowry *et al.* (26), with BSA as the standard.

Assay for [^{3}H]*N*-methylcarbamyl-PAF and [^{3}H]C_{16}-PAF binding. The binding of [^{3}H]C_{18}-PAF or [^{3}H]*N*-methylcarbamyl-PAF to receptors in isolated membranes was performed as described previously (9, 16). For saturation isotherm binding studies, 100 μg of membrane protein were incubated with 0.5–6 nM [^{3}H]C_{18}-PAF or [^{3}H]*N*-methylcarbamyl-PAF. Nonspecific binding was determined with a 1000-fold excess of C_{16}-PAF. The equilibrium dissociation constant (K_D) and the maximal detectable receptor number (B_{max}) were calculated by using program EBDA (Elsevier-Biosoft, Cambridge, UK) on an IBM-AT computer. To displace the binding of *N*-[^{3}H]methylcarbamyl-PAF, 100 μg of membrane protein were added to a final 1-ml solution containing 1 pmol (1 nM final concentration) of *N*-[^{3}H]methylcarbamyl-PAF and a known amount of C_{16}-PAF, enantio-C_{16}-PAF, or L-659,989, in 10 mM $MgCl_2$, 10 mM Tris, 0.25% BSA, pH 7.0. After 4 hr of incubation at 0°, the mixture was filtered through a Whatman GF/C fiber glass filter to separate the bound and unbound tritium-labeled ligand. The binding inhibition was normalized as the percentage of inhibition by assigning the total binding in the absence of inhibitor to be 0% inhibition and total binding in the presence of 1 μM C_{16}-PAF to be 100% inhibition (21). The ED_{50} value was defined as the concentration of the inhibitor needed to obtain 50% of the specific binding.

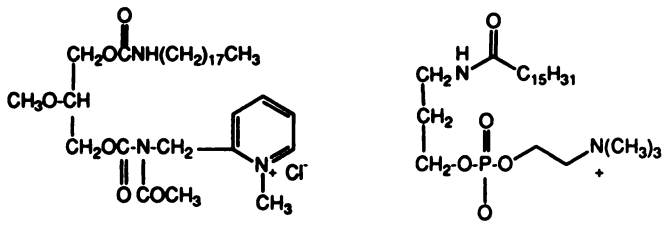
To calculate the equilibrium inhibition constant (K_i) of the compound from the competitive inhibition of [^{3}H]*N*-methylcarbamyl-PAF binding, IC_{50} was determined from an indirect Hill plot (27). The K_i value was then calculated from the Cheng and Prusoff equation (28), as described previously (29).

Treatment of human platelet membranes with neuraminidase and β-*N*-acetylglucosaminidase. The platelet membranes at a concentration of 1 mg/ml were incubated with neuraminidase (0.05 unit/ml; Sigma) and β-*N*-acetylglucosaminidase (0.1 unit/ml; Sigma) in a phosphate-citrate buffer (8.3 mM), pH 5.0, at 25° for 3 min. No further sialic acids were released with a longer incubation. The released sialic acid was determined by following the published procedure (30). After incubation, the enzymes were removed by layering of the membranes on a discontinuous sucrose density gradient (0.25 and 1.5 M) and centrifugation at 100,000 × *g* for 1 hr at 4°. The membranes at the interface between 0.25 and 1.5 M were used for the binding assay.

Preparation of gel-filtered human and rabbit platelets and monitoring of platelet aggregation. Human blood (9 volumes) was drawn from an arm vein of healthy volunteers, and rabbit blood was drawn from an ear artery directly into plastic syringes containing 1 volume of 3.8% sodium citrate. Gel-filtered human and rabbit platelets were prepared as described previously (9, 21). The aggregation of human or rabbit platelets was performed using a Chronolog Dual Aggregometer (Havertown, PA), at 37° with continuous stirring. The percentage of platelet aggregation was calculated from the maximal transmittance change by assigning the transmittance of the unstimulated platelet suspension to be 0% and that of buffer solution to be 100% aggregation. To avoid platelet aggregation due to cyclooxygenase metabolites, the isolated platelets were preincubated with aspirin at 100 μM for 15 min. Creatine phosphate (0.7 mM) and creatine phosphokinase (39.3 units/ml) were added before PAF stimulation (21). The ED_{50} was defined as the concentration required to reach 50% of the maximal aggregation.

Binding of [^{3}H]*N*-methylcarbamyl-PAF to washed human platelets. Washed human platelets were prepared as described by Alam *et al.* (31), except that the isolation was performed at room temperature. The prepared human platelets in Tris-Tyrode's albumin buffer were incubated at 37° for 15 min in either the presence or absence of L-648,611. The platelets (final concentrations of 6–10 × 10^8 platelets/ml) were then added to an incubation mixture containing 0.1–15 nM [^{3}H]*N*-methylcarbamyl-PAF in Tris-Tyrode's albumin buffer at pH 7.4, in the presence or absence of 30 μg WGA/ml. Experimental procedures and data calculation were performed as described for the isolated membrane system, except that the cells were incubated for 2 hr and a 1000-fold excess of L-659,989 was used to measure the nonspecific binding. Excess C_{16}-PAF interfered with the determination of total binding and nonspecific binding of [^{3}H]PAF to washed platelets in the presence of L-648,611. With concentrations of [^{3}H]*N*-methylcarbamyl-PAF above 5 nM, the nonspecific binding is larger than the total binding. This could be due to the enhanced detergent effect in the presence of C_{16}-PAF (>5 μM) and L-648,611 (80 μM). In the control experiments, no differences in the K_D and B_{max} values were observed using either excess C_{16}-PAF or excess L-659,989 to measure the nonspecific binding.

Incorporation of [^{3}H]acetate into PAF in washed human platelets. Washed human platelets, prepared as described above, were either used directly for the studies of PAF synthesis or pretreated with 2 mM PMSF at 37° for 10 min; the PMSF was removed by centrifugation at 1800 × *g* for 6 min (32). For the study of PAF synthesis by washed human platelets, procedures described by Doebber and Wu (33) were followed. [^{3}H]Acetate (50 μCi) was added, 15 min before the addition of 30 μg of WGA to each tube, to a final volume of 1 ml containing 10^9 platelets in Tris-Tyrode's albumin buffer at pH 7.4. After incubation for a time interval as indicated, the platelet suspensions were then transferred to glass tubes containing 3.8 ml of methanol/chloroform (2:1, v/v). The [^{3}H]PAF was separated from [^{3}H]acetate by two sequential extractions, utilizing the procedure of Bligh and Dyer



CV-6209 L-648,611

Fig. 1. Structural formulae of CV-6209 and L-648,611.

