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Characterization of Platelet-Activating Factor (PAF) Receptor by Specific Binding of [^{3}H]L-659,989, a PAF Receptor Antagonist, to Rabbit Platelet Membranes: Possible Multiple Conformational States of a Single Type of PAF Receptors

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SUMMARY

(*trans*)-2-(3-Methoxy-5-methylsulfonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)tetrahydrofuran (L-659,989) is a potent and orally active platelet-activating factor (PAF)-specific and competitive receptor antagonist. The 2,5-tritium-labeled L-659,989 ([^{3}H]L-659,989) specifically binds to rabbit platelet membranes with an equilibrium dissociation constant (K_D) of 1.60 ± 0.20 nM in 10 mM $MgCl_2$. Several selected PAF analogs and PAF receptor antagonists show equilibrium inhibition constants roughly similar to those found in the specific [^{3}H]PAF binding assay. Other pharmacological agents with no PAF antagonistic activities do not inhibit the specific binding of [^{3}H]L-659,989. K^+ and divalent cations such as Mg^{2+}, Ca^{2+}, and Mn^{2+} potentiate the specific [^{3}H]L-659,989 binding. Na^+ and Li^+ also enhance but GTP shows no effect on the specific binding of [^{3}H]L-659,989. However, Ni^{2+} inhibits the specific binding. Scatchard analysis demonstrates that the potentiating effect of these cations is due to an increase in the detectable receptor number for L-659,989. In 10 mM $MgCl_2$ [^{3}H]L-659,989 shows higher receptor number than [^{3}H]PAF. Under various ionic conditions with or without GTP, in which [^{3}H]L-659,989 binding remains approximately the same, C_{16}-PAF shows different potencies in competing against the specific [^{3}H]L-659,989 binding. These results demonstrate the existence of multiple conformational states of the PAF-specific receptor. The variation in the detectable receptor number under different conditions is due to the coexistence of the high and low affinity states and the fact that the low affinity state(s) of the receptor with K_D value(s) possibly in the micromolar range cannot be detected in the Scatchard analysis with the radioligand at nanomolar concentrations. In the presence of 150 mM NaCl and 1 mM GTP, receptors exist in a single conformational state with an equilibrium dissociation constant (K_B) of 0.931 μM for PAF.

PAF is a potent phospholipid mediator with a wide range of biological activities, which include aggregation and degranulation of platelets and neutrophils, bronchoconstriction, vascular permeability, and hypotension (1). Since its structure was identified as 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine (2–4), a large variety of chemical analogs have been synthesized. The structural requirement for PAF biological actions is highly specific. Alterations of the chemical groups, e.g., the alkyl ether group at C_1, acetyl group at C_2, or phosphorylcholine at C_3, or the stereochemical configuration of the molecule ivariably lead to significant reductions of potency (5).

A cell surface receptor that specifically binds PAF is generally believed to mediate various cellular responses to PAF. Indeed, specific receptors for PAF have been reported in a variety of cell membranes, including those from human (6–8) and rabbit (9, 10) platelets, human neutrophils (11–13), and plasma membranes from several types of tissues (9, 14, 15). However, whether there exists a single type of receptors or more in the same type of cells is still controversial. Furthermore, the equilibrium dissociation constant (K_D) of the high affinity binding sites varies widely from one study to another (5).

With isolated membranes, we have previously reported that a single type of PAF-specific receptor was found in human (8) and rabbit (9, 16) platelets, human PMNs (13), human lung tissue (14), rat live tissue (15), and rat peritoneal PMNs (cited in Ref. 13), with an equilibrium dissociation constant about 0.5

ABBREVIATIONS: PAF, platelet-activating factor; [^{3}H]L-659,989, (*trans*)-[2,5-[^{3}H]-2-(3-methoxy)-5-methylsulfonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl) tetrahydrofuran; C_{16}-PAF, 1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine; C_{18}-PAF, 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine; enantio-PAF, 3-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine; acetylamino-PAF, 1-*O*-hexadecyl-2-acetylamino-2-deoxy-*sn*-glycero-3-phosphorylcholine; azido-PAF, 1-*O*-hexadecyl-2-azido-2-deoxy-*sn*-glycero-3-phosphorylcholine; L-652,731, (*trans*)-2,5-bis-(3,4,5-trimethoxyphenyl)tetrahydrofuran; L-653,150, (*trans*)-2,5-bis-(3,4,5-trimethoxyphenyl)tetrahydrothiophene; L-651,142, (*E*)-(*trans*)-1-[(4′-aminosulfonyl)-phenyl]methylene-5-methoxy-2-methyl-1*H*-indene-3-(2′-methoxy)ethane; Ono-6240, 1-*O*-hexadecyl-2*RS*)-*O*-ethyl-3-*O*-(7-thiazolioheptyl)-glycerol chloride; CV-3988, rac-3-(*N*-*n*-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethyl phosphate; PMN, polymorphonuclear leukocyte; BSA, bovine serum albumin.

nM under identical ionic conditions. Specific [^{3}H]PAF binding to rabbit platelet membranes is specifically inhibited by Na^+ ions and GTP but is potentiated by K^+ and divalent cations, such as Mg^{2+}, Ca^{2+}, and Mn^{2+} (16). Similar ionic effects have also been found in human platelets (13). The Na^+ inhibitory effect was found not only to increase the equilibrium dissociation constant of the PAF receptor but also to decrease the detectable number of receptor sites. On the other hand, the increase in specific [^{3}H]PAF binding by divalent cations is attributed partially to the increase in the PAF affinity for its receptor and partially to the increase in the total number of detectable receptor sites (16). Multiple conformational states of the specific PAF receptor are therefore suggested. Here, we characterize the specific binding of L-659,989, a PAF-specific and competitive receptor antagonist, and confirm the existence of multiple conformational states of a single type of receptor in rabbit platelets.

Experimental Procedures

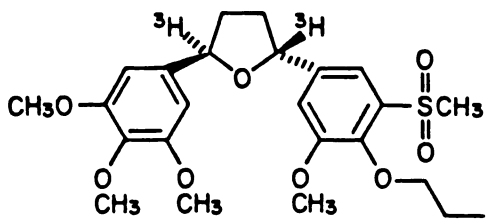
Materials

The tritium labeled PAF (1-*O*-[1,2-^{3}H]alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine) was purchased from DuPont-NEN (Boston, MA), with a specific activity of 49.5 Ci/mmol, in which 1-*O*-hexadecyl was greater than 98% (DuPont-NEN Technical Service). [^{3}H]L-659,989 was prepared using the route described previously (17), with a specific activity of 16.01 Ci/mmol. The unlabeled C_{16}-PAF, C_{18}-PAF, and enantio-PAF were obtained from BACHEM (Torrance, CA). Kadsurenone (18–20), dihydrokadsurenone (21), L-652,731 (22), L-653,150 (22), L-651,142 (8), Ono-6240 (8), and CV-3988 (8) were synthesized, either as previously described or in Merck for our on-going project. Ginkgolide B (BN-52021) isolated from the *Ginkgo biloba* tree (23) was a gift from Dr. P. Braquet (IHB-IPSEN, Institute for Therapeutic Research, Le-Plessis-Robinson, Paris, France). Acetylamino-PAF and azido-PAF were also synthesized in Merck and their detailed chemical synthesis have been published (24).

Methods

Preparation of rabbit platelet membranes. Rabbit platelets were prepared as previously described (9). The cells were then lysed in a Na^+-free medium containing 5 mM $MgCl_2$, 10 mM Tris, and 2 mM EDTA at pH 7.0 (16, 25). The lysed membranes were then further fractionated with a 0.25, 1.03, and 1.5 M discontinuous sucrose density gradient at a speed of 63,500 × *g* for 2 hr. The membranes fractionated at the interface of 0.25 and 1.03 M (membrane fraction A) and of 1.03 and 1.5 M (membrane fraction B) were collected separately (21). Membrane fraction B contained more receptor sites (21) and was therefore used throughout the experiments. The protein content in the prepared membranes was determined by the method of Lowry *et al.* (26) with BSA as the standard. The prepared membranes were stored −80° and thawed before use.

