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High Affinity Specific Binding Sites for Tritiated Platelet-Activating Factor in Canine Platelet Membranes: Counterparts of Platelet-Activating Factor Receptors Mediating Platelet Aggregation

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SUMMARY

In canine platelet membranes, tritiated platelet activating factor (PAF) labels in a saturable and reversible manner a single population (n_H = 0.97) of binding sites. The affinity of this binding was high (K_d = 0.23 ± 0.02 nM, n = 4, and 0.21 ± 0.05, n = 8, determined by kinetics or saturation experiments, respectively), and the density of binding sites (B_{max}) was 911 ± 31 fmol/mg of protein (n = 8). [^{3}H]PAF binding was entirely reversed by unlabeled PAF (10 μM). [^{3}H]PAF exhibited stereoselective discrimination inasmuch as it was poorly displaced by enantio-PAF, the PAF enantiomer that does not occur naturally. Furthermore, the displacing potency of the (+)-enantiomer of the PAF antagonist 52770 RP against [^{3}H]PAF was 45 times higher than that of the (−)-enantiomer. [^{3}H]PAF binding displayed a remarkable specificity in that it was not affected by a variety of classical pharmacological agents. However, this binding was displaced by several PAF receptor antagonists such as 59227 RP, CV-6209, Ro 19-3704, 52770 RP, brotizolam, WEB 2086, SRI 63-441, L-652,731, alprazolam, triazolam, and BN 52021. The K_i of the 16 studied antagonists ranged from 7.9 nM (59227 RP, most potent) to 16.8 μM (BN 52021, least potent). The possible biological significance of our binding procedure was assessed by correlating the potencies of 16 PAF antagonists as [^{3}H]PAF displacers in dog platelet membranes and as inhibitors of PAF-induced platelet aggregation in washed canine platelets. This analysis revealed the existence of a highly significant correlation (r = 0.82, $p < 0.001$) between biochemical and functional tests. However, two compounds (Ro 19-3704 and BN 52021) were found to be located outside the confidence limits when the probability level of belonging to the regression line was set at 0.01. In conclusion, this study provides evidence that [^{3}H]PAF binding in canine platelet membranes exhibits the required properties for a valid binding procedure. Furthermore, the labeled sites are likely to be the counterparts of platelet receptors that, when activated by PAF, induce aggregation.

PAF is a phospholipid mediator of a variety of pathophysiological effects such as inflammation, allergic reactions (1), increased vascular permeability (2), platelet aggregation (3), chemotaxis, enzyme secretion, bronchoconstriction (4), hypotension, and cardiac dysfunction (5).

Specific binding sites for [^{3}H]PAF have been demonstrated in human platelets and lysed or whole platelets from rabbit (6, 7). Interestingly, this binding is displaceable by compounds of diverse chemical structure that have the ability to inhibit PAF-evoked platelet aggregation, bronchospasm, and hypotension. The potency of PAF, as a platelet aggregant, differs substantially in the animal species studied (the decreasing rank order being guinea pigs > rabbits > dogs > humans) (9). For instance, PAF is 20-fold less potent as an aggregating agent in dog than rabbit platelets. Interestingly, rat platelets, which lack [^{3}H]PAF binding sites, do not aggregate when exposed to PAF (10).

The aim of this report is to describe the characterization of high affinity specific binding sites for [^{3}H]PAF on canine platelet membranes and to provide evidence that there is a pharmacological similarity between these sites and PAF receptors mediating platelet aggregation. A brief report on the binding of [^{3}H]PAF to washed dog platelets was published a few years ago, but it lacks sufficient technical information for reproducibility and does not mention all the properties of this binding (11).

Our interest in studying [^{3}H]PAF binding in dog platelets derives from the fact that in this experimental animal PAF can produce dramatic cardiovascular effects (hypotension, fall in cardiac output and renal blood flow, myocardial ischemia, etc.) (12–16). Thus, the availability of a sensitive [^{3}H]PAF-binding technique for dog platelets (which are often implicated in the functional effects of PAF) may be a useful tool in the investi-

ABBREVIATIONS: PAF, platelet-activating factor; PRP, platelet-rich plasma; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BSA, bovine serum albumin; *df*, degrees of freedom.

gation of the role of PAF in pathophysiological processes and for the interspecies characterization of PAF antagonists.