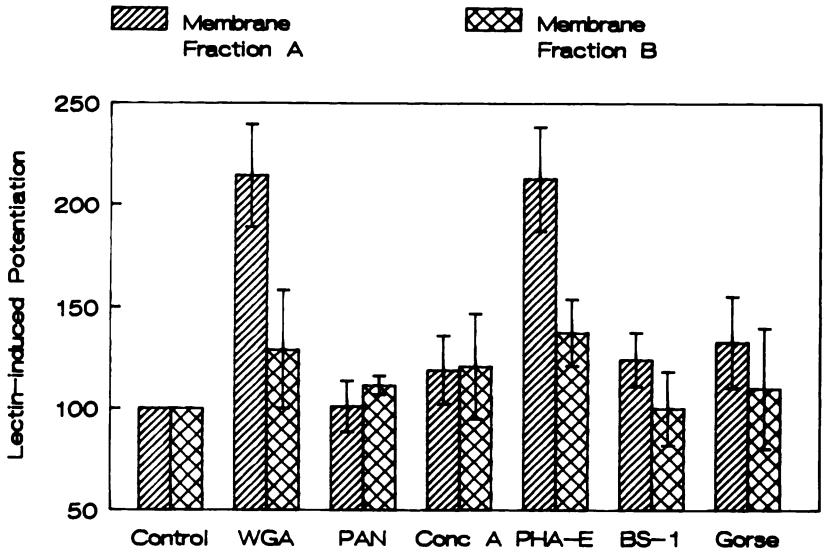


Fig. 2. Potentiation effects of [^{3}H]*N*-methylcarbamyl-PAF binding to membrane fraction A and membrane fraction B from lysed human platelet membranes. Lectins were added to an incubation mixture containing 100 μg of membrane protein and 1 nM [^{3}H]*N*-methylcarbamyl-PAF, in 10 mM $MgCl_2$, 10 mM Tris, 0.25% BSA, pH 7.0. The dosages of lectins used were WGA, 31 μg/ml; PAN, 0.8 μg/ml; Conc A, 62 μg/ml; PHA-E, 16 μg/ml; BS-1, 31 μg/ml; and Gorse, 8 μg/ml. The data were normalized so that the specific binding in the absence of lectin was 100%. The *data point error bars* are the mean standard deviations of four independent experiments. In each experiment, quadruplicate determinations were performed.

(34). The [^{3}H]PAF was identified by thin layer chromatography with the solvent system of methanol/chloroform/water/acetic acid (25:50:4:8) (35). A [^{3}H]PAF standard that was added to 3.8 ml of methanol/chloroform containing platelet suspensions was processed in the same manner; this was used for the identification of PAF and the determination of the percentage of recovery from the separation procedures. One-centimeter sections of each lane were scraped and counted for tritium activity.

Stimulation of acetyltransferase activity in washed human platelets. For the WGA induction of acetyltransferase in washed human platelets, the procedures described by Doebber and Wu (33) were followed. One hundred microliters of WGA in Tris-Tyrode's albumin buffer was added to 400 μl of washed human platelets (1.25×10^9 platelets/ml). The reaction mixture was then incubated at 37° for an indicated time. Then, 0.5 ml of 0.1 M Tris·HCl (pH 7.2) was added to the cell suspension, and the suspension was sonicated for 10 sec (Sonicator W-375; Heat-System Ultrasonics, Inc.). Sonicated cells (400 μl) were mixed with 600 μl of 0.1 M Tris·HCl, pH 7.2, containing substrates to result in final concentrations of 5 μM lyso-PAF and 100 μM [^{3}H]acetyl-coenzyme A (0.3 μCi). After 10 min of incubation, the reaction was stopped by the addition of 3.8 ml of a 2:1 mixture of methanol/chloroform. The synthesized [^{3}H]PAF was separated from [^{3}H]acetyl-coenzyme A by three sequential extractions, following the procedure of Bligh and Dyer (34). The lower chloroform phase was collected, dried under argon gas, and counted for radioactivity. A [^{3}H] C_{16}-PAF standard and a [^{3}H]acetyl-coenzyme A sample without cell homogenates were processed in the same manner for the determination of the percentage of recovery and the background for the separation procedures.

Inhibition of PAF acetylhydrolase activity by PMSF. PAF acetylhydrolase activity was determined by measuring [^{3}H]acetate release from [*acetyl*-2′-^{3}H]C_{16}-PAF (NEN-DuPont), following the procedures described by Miwa *et al.* (36). [^{3}H]Acetyl-PAF was suspended in Tris-Tyrode's albumin buffer, pH 7.4, and unlabeled C_{16}-PAF was added to reach the desired concentrations. Incubation was initiated by the addition of either 50 μl of homogenates of human platelets or 25 μl of human serum and 25 μl of Tris-Tyrode's albumin buffer, with or without 2 mM PMSF, to 50 μl of [^{3}H]acetyl-PAF. The reaction was incubated at 37° for 2, 5, 10, 20, or 30 min and then stopped by the addition of 100 μl of 14% trichloroacetic acid. The reaction mixture was left to stand at 0° for 10 min and then centrifuged with an Eppendorf centrifuge for 5 min. A 100-μl aliquot of the supernatant was mixed with 10 ml of Aquasol II, to count the radioactivity. Human serum samples were obtained by centrifugation of the venous blood at $750 \times g$ for 15 min, and homogenates of human platelets were obtained by sonication of the washed human platelets for 15 sec (Sonicator W-375; Heat-System Ultrasonics, Inc.). The control values of the released [^{3}H]acetic acid were obtained by the addition of serum or platelet homogenate to a reaction mixture of 50 μl of [^{3}H]acetyl-PAF and 100 μl of 14% trichloroacetic acid.

Results

Effects of lectins on the binding of [^{3}H]*N*-methylcarbamyl-PAF to human platelet membranes. Fig. 2 shows the effects of several selected lectins on the binding of *N*-methylcarbamyl-PAF to specific receptor sites on human platelet membranes. The dosages of lectins used here were about 4 times those used to initiate the agglutination of the red blood cells (Sigma technical data). WGA and PHA-E significantly potentiated the specific binding of [^{3}H]*N*-methylcarbamyl-PAF to human platelet membranes, whereas Conc A, PNA, Gorse, and BS-1 showed no effects on the binding. As also shown in Fig. 2, the potentiation effect on membrane fraction A was higher than that on fraction B. Membrane fraction A has also been shown to contain higher activity of alkaline phosphates, lower activity of antimycin-insensitive NADH-cytochrome *c* reductase (11, 37), and lower density of receptors for inositol-1,4,5-trisphosphate (38) than membrane fraction B. Therefore, membrane fraction A is likely a plasma membrane-enriched fraction and membrane fraction B is enriched with the dense tubular system. Unless otherwise specified, membrane fraction A was used throughout.