Inhibition of [^{3}H]PAF binding to rabbit platelet membranes. Binding of [^{3}H]PAF to rabbit platelet membranes was performed as previously described (9). To look at the inhibition of [^{3}H]PAF binding, membrane protein, 100 μg, was added to a final 1-ml solution containing 1 pmol of [^{3}H]PAF and a known concentration of compound, in a medium containing 10 mM $MgCl_2$, 10 mM Tris, and 0.25% BSA at pH 7.0. The bound and unbound [^{3}H]PAF were separated through a Whatman GF/C filter under the house vacuum. No [^{3}H]PAF degradation was found in the filter-bound materials even after 3–4 hr of incubation at 0° (8). Total binding, specific binding, and nonspecific binding were defined as before (9). The inhibition by the compound was normalized as per cent inhibition by the equation described elsewhere (19). The ED_{50} value was defined as the concentration of inhibitor required to achieve 50% inhibition of the specific binding. The equilibrium inhibition constant (K_i) was calculated from the Cheng and Prusoff equation (27), with the IC_{50} value derived from the indirect Hill plot as described previously (8, 25).



[^{3}H] L-659,989

Binding of [^{3}H]L-659,989 and its inhibition by PAF, PAF analogs, and PAF receptor antagonists. Binding of [^{3}H]L-659,989 to rabbit platelet membranes was performed as described for [^{3}H]PAF binding (9, 16). To assess the affinity of [^{3}H]L-659,989 binding and the number of recognition sites on the isolated rabbit platelet membranes, 100 μg of membrane protein was incubated with 0.1–10 nM [^{3}H]L-659,989 in a incubation medium containing 10 mM Tris, 0.25% BSA, pH 7.0, and the specific ions at a known concentration. A 3–4-hr incubation at 0° is required to reach binding equilibrium in 10 mM $MgCl_2$. The nonspecific binding was determined from total binding of [^{3}H]L-659,989 in the presence of excess (1000-fold) unlabeled L-659,989, ranging from 0.1 to 10 μM. The bound and unbound [^{3}H]L-659,989 were separated through a Whatman GF/C fiber glass filter under the house vacuum after a 4-hr incubation at 0°. No detectable degradation of [^{3}H]L-659,989 was observed even up to 4 hr of incubation at 0°.

To look at the inhibition of [^{3}H]L-659,989 binding by PAF, PAF analogs, or PAF receptor antagonists, 100 μg of membrane protein was added to a final 1-ml solution containing 1 pmol (1 nM final concentration) of [^{3}H]L-659,989 and a known amount of inhibitor in a defined medium. After a 4-hr incubation at 0°, the bound and unbound [^{3}H]L-659,989 were separated as described above. The ED_{50}, IC_{50}, and K_i values were defined and calculated as above for [^{3}H]PAF binding.

Data presentation. Unless otherwise specified, all data are presented as the mean ± standard deviation of two to four independent experiments. In each experiment, duplicate determinations were performed.

Results

Specific binding of [^{3}H]L-659,989 to rabbit platelet membranes. Fig. 1A shows the binding isotherm of [^{3}H]L-659,989 to rabbit platelet membranes (membrane fraction B) in an incubation medium of 10 mM $MgCl_2$, 10 mM Tris, and 0.25% BSA at pH 7.0. The nonspecific binding increased linearly with the concentration of [^{3}H]L-659,989. Unlike the specific binding of [^{3}H]PAF to isolated membranes, the specific binding of [^{3}H]L-659,989 was almost as high as the total binding. It was readily saturable and reached near maximum around 10 nM [^{3}H]L-659,989. Scatchard analysis (28) of the specific binding in 10 mM $MgCl_2$ indicated a single high affinity binding sites (Fig. 1B). For comparison, the Scatchard analysis of a representative experiment of [^{3}H]PAF saturation isotherm binding studies with the same membrane preparation under the identical ionic conditions is also given in Fig. 1B. The equilibrium dissociation constant (K_D) of [^{3}H]L-659,989 from several repeated experiments was found to be 1.60 ± 0.20 nM (six experiments). The maximal number (B_{max}) of receptor sites was estimated to be 4.27 ± 0.34 pmol/mg of protein (six experiments), which is about 25% higher than the B_{max} value of [^{3}H]PAF binding sites (3.19 ± 0.39 pmol/mg of protein; three

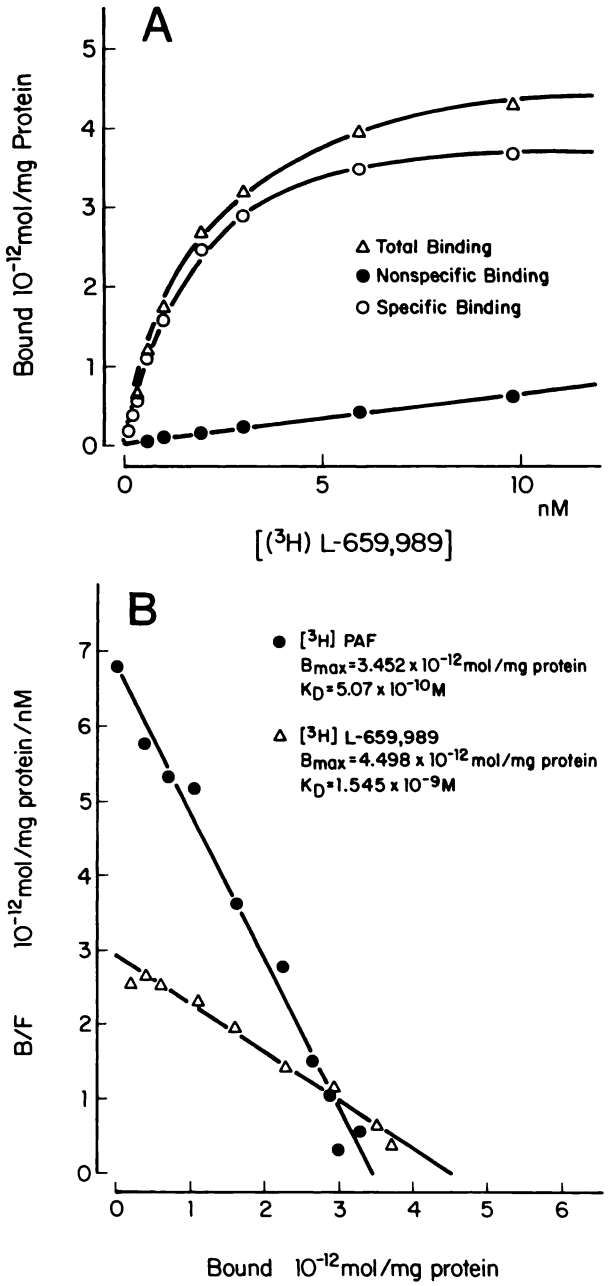


Fig. 1. Binding of [^{3}H]L-659,989 to rabbit platelet membranes. A, Saturation isotherms for [^{3}H]L-659,989 binding. Binding was measured over a radioligand concentration range of 0.1–10 nM. The assay was performed with 100 μg of membrane protein per tube in 10 mM $MgCl_2$, 10 mM Tris, and 0.25% BSA at pH 7.0 and 0°. Each *point* is the average of triplicate determinations. The nonspecific binding is low even at 10 nM [^{3}H]L-659,989. B, Scatchard analysis of binding of [^{3}H]L-659,989 and [^{3}H]PAF to rabbit platelet membranes. The data point for [^{3}H]L-659,989 was calculated from the data presented in A. Binding of [^{3}H]PAF was measured over a radioligand concentration range of 0.1–10 nM. The experimental conditions were exactly identical to those for [^{3}H]L-659,989 described in A.

experiments) assayed under the identical ionic conditions. The mean K_D value of the [^{3}H]PAF binding to rabbit platelet membranes in these experiments was 0.57 ± 0.07 nM (three experiments), which is not different from the K_D value, 0.53 ± 0.063 nM reported previously (16). The Hill coefficient (n_H) was calculated to be 1 (data not shown), suggesting that there is no positive or negative cooperativity in the binding of [^{3}H] L-659,989 in rabbit platelet membranes.

Modulation of [^{3}H]L-659,989 binding to rabbit platelet membranes. Fig. 2 shows the effects of monovalent (Fig. 2A) and divalent cations (Fig. 2B) on the specific binding of [^{3}H]L-659,989 to rabbit platelet membranes. Unlike the [^{3}H] PAF binding, in which the monovalent cations Na^+ and Li^+ inhibited but K^+ potentiated the binding (16), here, Na^+, Li^+, and K^+ ions all potentiated the specific binding of [^{3}H]L-659,989 to rabbit platelet membranes. Maximal ionic effects were achieved at around 100 mM, with a sharp decrease in the potentiation caused by either increasing or decreasing the monovalent cation concentration. At 100 mM concentration, the rank order of potency was $Na^+ > K^+ > Li^+$. In a similar manner, the divalent cations Mg^{2+}, Ca^{2+}, and Mn^{2+} potentiated the specific binding of [^{3}H]L-659,989 by about 2-fold (Fig. 2B). Similar to the potentiation of the specific [^{3}H]PAF binding (16), Mg^{2+} ions were slightly more effective than the other two cations and the maximal stimulation was at 10 mM. Ni^{2+} showed no effects on the specific [^{3}H]PAF binding (29), whereas it inhibited the specific [^{3}H]L-659,989 binding, with a maximal effect around 1 mM.