Potentiation effects of WGA on the binding of [^{3}H]*N*-methylcarbamyl-PAF to human platelet membranes. Fig. 3 shows the dose dependence of the effects of WGA on the binding of [^{3}H]*N*-methylcarbamyl PAF to membrane fraction A of human platelet membranes. WGA increased both the total binding and the nonspecific binding in membrane fraction A. However, no detectable increase in the nonspecific binding was observed when the experiments were performed with membrane fraction B. With membrane fraction A, the potentiation of specific binding reached a maximum at or around 30 μg/ml. At 30 μg/ml, the specific binding was potentiated almost 5-fold in this membrane preparation. Similar to the previous report on the variable number of receptors for PAF (11), the potentiation effect by WGA on the specific binding also varied (2- to 5-fold) from one membrane preparation to another.

A more detailed analysis of WGA potentiation is shown in

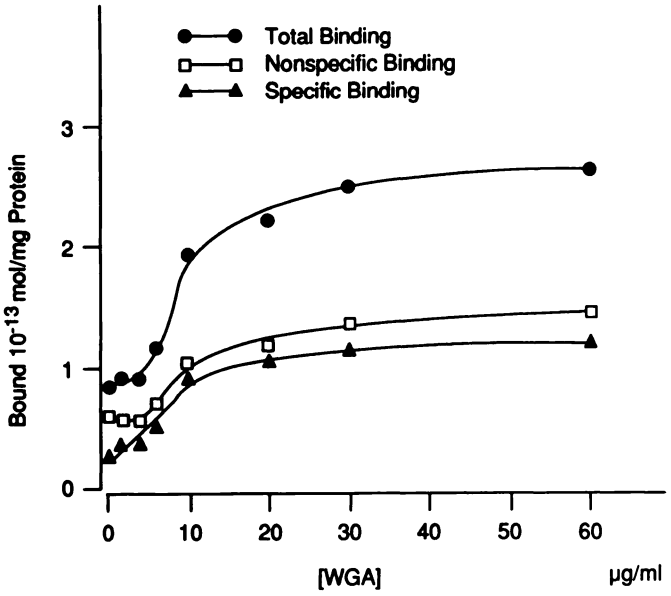


Fig. 3. Dependence on WGA concentration of the specific binding of [^{3}H] *N*-methylcarbamyl-PAF to human platelet membranes. WGA was added to the incubation mixture as described in the legend to Fig. 2. Each *data point* represents the mean of quadruplicate determinations.

Fig. 4 and summarized in Table 1 for several membrane preparations. As demonstrated in Scatchard plots (39), the WGA potentiation effect was apparently due to an increase in the maximal number of receptor sites (B_{max}) but not in the equilibrium dissociation constant (K_D). In the presence of 30 μg WGA/ml, as summarized in Table 1, the number of specific receptor sites for *N*-methylcarbamyl-PAF or C_{18}-PAF was increased 2- to 3-fold, but with no significant alteration in the K_D values. In the same membrane preparation (membrane 334A in Table 1), [^{3}H]C_{18}-PAF was found to have a significantly higher number of maximal binding sites than [^{3}H]*N*-methylcarbamyl PAF ($p = 0.0048$). However, in the presence of 30 μg WGA/ml, the maximal detectable number of receptor sites for [^{3}H]C_{18}-PAF was not significantly different from that for [^{3}H]*N*-methylcarbamyl PAF.

ADP (100 μM), thrombin (1 unit/ml), and collagen (0.2 mg/ml) showed no effects on the specific or nonspecific binding of [^{3}H]C_{18}-PAF to isolated membranes and showed no effects on the WGA-induced potentiation of the [^{3}H]C_{18}-PAF binding. These results exclude the possibility that the increase in binding sites is not due to a specific effect of the lectin but is a component of activation of platelets.

Specificity of the WGA-potentiated specific binding of *N*-methylcarbamyl-PAF. The WGA-potentiated specific binding of [^{3}H]*N*-methylcarbamyl-PAF was specifically inhibited by *N*-acetyl-D-glucosamine, with an ED_{50} value of 7 mM (Fig. 5). D-Glucosamine and *N*-acetyl-D-galactosamine were less potent than *N*-acetyl-D-glucosamine, with ED_{50} values of 30 and 100 mM, respectively. L-Fucose at similar concentrations showed no inhibition of the WGA-potentiated binding of [^{3}H] *N*-methylcarbamyl-PAF.

Effects on the K_D and B_{max} values of human platelet membranes treated with neuraminidase and β-*N*-acetylglucosaminidase. As demonstrated in Table 2, human platelet membranes treated with neuraminidase and β-*N*-acetylglucosaminidase to remove both sialic acid and *N*-acetylglucosamine had a K_D identical value to the K_D values of membranes that were either untreated (Table 1) or treated with phosphate-citrate buffer, pH 5.0, for 3 min at 25°. However, the B_{max} value of the enzyme-treated membranes was significantly higher than that of the control membranes, increased from 5.66 in the control membranes to 12.16×10^{-14} mol/mg of protein in the enzyme-treated membranes, which is roughly the same value as that for membranes in the presence of 30 μg WGA/ml during the binding assay (Table 2). WGA showed no further significant effects on the specific or nonspecific binding of [^{3}H]C_{18}-PAF in the enzyme-treated membranes.

Na^+ effects on the binding of [^{3}H]*N*-methylcarbamyl-PAF in the presence or in the absence of WGA. It had been demonstrated previously that Na^+ specifically inhibited the binding of [^{3}H]PAF to both rabbit (16) and human platelet membranes (13). Here, Na^+ showed an identical inhibitory activity on the specific binding of *N*-methylcarbamyl-PAF, with an ED_{50} value of approximately 5 mM (data not shown) either with or without WGA. A similar ED_{50} value for Na^+ inhibition of the [^{3}H]PAF binding to rabbit platelet membranes has been reported previously (16).

Inhibition of WGA-potentiated binding of [^{3}H]*N*-methylcarbamyl-PAF by C_{16}-PAF, enantio-C_{16}-PAF, and L-659,989. C_{16}-PAF, enantio-C_{16}-PAF and L-659,989, a specific PAF receptor antagonist (22), competed with the binding of [^{3}H]*N*-methylcarbamyl-PAF to human platelet membranes as potently as with the binding of [^{3}H]PAF to human platelet membranes (11, 22). C_{16}-PAF, enantio-C_{16}-PAF, and L-659,989 also fully displaced the WGA-potentiated binding of [^{3}H]*N*-methylcarbamyl-PAF, with similar potencies as in control membranes.

WGA potentiation of the PAF-induced aggregation of rabbit and human platelets. PAF induced aggregation of human and rabbit platelets with ED_{50} values of about 4×10^{-9} and 9×10^{-10} M, respectively (Fig. 6). With a concentration of WGA below the threshold for platelet aggregation, PAF-induced platelet aggregation was potentiated by WGA (Fig. 6).The potentiation effects of WGA on PAF-induced aggregation were partly due to an increase in the PAF potency (leftward shifting of the dose-response curves) and partly due to an increase in the maximal aggregation response induced by PAF. In the presence of WGA, the PAF concentrations required to reach an equivalent aggregation of human and rabbit platelets were shifted to 2×10^{-9} and 2×10^{-10} M, respectively. The maximal aggregation responses were increased from 10 and 56 to 22 and 67% aggregation for human and rabbit platelets, respectively. WGA-induced increases in the maximal aggregation response of platelets induced by PAF indicated an increase in the receptor number of the functional PAF receptors. A shift of the PAF dose-response curve to the left may correspond to PAF synthesis in platelets (see below).