More detailed analysis of Na^+ and Mg^{2+} potentiation of [^{3}H] L-659,989 binding is shown in Fig. 3 and summarized in Table 1. The potentiation effects of Na^+ and Mg^{2+} are apparently due to the increase in the maximal detectable number of binding sites, with a minimal alteration in the equilibrium dissociation constant. The total number of detectable receptor sites was doubled in the presence of either 150 mM NaCl or 10 mM $MgCl_2$, as compared with that in the absence of ions (Table 1).

Specificity of [^{3}H]L-659,989 binding sites. Figs. 4 and 5 show the normalized per cent inhibitions of the specific [^{3}H] L-659,989 binding to rabbit platelet membranes by PAF, PAF analogs, and PAF receptor antagonists, which have been previously characterized in the inhibition of specific [^{3}H]PAF binding to rabbit and human platelets (8), human neutrophils (13) and human lung membrane fragments (8, 14). These PAF analogs and receptor antagonists, as well as PAF, fully displaced the specific [^{3}H]L-659,989 binding to rabbit platelet membranes. The calculated equilibrium inhibition constants (K_I) to inhibit the specific [^{3}H]L-659,989 binding were very similar to the K_I values obtained for the inhibition of the specific [^{3}H]PAF binding to rabbit platelets (see Table 2). An excellent correlation ($r = 0.997$, *slope* = 0.94) exists between the potencies of the compounds in displacing [^{3}H]PAF from its binding sites and in inhibiting the specific [^{3}H]L-659,989 binding (Fig. 6).

Three different shapes of the competition profile are demonstrated in Figs. 4 and 5. The competition curves of PAF receptor antagonists, except Ono-6240, as shown in Fig. 5, belonged to the "normal steepness" class. The cooperativity index (R_s), defined as the concentration range of competitor that proceeds from 10% to 90% competition of the specific binding, was around 81 (see Ref. 30 for the discussion of cooperativity index). The pseudo-Hill coefficients, as obtained from the slope of the indirect Hill plot, were very close to −1 (varying from 0.96 to 1.05) (Fig. 5B). However, the competition curve of Ono-6240 showed positive cooperativity, with a R_s value of 17, which belonged to a "steep competition" class. The pseudo-Hill coefficient of Ono-6240, as shown in Fig. 5B, was −1.56. Enantio-PAF showed a normal steepness competition curve (Fig. 4), with a pseudo-Hill coefficient equal to −1.1. However, C_{16}-PAF, C_{18}-PAF, acetylamino-PAF, and azido-PAF

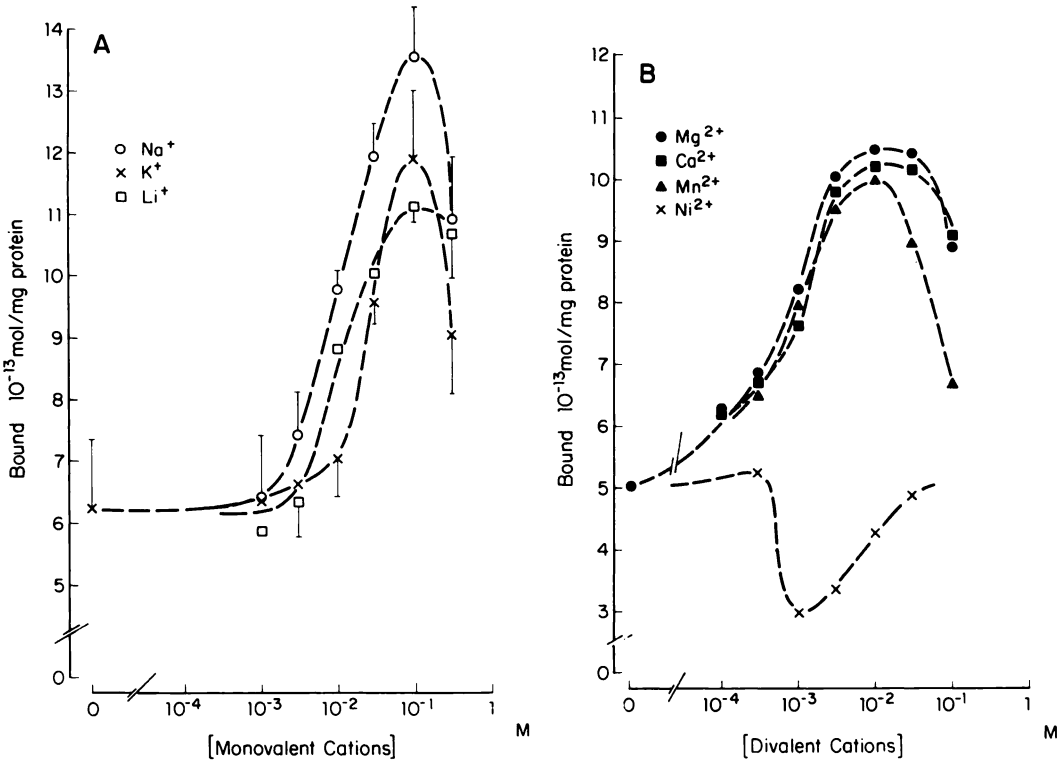


Fig. 2. Effects of monovalent (A) and divalent (B) cations on specific [^{3}H]L-659,989 binding to rabbit platelet membranes. Na^+, Li^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, or Ni^{2+} was added to the reaction mixture, which contained about 0.11 mM $MgCl_2$ which resulted from the addition of 21 μl of the membranes prepared in 5 mM $MgCl_2$ (9). The *data points* are the mean and the *error bars* are the standard deviation of three independent experiments. In each experiment, duplicate determinations were performed.

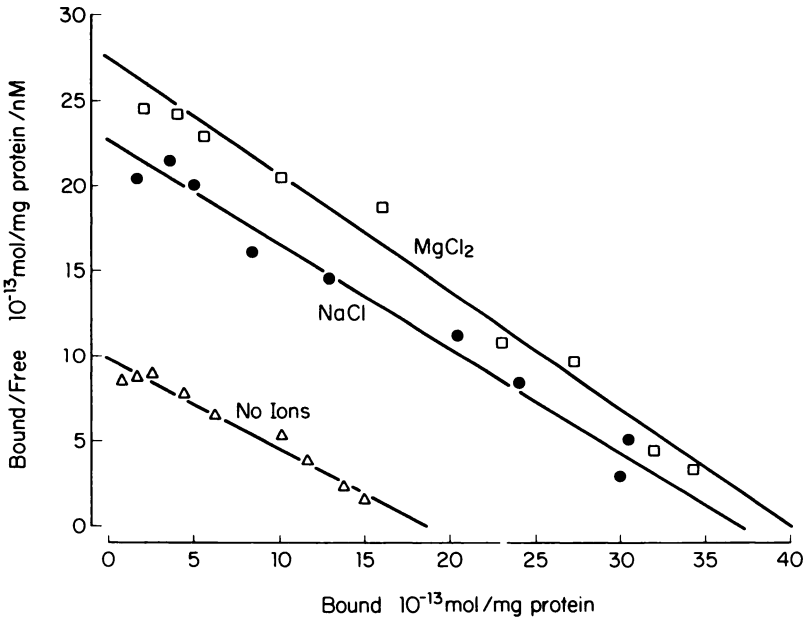


Fig. 3. Scatchard analysis of the saturation isotherms of the specific [^{3}H]L-659,989 binding with no addition of ions (△), 150 mM NaCl (●), and 10 mM $MgCl_2$. The specific [^{3}H]L-659,989 binding to rabbit platelet membranes was measured over a radioligand concentration range of 0.1 to 10 nM. The assay was performed with 100 μg of membrane protein per tube in 10 mM Tris, 0.25% BSA, with no addition of ions, 10 mM $MgCl_2$, or 150 mM NaCl, at 0°.

showed "shallow" displacement curves, with R_s values > 81 (Fig. 4). The indirect Hill plots of acetylamino-PAF and azido-PAF showed straight lines with pseudo-Hill coefficients of −0.61 and −0.53, respectively (Fig. 4B). Two straight lines with slopes equal to −1 and −0.5 better fit the data of C_{16}-PAF and C_{18}-PAF, suggesting that multiple equilibria are occurring in the competition binding incubation in 10 mM $MgCl_2$.