Biosynthesis of PAF in human platelets induced by WGA. WGA also induced aggregation of human (>2 μg/ml) and rabbit platelets (>50 μg/ml), even in the presence of an ADP scavenger (creatine phosphate/creative phosphokinase, 0.7 mM/39.5 units/ml) and the cyclooxygenase inhibitor aspirin (100 μM). In human platelets, the aggregation reached a maximum at 30 μg/ml WGA (Fig. 7). WGA also induced an increase in the acetyltransferase activity in human platelets. The increase in the acetyltransferase activity was observed even at dosages at which little or no measurable aggregation was observed (Fig. 7). The aggregation induced by WGA was not sensitive to a PAF receptor antagonist, L-659,989 (22), even up

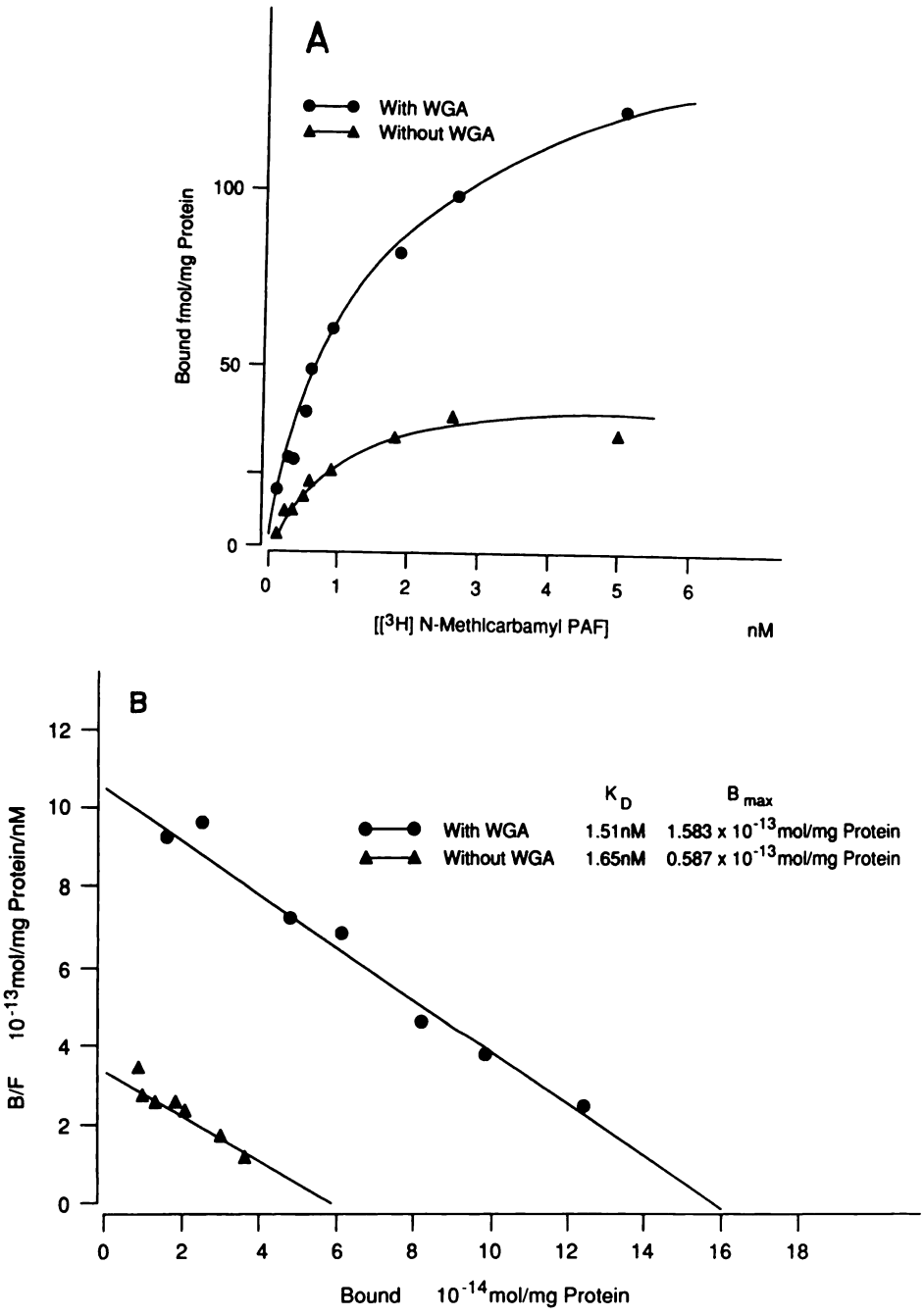


Fig. 4. A, Saturable binding of [^{3}H]*N*-methylcarbamyl-PAF to human platelet membranes in the presence (●) and in the absence (▲) of 30 μg WGA/ml. One hundred micrograms of membrane protein were added to tubes containing [^{3}H]*N*-methylcarbamyl-PAF ranging from 0.2 to 6 nM, in an assay medium of 10 mM $MgCl_2$, 10 mM Tris, 0.25% BSA, pH 7.0. Each *data point* is the mean of quadruplicate determinations. B, Scatchard plots of the specific [^{3}H]*N*-methylcarbamyl-PAF binding to human platelet membranes in the presence (●) and in the absence (▲) of 30 μg WGA/ml. The data points were calculated from the data presented in A. *B/F*, bound/free.

TABLE 1

Equilibrium dissociation constants (K_D) and maximal binding sites (B_{max}) for tritium-labeled C_{18}-PAF and *N*-methylcarbamyl-PAF

Ligand	Membrane	WGA	K_D	B_{max}	Repeated experiments
		μg/ml	nM	10^{-14} mol/mg of protein	
N-Methylcarbamyl-PAF	334A	30	1.98 ± 0.17	9.23 ± 0.22	(n = 2)
		0	1.69 ± 0.26	3.11 ± 0.59	(n = 4)
	280A	30	1.55 ± 0.06	18.34 ± 2.4	(n = 4)
		0	1.53 ± 0.13	5.71 ± 0.3	(n = 4)
C_{18}-PAF	334A	30	1.62 ± 0.27	10.55 ± 0.95	(n = 3)
		0	1.54 ± 0.04	5.45 ± 1.1	(n = 3)
	306A	30	1.75 ± 0.28	30.6 ± 7.4	(n = 4)
		0	1.72 ± 0.24	14.1 ± 2.5	(n = 4)

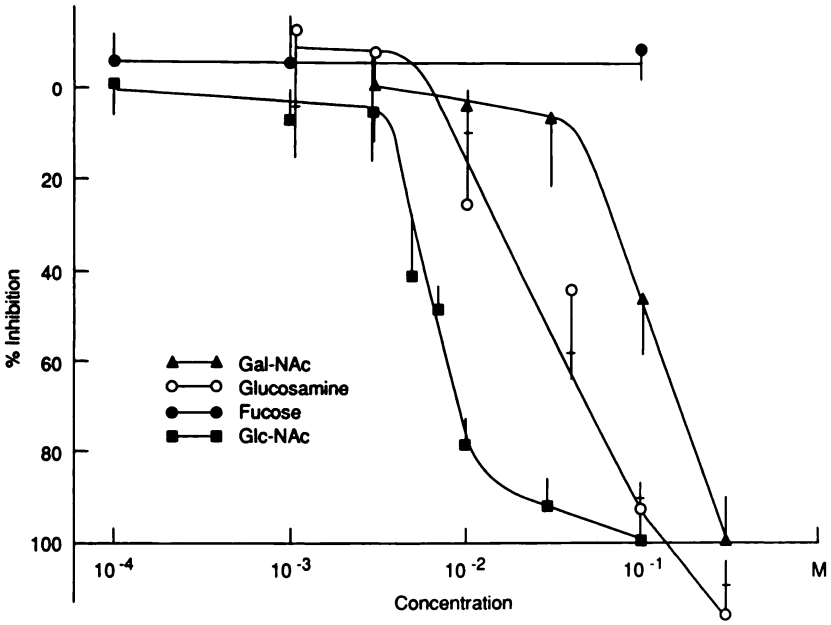


Fig. 5. Inhibition of WGA-potentiated specific binding of [^{3}H]*N*-methylcarbamyl-PAF to human platelet membranes by *N*-acetyl-D-glucosamine (■), D-glucosamine (○), L-fucose (●), and *N*-acetyl-D-galactosamine (▲). The assay was performed with 100 μg of membrane protein and 1 nM [^{3}H]*N*-methylcarbamyl-PAF, in 10 mM $MgCl_2$, 10 mM Tris, 0.25% BSA, pH 7.0, in a final volume of 1 ml. The data were normalized so that the specific binding with 30 μg/ml WGA was defined as 0% inhibition and the specific binding in the absence of WGA was 100% inhibition. The *data points* and *error bars* are the means and the standard deviations from three or four experiments.

TABLE 2

Equilibrium dissociation constants (K_D) and maximal binding sites (B_{max}) for [^{3}H]C_{18}-PAF in human platelet membranes (membrane 324A) treated with neuraminidase (0.05 unit/ml) and β-*N*-acetylglucosaminidase (0.2 unit/ml)

Compound	K_D	B_{max}
	nM	10^{-14} mol/mg of protein
Control	1.74 ± 0.08	5.66 ± 1.27 (*n* = 3)
Control + 30 μg/ml WGA	1.67 ± 0.15	12.16 ± 2.16 (*n* = 3)
Enzyme-treated	1.72 ± 0.07	11.27 ± 1.28 (*n* = 3)

to a concentration of 12 μM. However, the aggregation could be partially blocked by L-648,611, an inhibitor of PAF acetyltransferase (23, 24), and CV-6209, a specific PAF receptor antagonist (25) (Fig. 8). Here, L-648,611 at 80 μM showed no significant inhibition of the PAF-induced aggregation of human platelets (Fig. 8). These results suggest that the inhibitory effect of L-648,611 on WGA-induced aggregation is not due to the possible detergent effect of L-648,611 and that PAF synthesis may be involved in the WGA-induced platelet aggregation.

Indeed, PAF was synthesized in the WGA-activated platelets that were either pretreated with 2 mM PMSF or not treated. As shown in Fig. 9, PAF synthesis induced by WGA reached a maximum after approximately 2–3 min and then declined even before the maximal response of platelet aggregation was reached. A much larger amount of PAF was synthesized in the presence of 2 mM PMSF. Therefore, PMSF may act as an inhibitor of the intracellular acetylhydrolase, as originally suggested by Touqui *et al.* (32). Indeed, PMSF is a competitive inhibitor of PAF acetylhydrolase either from human plasma or from washed human platelets. In the double-reciprocal plot of enzyme kinetics, the intercept on the *y*-axis is the same in the presence and absence of inhibitor, although the slope is different (data not shown). Also, as demonstrated in Fig. 10, the WGA-induced PAF synthesis can be blocked by pretreatment of washed platelets with the acetyltransferase inhibitor L-648,611. At a concentration higher than 90 μM, the amount of PAF synthesized is significantly lower than that in the unstimulated human platelets.

Effects of WGA and L-648,611 on the binding of [^{3}H] *N*-methylcarbamyl-PAF to washed human platelets. As described above, WGA potentiated the binding of [^{3}H]C_{18}-PAF and [^{3}H]*N*-methylcarbamyl-PAF in isolated membranes, and the potentiation effect was due to an increase in the B_{max} values, with no significant changes in the K_D values. However, in washed human platelets, the receptor number/platelet was not increased in the presence of 30 μg WGA/ml. Washed human platelets had 72 ± 2 receptors/platelet and the receptors had a K_D value of 2.67 ± 0.73 nM (four experiments) (Table 3). In the presence of 30 μg/WGA ml, the receptor number/platelet decreased to 58. Decrease in the receptor number/platelet might be due to the PAF synthesis in the WGA-activated platelets. Indeed, the receptor number/platelet increased more than 2-fold in the WGA-activated platelets in the presence of 80 μM L-648,611, a PAF synthesis inhibitor. L-648,611 alone showed no effect on the K_D or B_{max} values.

Discussion

Here, we have demonstrated that WGA potentiated the binding of [^{3}H]C_{18}-PAF and [^{3}H]*N*-methylcarbamyl-PAF to isolated human platelet membranes. The potentiation effects arise from increases in the PAF receptor number and can be specifically blocked by those saccharides that specifically bind to the lectins. Increases in the B_{max} value can also be detected by treating membranes with neuraminidase and β-*N*-acetylglucosaminidase. The receptors exposed by WGA seem to be identical to those present in the absence of WGA. They exhibit exactly the same K_D value, the same Na^+ inhibitory effects, an equal capacity to distinguish between the biologically active C_{16}-PAF and the inactive enantio-C_{16}-PAF, and an equal binding affinity for the PAF receptor antagonist L-659,989.