Such multiple equilibria of C_{16}-PAF or C_{18}-PAF in the binding of [^{3}H]L-659,989 can be further demonstrated by the saturation isotherm studies. In the presence of unlabeled PAF, <1 nM, PAF decreased the affinity of [^{3}H]L-659,989 binding to rabbit platelet membranes, with no appreciable decrease in the number of maximal detectable sites. However, when the PAF concentration was higher than 3 nM, the receptor number of [^{3}H]L-659,989 decreases with increasing concentrations of PAF. Fig. 7 shows such an experiment, and Table 3 summarizes the results from three repeated experiments. In the presence of 30 nM PAF, the B_{max} dropped from 3.839 ± 0.08 pmol/mg of protein (mean ± standard deviation) without unlabeled PAF to 2.046 ± 0.23 pmol/mg of protein (mean ± standard deviation), nearly 50% lower than that in the absence of competitor.

Pharmacological specificity of [^{3}H]L-659,989 binding sites on rabbit platelets. The inhibition of [^{3}H]L-659,989 by different compounds of several pharmacological classes was performed in order to further confirm the binding specificity of L-659,989 toward rabbit platelets. Compounds tested included

TABLE 1

Effects of Mg^{2+} and Na^+ on the equilibrium dissociation constant (K_D) of [^{3}H]L-659,989 binding and the maximal number of binding sites (B_{max})

Numbers in parentheses, number of experiments.

Conditions	K_D	B_{max}
	nM	pmol/mg of protein
10 mM $MgCl_2$	1.60 ± 0.20	4.27 ± 0.34 (n = 6)
150 mM NaCl with 0.2 mM $MgCl_2$	1.50 ± 0.20	4.09 ± 0.52 (n = 2)
0.2 mM $MgCl_2$[a]	1.89 ± 0.01	2.16 ± 0.33 (n = 2)

[a] The presence of 0.2 mM $MgCl_2$ in the assay is a result of the addition of 36 μl membranes prepared in 5 mM $MgCl_2$, which is required during the membrane preparations to maintain the high affinity of the PAF receptor for [^{3}H]PAF.

a variety of agonists and antagonists of catecholamine (epinephrine, norepinephrine, isoproterenol, yohimbine, and propranolol), histamine (diphenhydramine, pyrilamine, and cimetidine), serotonin (cyproheptadine and methylsergide), calmodulin (trifluoperazine and chloropromazine), and benzodiazepine (alprazolam and diazepam), a Ca^{2+}-channel blocker (verapamil), and several enzyme inhibitors (aspirin, sulindac, indomethacin, dexamethasone, and papaverine). With the exception of alprazolam and chlorpromazine, none of the compounds tested at 5 to 10 μM showed a significant inhibition of the specific [^{3}H]L-659,989 binding to rabbit platelet membranes. These results are consistent with those of [^{3}H]PAF or [^{3}H] dihydrokadsurenone binding (21), in that alprazolam and chlorpromazine, but not diazepam and trifluoperazine, showed moderate activity.

Effects of ions and GTP on the binding competition of [^{3}H]L-659,989 by C_{16}-PAF and receptor antagonists. NA^+ and GTP decreased the potency of C_{16}-PAF to compete against the binding of [^{3}H]L-659,989 to rabbit platelet membranes (Fig. 8A). In the presence of 10 mM $MgCl_2$, the ED_{50} of C_{16}-PAF to compete against the L-659,989 binding was 1.4×10^{-9} M, whereas, in the presence of 150 mM NaCl, the ED_{50} value was shifted to about 1 μM, almost 1000-fold higher than that in 10 mM $MgCl_2$. GTP (1 mM) also significantly shifted the PAF competition curves to the right, from an ED_{50} value of 1.4 nM to 10 nM in the presence of 10 mM $MgCl_2$ and from 1 μM to 2 μM in the presence of 150 mM NaCl, whereas GTP at 1 mM showed no effects at all on the binding of [^{3}H]L-659,989 to rabbit platelet membranes. A noticeable biphasic competition curve by PAF was observed for [^{3}H]L-659,989

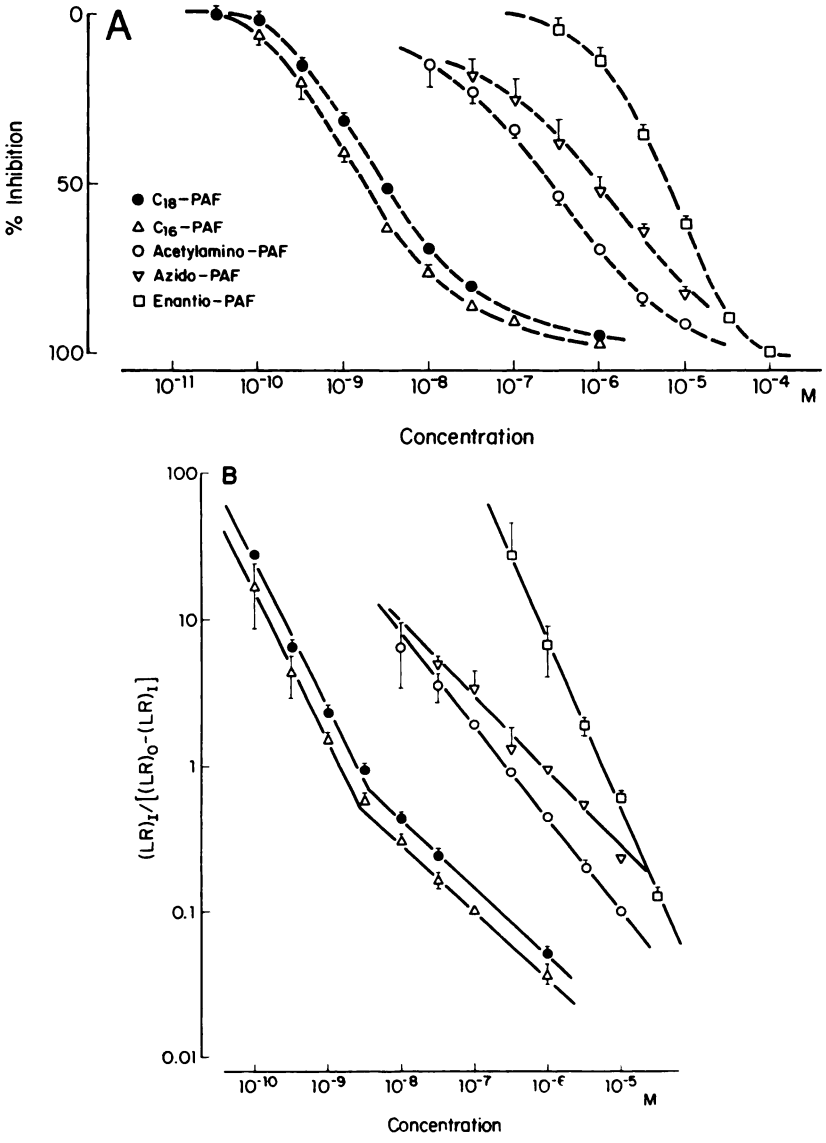


Fig. 4. Inhibition of specific [^{3}H]L-659,989 binding to rabbit platelet membranes by PAF or PAF analogs in 10 mM $MgCl_2$. A, Normalized per cent inhibitions of [^{3}H]L-659,989 binding to rabbit platelet membranes by C_{16}-PAF (△), C_{18}-PAF (●), acetylamino-PAF (○), azido-PAF (▽), and enantio-PAF (□). Membrane protein (100 μg) and 1 nM [^{3}H]L-659,989 were used in each tube. *Data points* and *error bars* represent the mean and standard deviation of two experiments. Duplicate determinations were performed in each experiment. B, Indirect Hill plots of inhibitory results on specific [^{3}H]L-659,989 binding to rabbit platelet membranes by PAF or PAF analogs. *Data points* were calculated from the same experiment presented in A. $(LR)_o$ is the amount of [^{3}H]L-659,989 specifically bound in the absence of inhibitor; $(LR)_I$ is the amount of [^{3}H]L-659,989 specifically bound in the presence of inhibitor. (△), C_{16}-PAF; (●), C_{18}-PAF; (○), acetylamino-PAF; (▽), azido-PAF; and (□), enantio-PAF.