The WGA-potentiated binding of [^{3}H]*N*-methylcarbamyl-PAF can be specifically blocked by *N*-acetyl-D-glucosamine, and the inhibitory activity is more potent than that of two other structural analogs, *N*-acetyl-D-galactosamine and D-glucosamine. L-Fucose shows no activity at even up to 100 mM concentration. This order of inhibitory potency is identical to the binding specificity of WGA for various sugars (40). [^{3}H]*N*-Methylcarbamyl-PAF shows no specific binding to WGA itself. The potentiation effect in membrane fraction A is higher than that in membrane fraction B, and WGA shows no potentiation

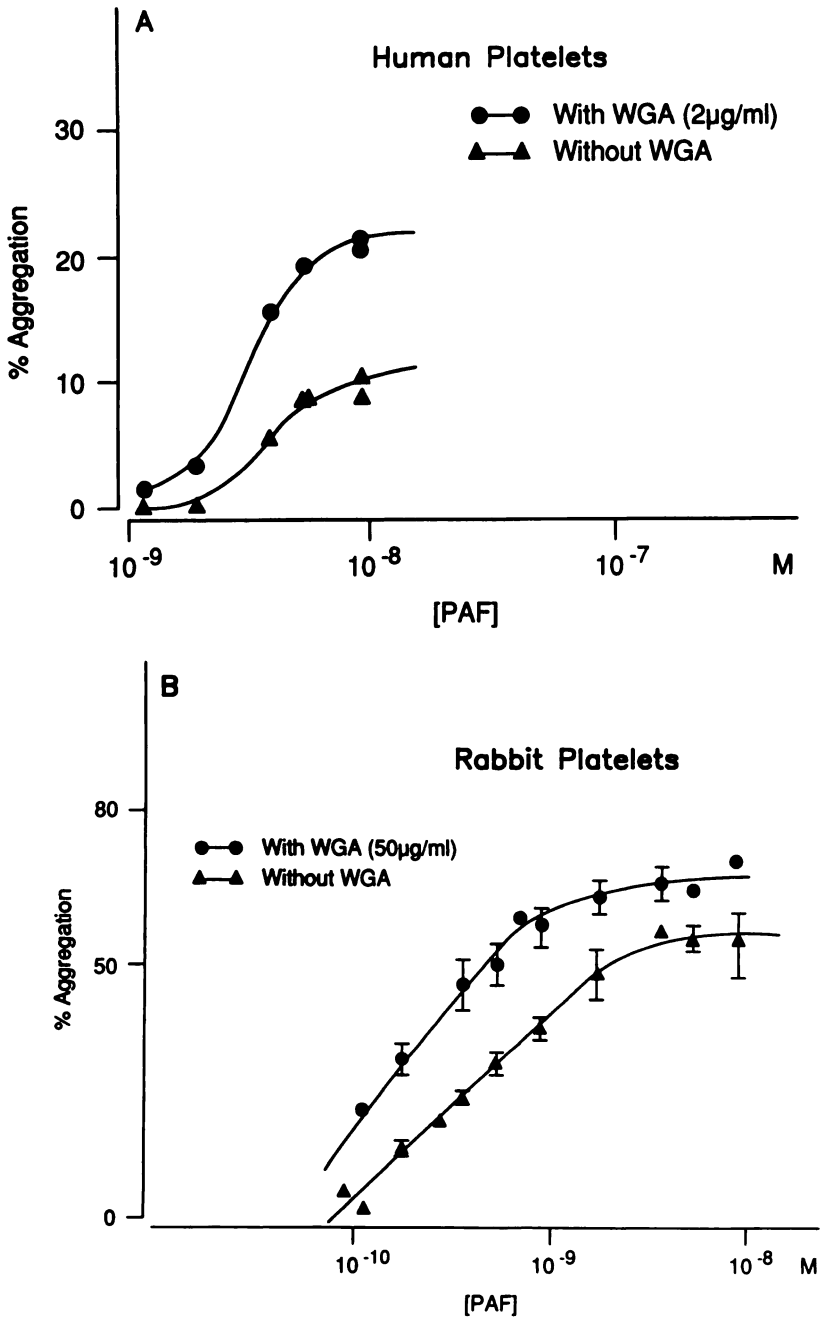


Fig. 6. A, Potentiation effects on PAF-induced aggregation of rabbit platelets of 50 μg WGA/ml. B, Potentiation effects on PAF-induced aggregation of human platelets of 2 μg WGA/ml.

effects on those membranes pretreated with neuraminidase and β-*N*-acetylglucosaminidase. These results suggest that the potentiation effect on the binding caused by WGA is not due to WGA itself but is due to the binding of WGA to one of the membrane glycoproteins or glycolipids, which may then modulate the conformation of the PAF receptor.

Two possible mechanisms could account for the binding potentiation by WGA or PHA-E. The first is that the PAF receptor itself is a glycoprotein. In this case, WGA or PHA-E would bind to the PAF receptor and change the conformation of those receptors in the low affinity state to the high affinity state. It has been reported previously that membrane fraction B contains more receptor sites than membrane fraction A. PAF receptors from either membrane fraction A or membrane fraction B show identical K_D values for [^{3}H]*N*-methylcarbamyl-PAF and [^{3}H]PAF, as well as identical K_i values for several selected PAF receptor antagonists with different structures (11). Here, we have demonstrated that WGA or PHA-E potentiated the binding preferentially in membrane fraction A and that the membranes pretreated with neuraminidase and β-*N*-acetylglucosamine showed K_D and B_{max} values similar to those of membranes in the presence of WGA. These results suggest that WGA modulation sites may not be on the PAF receptor itself.

The second possibility is that WGA or PHA-E binds not to PAF receptors but to an intrinsic inhibitor of PAF receptors. The binding of WGA to the saccharide substituent of such an endogeneous glycolipid or glycoprotein could induce either dissociation from or association with the PAF receptor. Indeed, the fact that membrane fraction A from human platelets shows greater potentiation of binding than membrane fraction B supports the idea that an endogeneous modulator exists. However, we cannot rule out the possibility that an internal pool of receptors lacks posttranslational modification with carbohydrate groups.

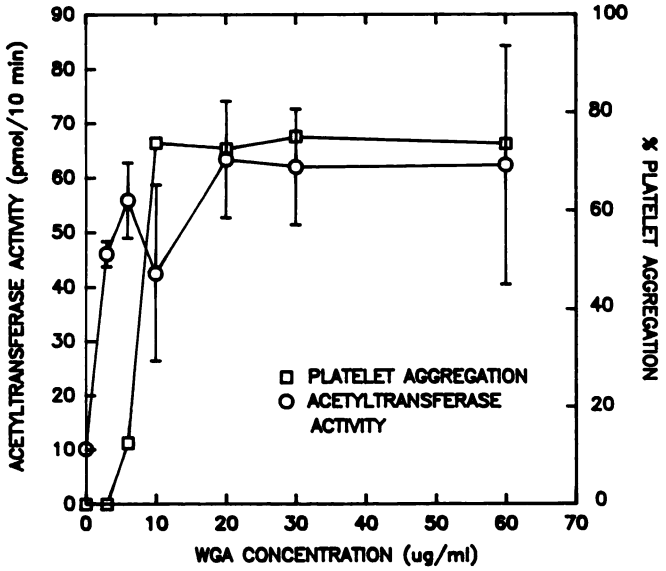


Fig. 7. WGA-induced aggregation of (□) and WGA-induced increase in acetyltransferase activity from (○) washed human platelets at 37°. The WGA-induced aggregation of human platelets was monitored as described in Experimental Procedures. For the WGA induction of acetyltransferase activity, human platelets were stimulated with WGA, at the indicated concentration, at 37°. After a 2-min stimulation with WGA, the cell suspension was diluted with 0.5 ml of 0.1 M Tris·HCl, pH 7.2 (0°), followed by sonication for 10 sec. The acetyltransferase activity was then quantitated by measurement of the synthesized [^{3}H]PAF after 10 min of incubation at 37° with 5 μM lyso-PAF and 100 μM [^{3}H]acetyl-coenzyme A (0.3 μCi). The *data points* and *error bars* for the PAF synthesis are the means and standard deviations of triplicate determinations.

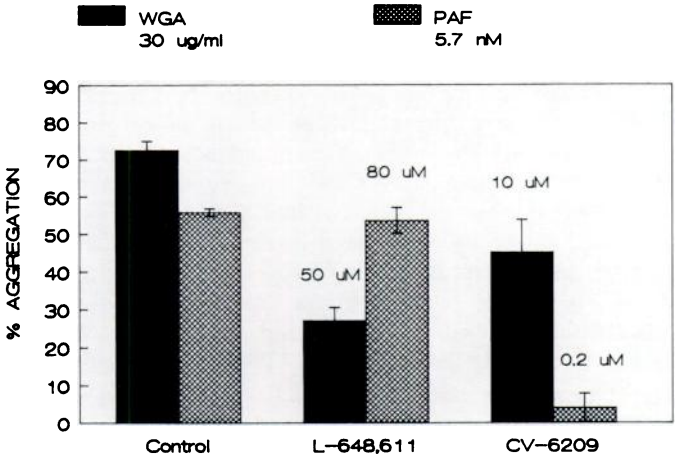


Fig. 8. Inhibition of WGA- and PAF-induced aggregation of gel-filtered human platelets. L-648,611 and CV-6209 were solubilized in dimethyl sulfoxide and were added 15 min before WGA stimulation. The control was pretreated with dimethyl sulfoxide. The inhibition of WGA-induced aggregation depended on the time interval between the addition of inhibitor and the addition of WGA. Maximal inhibition occurred with about 15 min of pretreatment. For the inhibitory effects of compounds on PAF-induced aggregation, the compound was added 1 min before PAF stimulation. The *data points* and *error bars* represent the means and standard deviations from three or four experiments.

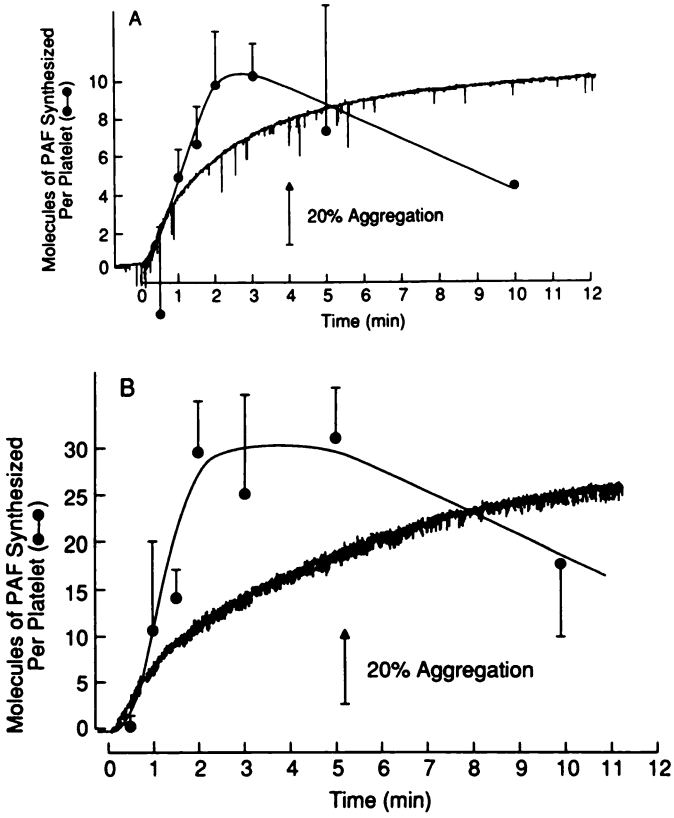


Fig. 9. WGA-induced PAF synthesis and aggregation in human platelets either untreated (A) or pretreated with 2 mM PMSF (B). Platelet aggregation and PAF synthesis were induced with 30 μg/ml WGA. The *data points* and *error bars* are the means and standard deviations of triplicate determinations. Results shown are representative of three different experiments.

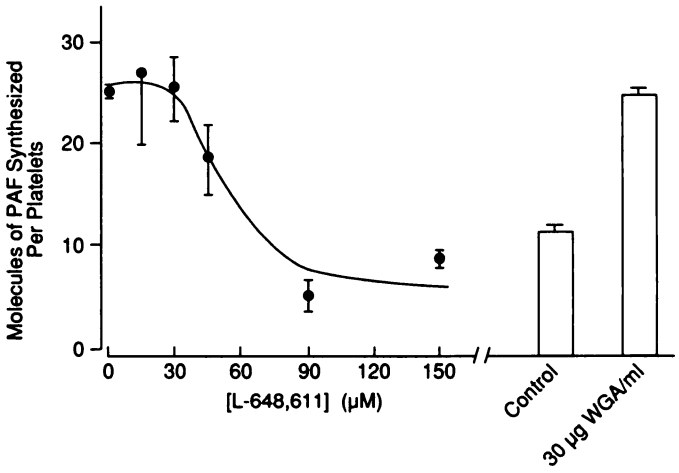


Fig. 10. Inhibition by L-648,611 of WGA-induced PAF synthesis in human platelets pretreated with 2 mM PMSF. L-648,611 in dimethyl sulfoxide was added 15 min before WGA (30 μg/ml) stimulation. The control was pretreated with dimethyl sulfoxide. The *data points* and *error bars* are means and standard deviations of triplicate determinations.

Na^+ specifically inhibits the binding of [^{3}H]PAF to both rabbit (16) and human platelet membranes (13) and may convert PAF receptors in a high affinity state into a low affinity state, with a K_D value possibly in the micromolar range (17). However, Na^+ shows no differences in inhibiting the binding to either PAF receptors or WGA-exposed receptors, suggesting that WGA may not bind to the Na^+ regulatory sites.