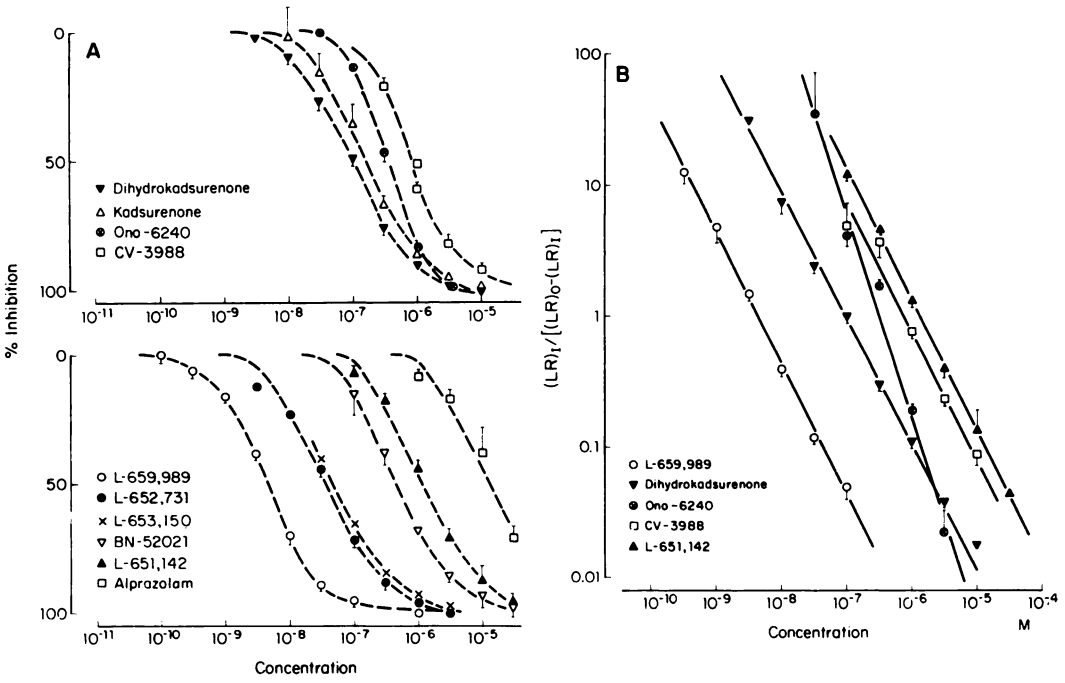


Fig. 5. Inhibition of [^{3}H]L-659,989 binding to rabbit platelet membranes by PAF receptor antagonists in 10 mM $MgCl_2$. A, Normalized per cent inhibitions of [^{3}H]L-659,989 binding by PAF receptor antagonists. The experimental conditions were identical to those in Fig. 4. B, Indirect Hill plots of inhibitory results on specific [^{3}H]L-659,989 binding to rabbit platelet membranes by PAF receptor antagonists. $(LR)_o$ and $(LR)_i$ are as defined in Fig. 4B. Data points were calculated from the same experimental results presented in Fig. 5A.

TABLE 2

Equilibrium inhibition constants (K_i) of PAF agonists and antagonists on the specific binding of [^{3}H]L-659,989 and [^{3}H]PAF to rabbit platelet membranes

Compound	K_i	
	[^{3}H]PAF[a]	[^{3}H]L-659,989
	M	
1. C_{16}-PAF	$5.37 \pm 0.32 \times 10^{-10}$	$9.726 \pm 0.78 \times 10^{-10}$
2. C_{18}-PAF	$1.06 \pm 0.14 \times 10^{-9}$	$1.705 \pm 0.11 \times 10^{-9}$
3. Acetylamino-PAF	$9.78 \pm 2.1 \times 10^{-8}$	$1.556 \pm 0.15 \times 10^{-7}$
4. Azido-PAF	$3.60 \pm 1.4 \times 10^{-7}$	$4.924 \pm 1.1 \times 10^{-7}$
5. Enantio-PAF	$3.51 \pm 0.7 \times 10^{-6}$	$3.292 \pm 0.40 \times 10^{-6}$
6. L-659,989	$1.1 \pm 0.19 \times 10^{-9}$	$2.599 \pm 0.08 \times 10^{-9}$
7. Dihydrokadsurenone	4.4×10^{-8}	$5.190 \pm 0.16 \times 10^{-8}$
8. Kadsurenone	$8.81 \pm 1.4 \times 10^{-8}$	$9.928 \pm 3.69 \times 10^{-8}$
9. L-652,731	$9.83 \pm 2.92 \times 10^{-9}$	$2.450 \pm 0.14 \times 10^{-8}$
10. L-653,150	$2.00 \pm 1.16 \times 10^{-8}$	3.245×10^{-8}
11. L-651,142	$8.39 \pm 6.5 \times 10^{-7}$	$8.134 \pm 1.76 \times 10^{-7}$
12. BN-52021	$2.23 \pm 0.76 \times 10^{-7}$	$3.103 \pm 0.23 \times 10^{-7}$
13. Ono-6240	$1.11 \pm 0.50 \times 10^{-7}$	$1.927 \pm 0.30 \times 10^{-7}$
14. CV-3988	$4.13 \pm 0.34 \times 10^{-7}$	$5.147 \pm 1.52 \times 10^{-7}$
15. Alprazolam	4.09×10^{-6}	$8.182 \pm 2.81 \times 10^{-6}$

[a] Data were either obtained from Ref. 10 or calculated from data presented in Ref. 21.

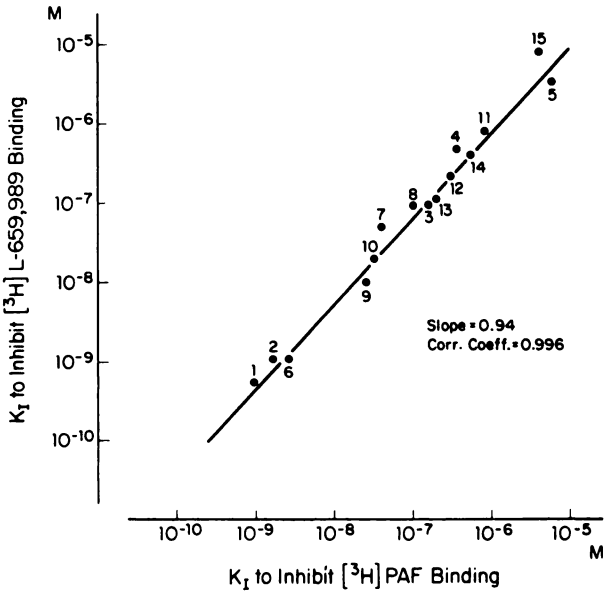


Fig. 6. Plot of K_i values to inhibit [^{3}H]L-659,989 binding against K_i values to inhibit [^{3}H]PAF binding to rabbit platelet membranes, listed in Table 3. The *numbers* in the figure corresponds to the compound number in Table 3. Inhibitions of [^{3}H]L-659,989 and [^{3}H]PAF binding were done in a medium of 10 mM $MgCl_2$, 10 mM Tris, and 0.25% BSA at pH 7.0 and 0°.

binding in the presence of 150 mM NaCl, whereas a normal steepness competition curve with a pseudo-Hill coefficient equal to −1 was observed in the presence of 150 mM NaCl and 1 mM GTP (Fig. 8B). A simple competitive antagonism C_{16}-PAF on the specific binding of [^{3}H]L-659,989 is therefore expected under this experimental condition. In fact, in the Scatchard plots of the saturation binding isotherms, C_{16}-PAF altered the slope of the apparent dissociation constant (K_D) of [^{3}H]L-659,989 to its receptor but not the intercept at the *x* axis or the maximal number of receptor sites (B_{max}). As shown in Fig. 9A, the K_D value is changed from 1.078 nM in the absence of C_{16}-PAF to 1.427, 2.173, and 4.633 nM in the presence of 0.3, 1, and 3 μM C_{16}-PAF, respectively, without a significant change in the B_{max}. Table 4 summarizes the results from three repeated experiments. In the Schild analysis, by plotting the logarithm of $(K_D'/K_D - 1)$ versus the logarithm of the C_{16}-PAF concentration, a unity slope (0.977) was found (Fig. 9B) where K_D' and K_D are the apparent and equilibrium dissociation constants of [^{3}H]L-659,989 in the presence and absence of C_{16}-PAF, respectively. The equilibrium dissociation constant (K_B) of C_{16}-PAF to PAF receptors obtained from the Schild plot (Fig. 9B) is found to be 9.31×10^{-7} M. On the other hand, for PAF receptor antagonists, no pronounced shift of the competition curves was observed, as shown in Fig. 10 for L-659,989 and kadsurenone after changing the ionic condition from 10 mM $MgCl_2$ to 150 mM NaCl or after the addition of 1 mM GTP.

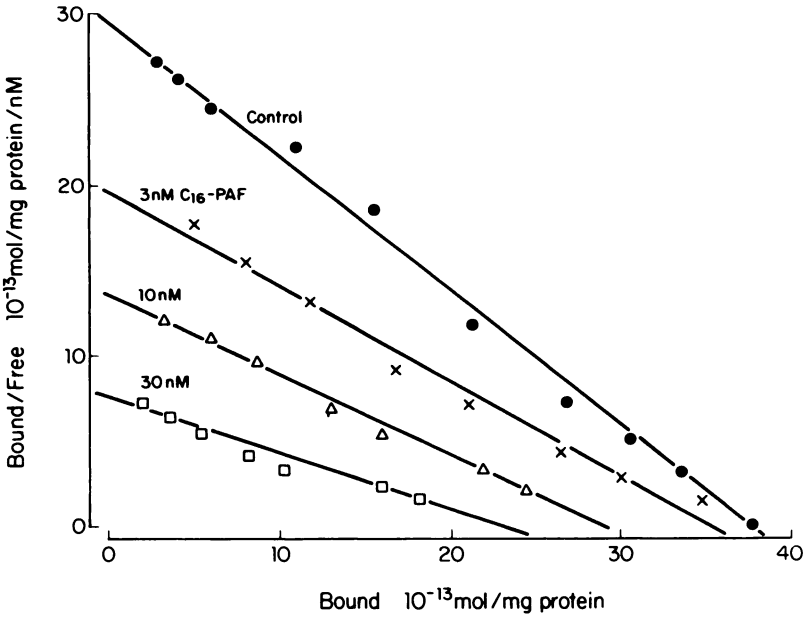


Fig. 7. Scatchard analysis of the saturation isotherms of the specific [^{3}H]L-659,989 binding in the absence (●) and in the presence of unlabeled PAF at 3 nM (×), 10 nM (△), and 30 nM (□). The specific binding of [^{3}H]L-659,989 was measured over a radioligand concentration range of 0.1 to 10 nM. The assay was performed with 100 μg of membrane protein in 10 mM $MgCl_2$, 10 mM Tris, and 0.25% BSA at pH 7.0 and 0°. The data points were calculated from the mean of duplicate determinations.

TABLE 3

Apparent dissociation constant (K_D') and the maximal detectable receptor sites (B_{max}) of [^{3}H]L-659,989 in the absence and presence of unlabeled C_{16}-PAF in 10 mM $MgCl_2$

Numbers in parentheses, number of experiments.

PAF Concentration	K_D'	B_{max}
nM	*nM*	*10^{-12} mol/mg of protein*
0	1.352 ± 0.08	3.839 ± 0.08 (n = 3)
3	1.85 ± 0.16	3.498 ± 0.08 (n = 3)
10	2.25 ± 0.18	2.854 ± 0.07 (n = 3)
30	2.82 ± 0.09	2.046 ± 0.23 (n = 3)

Similar results were also observed for L-652,731 and BN-52021 (data not shown).

Discussion

Here, we have demonstrated the binding of the tritium-labeled L-659,989 to rabbit platelet membranes. The specific binding is saturable, with a single type of high affinity binding sites in Scatchard plots. The equilibrium dissociation constant (K_D) is found to be 1.60 ± 0.20 nM in 10 mM $MgCl_2$. It is almost identical to the equilibrium inhibition constant (K_I), 1.10 ± 0.19 nM, calculated by the Cheng and Prusoff equation or the equilibrium dissociation constant (K_B), 1.49 nM, obtained from the Schild plot in saturation binding studies of [^{3}H]PAF in the presence or absence of unlabeled L-659,989 (31). The specific binding of [^{3}H]L-659,989 can be fully displaced by PAF and PAF analogs and also by several selected PAF receptors antagonists with different chemical structures. The K_I values of these PAF analogs, PAF receptor antagonists, and PAF against [^{3}H]L-659,989 binding are about the same as those K_I values against [^{3}H]PAF binding. The logarithm of K_I values against [^{3}H]L-659,989 binding correlated very well with those against [^{3}H]PAF. Also, similar to the results from [^{3}H]PAF binding or [^{3}H]dihydrokadsurenone binding (21), several selected pharmacologically defined drugs or agents, with a few similar exceptions, show no significant inhibition of [^{3}H]L-659,989. These results strongly indicate (a) that L-659,989 binds specifically to the PAF receptor, (b) that the L-659,989 binding sites, similar to those of PAF and dihydrokadsurenone, are distinct and structurally differentiated from other functionally related sites and other enzymatic active sites, and (c) that L-659, 989, L- 652,731, kadsurenone, and other receptor antagonists may share with PAF a common binding site in the PAF receptor.

In rabbit platelet membranes, it has been shown that Na^+ and Li^+ inhibit, but K^+ and divalent cations such as Mn^{2+}, Ca^{2+}, and Mg^{2+} potentiate, the specific [^{3}H]PAF binding (16). Similar ionic effects on the [^{3}H]PAF binding have been demonstrated in human platelet membranes (13) or, for other ligands, in a variety of membrane systems (32–35). Here, the specific [^{3}H]L-659,989 binding is not only potentiated by K^+, Mg^{2+}, Ca^{2+}, and Mn^{2+} but is also equally potentiated by Na^+ and Li^+. Ni^{2+} inhibits the specific [^{3}H]L-659,989 binding, even though it shows no effects on the specific [^{3}H]PAF binding to rabbit platelet membranes (31). Differences in the ionic modulation of [^{3}H]PAF and [^{3}H]L-659,989 binding to rabbit platelet membranes seem to be clear. Furthermore, such a clear change in the binding of agonist and antagonist caused by ionic conditions strongly suggested that the variation in the maximal number of binding sites under different ionic conditions results from a direct receptor effect rather than altered membrane structure.

The detectable receptor number for [^{3}H]L-659,989 in Scatchard graphs is significantly higher than that for [^{3}H]PAF in rabbit platelet membranes under an identical assay condition with 10 mM $MgCl_2$. L-659,989 is a PAF-specific and competitive receptor antagonist and can fully displace the specific [^{3}H]PAF binding to rabbit platelet membranes (31). It is also true that the unlabeled PAF can fully displace the specific binding of [^{3}H]L-659,989, as demonstrated here. Inasmuch as both [^{3}H]PAF and [^{3}H]L-659,989 show only a single type of receptor and they share a common binding site, the difference in the detectable number of receptor sites between PAF and L-659,989 is probably due to the existence of multiple conformational states of PAF receptors, not due to different receptors. Lower receptor numbers for [^{3}H]PAF result from the pitfalls of using Scatchard plots over a limited range of ligand concentrations. Scatchard graphs may be useful only to get approximate estimates of the