WGA increased not only the specific binding but also the nonspecific binding of [^{3}H]*N*-methylcarbamyl-PAF (Fig. 2) or [^{3}H]C_{18}-PAF to fraction A of human platelet membranes. The exact mechanism for this increase in the nonspecific binding is not yet known. However, no increase in the nonspecific binding

TABLE 3

Equilibrium dissociation constants (K_D) and receptor numbers/platelet for [^{3}H]*N*-methylcarbamyl-PAF in washed human platelets

Condition	K_D	Receptors/platelet
	nM	
Control	2.67 ± 0.73	72 ± 2 (n = 4)
+30 μg/ml WGA	2.03 ± 0.84	58 ± 19 (n = 4)
+80 μM L-648,611	3.97 ± 0.04	73 ± 4 (n = 2)
+30 μg/ml WGA and 80 μM L-648,611	3.88 ± 0.66	164 ± 27 (n = 3)

was observed when membrane fraction B was used for the binding studies. Furthermore, WGA showed little or no effects on either specific binding or nonspecific binding to those membranes pretreated with neuraminidase and β-*N*-acetylglucosaminidase. These results exclude the possibility that the WGA-induced increase in nonspecific binding may be dependent on PAF receptor specific binding.

It has been demonstrated before that the detectable receptor number for tritium-labeled L-659,989 in Scatchard graphs is significantly higher than that for [^{3}H]PAF, in rabbit platelet membranes under identical assay conditions with 10 mM $MgCl_2$ (17). The difference has been attributed to the coexistence of multiple conformational states of the PAF receptors (17). Here, the maximal detectable receptor number for [^{3}H]C_{18}-PAF binding to human platelet membranes is significantly higher than that for [^{3}H]*N*-methylcarbamyl-PAF, whereas both ligands detect identical numbers of receptor sites in the presence of WGA. This could again be due to the coexistence of multiple conformational states of the PAF receptors. PAF receptors are different between human platelets and PMNs (13). [^{3}H]C_{18}-PAF shows no significant difference in the K_D values between human platelet and human PMN membranes, whereas the K_D value for [^{3}H]*N*-methylcarbamyl-PAF in human PMN membranes is 5.18 ± 0.54 nM (mean ± standard deviation; eight experiments) (41), which is significantly different from the K_D value in human platelet membranes (Table 1). [^{3}H]*N*-Methylcarbamyl-PAF and [^{3}H]C_{18}-PAF may not bind to the PAF receptor at the same site. However, in the presence of WGA, the PAF receptor is modified such that it shows high affinity for either [^{3}H]C_{18}-PAF or [^{3}H]*N*-methylcarbamyl-PAF.

Human platelet membranes prepared by the freezing and thawing procedure (a) were routinely separable into two fractions on a discontinuous sucrose gradient. Based on several marker enzymes, as described in Results, membrane fraction A is likely a plasma membrane-enriched fraction, whereas membrane fraction B is enriched with the dense tubular system. However, membrane fraction B consistently contains more PAF receptor sites than membrane fraction A (11). It is clear that the intracellular membranes may contain PAF receptors. These intracellular PAF receptors may initiate cellular responses to PAF that is synthesized and retained intracellularly.

L-648,611 inhibited [^{3}H]PAF synthesis from lyso-PAF and [^{3}H]acetyl-coenzyme A in the presence of rat spleen microsomes (23). It also inhibited the synthesis of PAF in intact mouse peritoneal leukocytes stimulated by the calcium ionophore A23187 (24). In contrast, L-648,611 showed no inhibitory effects on the specific binding of [^{3}H]PAF to human and/or rabbit platelet membranes, on the activity of acetylhydrolase in human plasma, on the phospholipase A_2 activity in PAF-activated human neutrophils,[1] or on the PAF-induced aggregation of human platelets. Thus, the effects of L-648,611 on isolated enzyme and cell systems supported the classification of L-648,611 as a synthetic acetyltransferase inhibitor.

Lectins such as WGA, phytohemagglutinin, and *Ricinus communis* lectin have been shown to be strong inducers of both aggregation and release reaction for platelets (42, 43). The exact mechanism for lectin-induced platelet activation is still unknown, although it has been suggested that specific binding sites on the platelet surface may be involved in the induction of platelet activation (44). Here, 1) WGA induces aggregation of gel-filtered human platelets in the presence of a cyclooxygenase inhibitor (aspirin) and ADP scavenger (creatine phosphate/creatine phosphokinase), which can be blocked by a PAF synthesis inhibitor (L-648,611) and by a specific PAF receptor antagonist (CV-6209), 2) WGA increases the detectable number of PAF binding sites and potentiates the PAF-induced aggregation of rabbit and human platelets, and 3) WGA induces PAF synthesis and the PAF synthesis can be blocked by the PAF synthesis inhibitor L-648,611. These results suggest that PAF synthesis may be involved in the platelet activation induced by WGA, even though we cannot totally rule out the involvement of other possible mediators. Also, it should be noted that L-659,989 at 10 μM concentration shows no inhibitory effect on the WGA-induced aggregation of human platelets. A long term incubation of CV-6209 is required to demonstrate the inhibitory effect. These results suggest that the inhibitory effect of receptor antagonists may depend on the membrane permeability of the compound.

PAF synthesis in WGA-activated platelets could also explain our failure to detect an increase in the receptor number in washed platelets treated with WGA. Occupancy of PAF receptors has been found in a group of patients with septicemia (45), as well as in asthmatic patients after antigen challenge (46). A decrease in the receptor number in these patients has been proposed to be due to generation of PAF. Here, WGA increases the detectable receptor number in isolated platelet membranes. Also, the increase in the maximal aggregation response induced by PAF in the presence of WGA corresponds to an increase in the number of functional PAF receptors. However, in washed platelets the synthesized PAF may bind either to the extracellular receptor directly or to the ultracellular receptor and regulate the PAF receptor on the plasma membrane by an unknown mechanism. These receptors, therefore, cannot be detected in the radioligand binding assay. In the presence of L-648,611, by blocking the generation of PAF we indeed observed an increase in the PAF receptor number in WGA-activated human platelets.

In conclusion, WGA could interact with an endogeneous inhibitor and induce an increase in the receptor number in either isolated membranes or intact platelets. At the same time, WGA could also interact with its own receptor and induce the synthesis of PAF. The generated PAF could then activate the platelets. PAF could, therefore, act as an intracellular mediator, and the intracellular receptor may mediate the function of PAF that is synthesized and retained inside the cell. A similar conclusion has also been reported recently in human (47) and rabbit neutrophils (48), guinea pig peritoneal macrophages (48, 49), and bovine cultured aortic endothelial cells (48, 50).

Acknowledgments

The authors wish to thank My-Hanh Lam for technical help and Joan Kiliyanski and Pam Freshwater for typing this manuscript.

[1] Hwang, S. B., and M.-H. Lam, unpublished results.

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