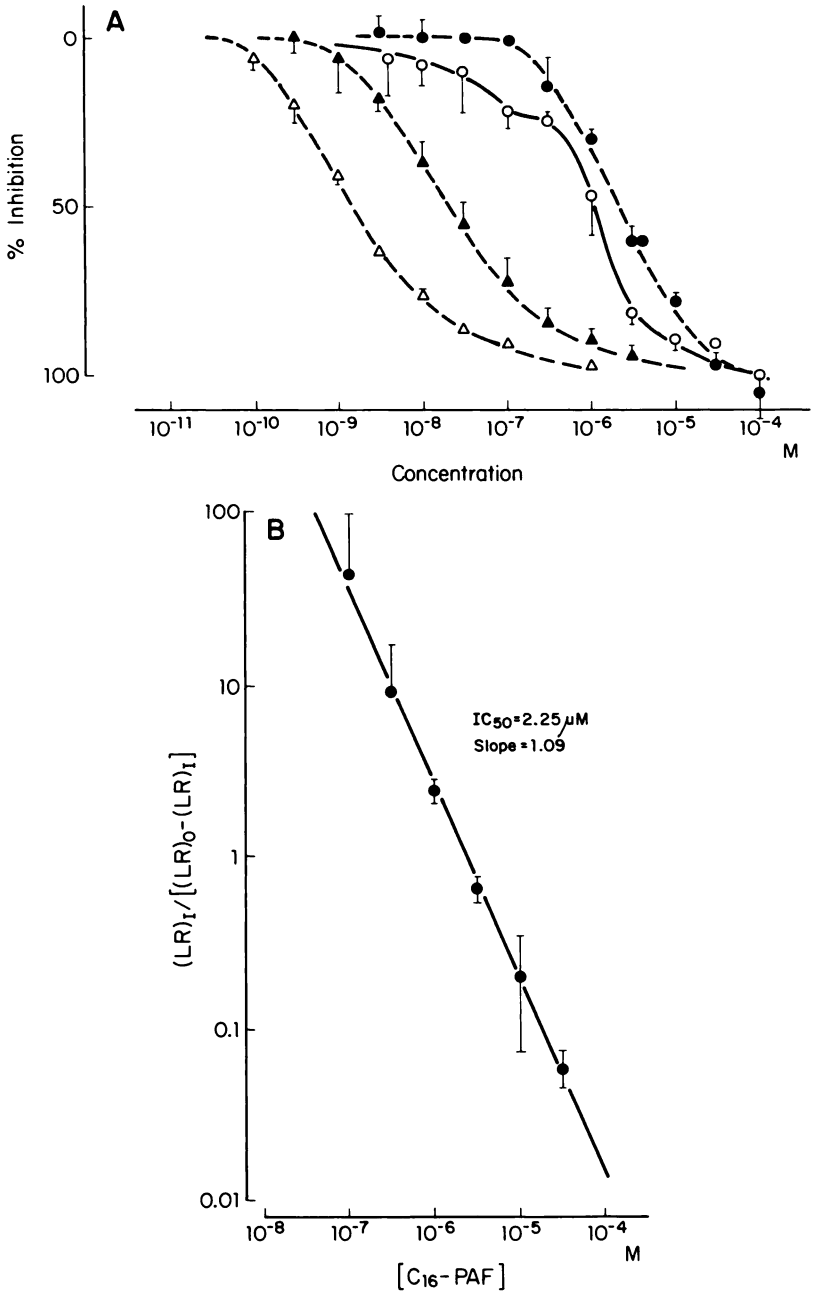


Fig. 8. A, Inhibition of [^{3}H]L-659,989 binding to rabbit platelet membranes by C_{16}-PAF. Membrane protein (100 μg) and 1 nM [^{3}H]L-659,989 was used in each tube in the assay medium, containing 10 mM Tris and 0.25% BSA at pH 7.0 with 10 mM $MgCl_2$ (△), 10 mM $MgCl_2$, and 1 mM GTP (▲), 150 mM NaCl (○), and 150 mM NaCl and 1 mM GTP (●). *Data points* and *error bars* are mean and standard deviation of two or three experiments. In each experiment, duplicate determinations were performed. B, Indirect Hill plot of inhibitory results on the specific [^{3}H]L-659,989 binding to rabbit platelet membranes by C_{16}-PAF in 150 mM NaCl and 1 mM GTP. $(LR)_0$ and $(LR)_I$ are as defined in Fig. 4B.

K_D and B_{max} for the highest affinity site. The low affinity sites for [^{3}H]PAF could not be detected in the concentration range of 0.1 to 10 nM for the saturation isotherms of [^{3}H]PAF binding studies here. A wide range of concentrations of ligand is required to collect the binding data so these Scratchard curves may be extrapolated accurately to the intercepts (28). However, this low affinity state could be detected in the displacement curves of [^{3}H]L-659,989 by C_{16}-PAF or C_{18}-PAF (Fig. 4). The biphasic competition curve becomes more pronounced under assay conditions of 150 mM NaCl, 10 mM Tris, and 0.25% BSA at pH 7.0; a significant inhibition can be observed at a C_{16}-PAF concentration as low as 3 nM (Fig. 8A), but a much higher concentration of C_{16}-PAF is required to fully displace the specific binding of [^{3}H]L-659,989 (ED_{50} ~ 1 μM).

We have also previously demonstrated that, in the presence of 10 mM $MgCl_2$, the detectable receptor number for [^{3}H]PAF in rabbit platelet membranes is about twice of that found either in the presence of 150 mM NaCl or in the absence of ions (16). Here, with no addition of ions, the detectable receptor number for [^{3}H]L-659,989 is only about 50% of that in the presence of either 10 mM $MgCl_2$ or 150 mM NaCl. Inasmuch as both L-659,989 and PAF bind to the same receptor and share a common binding site, the variation in the detectable receptor number under different ionic conditions should also be due to the coexistence of several conformational states of the PAF receptors. The low affinity state(s) with K_D value(s) possibly in the micromolar range failed to be detected in Scatchard plots with the radioligand in the nanomolar concentration. And because the receptors that cannot be detected by [^{3}H]PAF are detectable by [^{3}H]L-659,989, the lower detectable receptor number is not due to the receptor being either embedded into the membrane or translocated into the inner membrane surface.

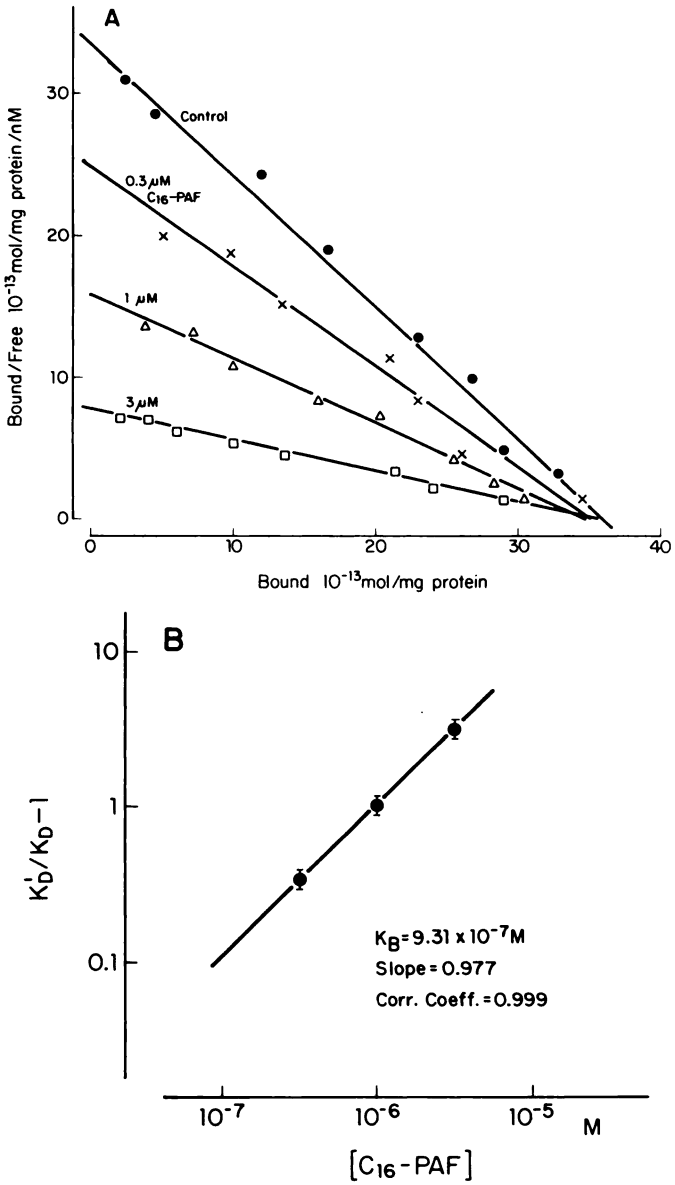


Fig. 9. A, Scatchard analysis of the saturation isotherms of the specific [^{3}H]L-659,989 binding to rabbit platelet membranes. The assay was measured with 100 μg of membrane protein over a radioligand concentration range of 0.1 to 10 nM in 150 mM NaCl, 1 mM GTP, 10 mM Tris, and 0.25% BSA at pH 7.0 and 0° in the absence (●) and in the presence of unlabeled C_{16}-PAF at the concentration of 0.3 μM (×), 1 μM (△), and 3 μM (□). B, Schild plot of the inhibitory results of [^{3}H]L-659,989 binding by C_{16}-PAF in the presence of 150 mM NaCl and 1 mM GTP. *Data points* are the mean and the *error bars* are the standard deviation of three independent experiments. The *straight line* was drawn and slope was calculated following the linear regression with a hand calculator.

TABLE 4

Apparent dissociation constant (K_D') and the maximal detectable receptor site (B_{max}) of [^{3}H]L-659,989 in the absence and presence of unlabeled C_{16}-PAF in 150 mM NaCl and 1 mM GTP

PAF concentration	K_D'	B_{max}
μM	nM	10^{-12} mol/mg of protein
0	1.052 ± 0.03	3.642 ± 0.12
0.3	1.405 ± 0.024	3.465 ± 0.16
1	2.115 ± 0.056	3.412 ± 0.18
3	4.391 ± 0.24	3.425 ± 0.19

As demonstrated previously, PAF stimulates GTPase activity in rabbit platelet (16) and in human platelet and PMN membranes (13). And GTP specifically decreases the specific [^{3}H] PAF binding to rabbit platelet membranes (16) and human PMN membranes (36). Here, GTP also shifts the competition curves of C_{16}-PAF to the right, i.e., GTP decreases the affinity of PAF for the receptor. Similar to that in 10 mM $MgCl_2$, a shallow steepness is still observed in 10 mM $MgCl_2$ with 1 mM GTP. Therefore, the PAF receptor is not in a single conformational state under these conditions. However, at 150 mM NaCl with 1 mM GTP, a normal steepness competition curve is obtained. The pseudo-Hill coefficient is −1, characteristic of ligand-receptor interactions describing a reversible bimolecular reaction that obeys mass action law. A single conformational state of PAF receptors with low affinity for PAF (K_B = 0.931 μM) is therefore observed under this experimental condition (150 mM NaCl and 1 mM GTP).

In order to determine whether the interaction of drug with a receptor is competitive or noncompetitive, saturation binding experiments are commonly performed. L-659,989, similar to kadsurenone (19, 20) and L-652,731 (25) showed a simple competitive inhibition of [^{3}H]PAF binding (31). In Scatchard plots, it decreased K_D without altering B_{max}. Schild regression is linear and has a slope not significantly different from unity for a considerable concentration range and a wide range of dose ratios (31). Here, in Scatchard plots of saturation isotherms of [^{3}H]L-659,989 binding in 10 mM $MgCl_2$, C_{16}-PAF (>3 nM) alters not only the apparent dissociation constant of [^{3}H]L-659,989 but also the maximal number of receptor sites (Fig. 7 and Table 3). The exact mechanism for this noncompetitive inhibition of C_{16}-PAF in the binding of [^{3}H]L-659,989 in 10 mM $MgCl_2$ is still not clear. But it could be due to the agonist properties of C_{16}-PAF, which could initiate the signal transduction and, thus, feedback modulate the conformation of the receptor that shows low affinity for L-659,989 and cannot be detected with the conventional Scatchard plots with the radioligand concentration used here. In fact, in the presence of 150 mM NaCl and 1 mM GTP, a simple competitive inhibition of [^{3}H]L-659,989 binding to rabbit platelet membranes by C_{16}-PAF is observed, i.e., decreased K_D without alteration of B_{max} and a linear Schild regression with a unit slope (Fig. 9). Coexistence of Na^+ and GTP could decouple the receptor-effector complex. The mechanism of signal transduction could not be initiated by PAF.

From the results demonstrated and discussed above, PAF receptors could exist in several distinct conformational forms. R_o is the state of the receptor in the absence of ions (Fig. 11), which shows low affinity for both [^{3}H]PAF and [^{3}H]L-659,989. Mg^{2+} and possibly other divalent cations, such as Ca^{2+} and Mg^{2+} converts the receptor from R_o to $R_{Mg^{2+}}$ state, possibly through the interaction between PAF receptor and guanine nucleotide-binding protein. $R_{Mg^{2+}}$ is the state with a high affinity for both agonist (PAF) and antagonist (L-659,989). GTP in the presence of Mg^{2+} converts $R_{Mg^{2+}}$ into $R_{Mg^{2+}\cdot GTP}$. PAF shows lower affinity for $R_{Mg^{2+}\cdot GTP}$ than for $R_{Mg^{2+}}$. Na^+ and possibly Li^+ convert R_o into R_{Na^+}, which shows low affinity for PAF (K_D in the micromolar range) but high affinity for L-659,989. GTP can similarly transform R_{Na^+} into $R_{Na^+\cdot GTP}$, possibly the lowest affinity state for PAF. L-659,989 seems to have identical affinity for receptor states in the form of $R_{Mg^{2+}}$, $R_{Mg^{2+}\cdot GTP}$, R_{Na^+}, and $R_{Na^+\cdot GTP}$ but shows low affinity for R_o. PAF could vary the

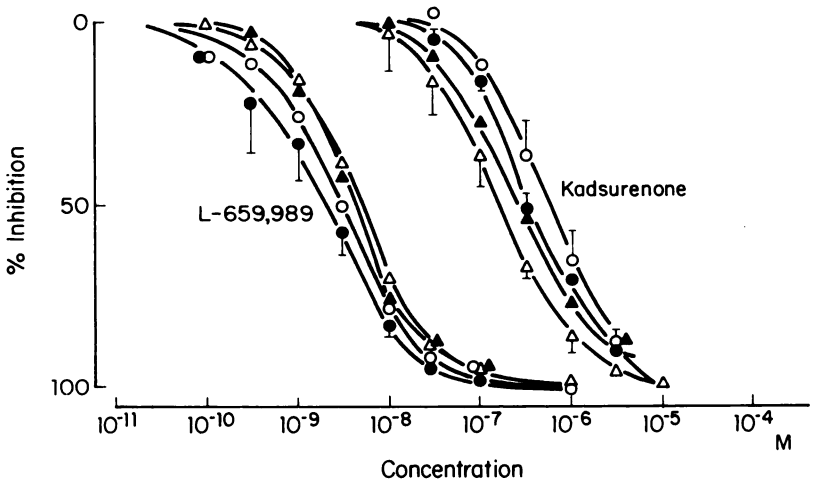


Fig. 10. Normalized per cent inhibition of [^{3}H]L-659,989 binding to rabbit platelet membranes by L-659,989 and kadsurenone in the assay medium containing 10 mM Tris and 0.25% BSA at pH 7.0 with 10 mM $MgCl_2$ (△), 10 mM $MgCl_2$, and 1 mM GTP (▲), 150 mM NaCl (○), and 150 mM NaCl and 1 mM GTP (●). *Data points* are mean and *error bars* are standard deviation of two or three experiments. In each experiment, duplicate determinations were performed.

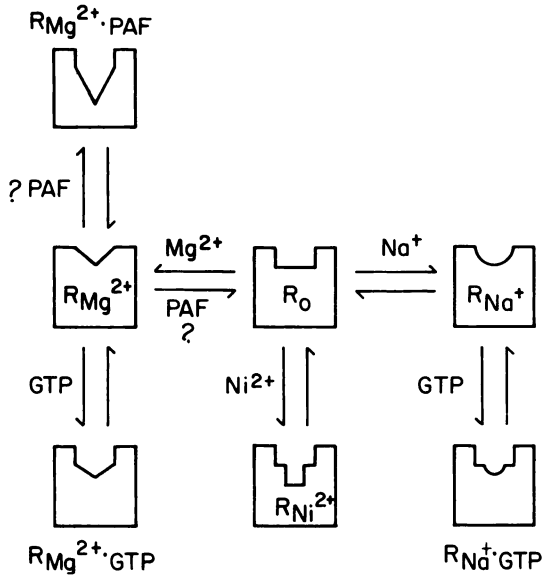


Fig. 11. Possible conformational states of PAF receptors in rabbit platelet membranes.

interaction between receptor and guanine nucleotide-binding protein, resulting in a transition from $R_{Mg^{2+}}$ to R_o, or to $R_{Mg^{2+}\cdot PAF}$, another conformational state of the receptor, which shows low affinity for L-659,989. Ni^{2+} shows no effects on the binding of [^{3}H]PAF to rabbit platelet membranes (31) but inhibits the specific [^{3}H]L-659,989 binding. Ni^{2+} could, therefore, induce another conformational state, $R_{Ni^{2+}}$. It should be noted that the above model for the multiple conformational forms of PAF receptors may be oversimplified. Using the isolated membranes for the characterization of membrane receptor functions has its own advantages and disadvantages. The isolated membrane is a much simpler system then the whole cell. Metabolism (10, 12) and probably internalization (10) of PAF may not happen in the isolated membrane system (8, 13). Also, different ionic or other assay conditions can easily be manipulated (13, 16, 20, 29, 32–36). However, the cytoplasmic proteins have been separated from the isolated membranes. A reversible association of the amphitropic proteins with membranes upon stimulation of agonist (37) could be missing, which may also control and modulate the receptor function in the living cell. Apparently, further experimental characterization is required to confirm these conformational states involved in the regulation of the function of PAF receptors.

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