Experimental Procedures

Materials. [^{3}H]PAF (1-*O*-[^{3}H]octadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine) with an activity of 80 Ci/mmol was obtained from Amersham International, France. Unlabeled PAF (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine), where alkyl refers to hexadecyl (PAF-C_{16}) or octadecyl (PAF-C_{18}), lyso-PAF-C_{18} (1-*O*-octadecyl-*sn*-glycero-3-phosphorylcholine), 2-*O*-methyl-lyso-PAF-C_{18} (1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine), 2-*O*-benzyl-lyso-PAF-C_{16} (1-*O*-hexadecyl-2-*O*-benzyl-*sn*-glycero-3-phosphorycholine), and enantio-PAF-C_{16} (3-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-1-phosphorylcholine) were purchased from BACHEM (Torrance, CA).

Drugs obtained as gifts were the following: Ro 19-3704 (Hoffmann-La Roche, Basel, Switzerland), RU 45703 (Prof. J. Godfroid, University of Paris VII), BN 52021 (I.H.B., Le Plessis, France), kadsurenone (Merck, Rahway, NJ), WEB 2086 and brotizolam (Boehringer Ingleheim, Ingelheim am Rhein, FRG) SRI 63-072 and SRI 63-441 (Sandoz, Basel, Switzerland), and CV-6209 (Takeda, Osaka, Japan).

52770 RP, 59277 RP, 5-hydroxytryptamine, CV-3988, diltiazem, dopamine, forskolin, haloperidol, histamine, indomethacin, isoproterenol, L-652,731, mepyramine, naloxone, and verapamil were provided by our Chemistry Department.

The chemical separation of (+)- and (−)-52770 RP was described in a previous publication on the characterization of [^{3}H]52770 RP, a novel ligand for PAF binding sites (7). 59227 RP is the (+)-enantiomer of *N*-(benzyl-3-phenyl)-3-(3-pyridinyl-1*H*,3*H*-pyrrolo[1,2-c]thiazole-7-carboxamide. Its chemical structure is close to that of 52770 RP (7), from which it differs by the presence of a benzyl-3-phenyl instead of a chloro-3-phenyl group.

The other drugs used were dipyridamole, quinidine, sulfinpyrazone (Sigma Chemical Co, St Louis, MO), atropine (Prolabo, Paris, France), phentolamine, imipramine (Ciba-Geigy Ltd, Basel, Switzerland), prazosin (Pfizer, UK), alprazolam, triazolam (Upjohn, Kalamazoo, MI), propranolol (ICI, Paris, France), and yohimbine (Ets Darasse, Paris, France).

Preparation of membranes from canine platelets. Beagle dogs (13–15 kg) were purchased from a breeding laboratory (C.E.D.S., Mezilles, France). Blood was withdrawn from a cannulated saphenous vein in conscious animals or from a femoral artery after induction of anesthesia with sodium pentobarbital (40 mg/kg intravenously) and collected into 10-ml plastic tubes containing 2 ml of anticoagulant (solution of ACD in distilled water: trisodium citrate, 74.8 mM; citric acid, 23.3 mM; and dextrose, 35.8 mM).

Blood was centrifuged three times at 100 × *g* for 10 min at room temperature to prepare PRP. PRP was subjected to cold (4°) centrifugation for 10 min at 16000 × *g*. The obtained platelet pellet was washed twice with a buffer solution (Tris·HCl, 50 mM; NaCl, 150 mM; EDTA, 20 mM; pH 7.4), lysed, and homogenized in a low ionic strength buffer solution (Tris·HCl, 5 mM; EDTA, 5 mM; pH 7.4). This suspension was centrifuged for 10 min at 39000 × *g*. The recovered pellet was washed with a buffer solution (Tris·HCl, 50 mM, pH 7.4) and finally resuspended in the same buffer (Tris-HCl, 50 mM; EDTA, 1 mM; $MgCl_2$, 2 mM; and BSA, 0.25%, w/v, pH 7.4) used for the binding assays. This membrane preparation was either used immediately or frozen in liquid nitrogen, stored at −80°, and used as needed over a period of up to 6 weeks during which no changes in binding sites could be detected.

[^{3}H]PAF binding assay. Membrane suspensions (30–40 μg) were dispensed in assay tubes containing variable concentrations of [^{3}H]PAF and a sufficient amount of assay buffer to give a final volume of 0.5 ml. They were incubated at 25° for a 60-min period, which was the time necessary to achieve equilibrium conditions as determined in preliminary saturation experiments. Then, the reaction was stopped by the addition of 1 ml of cold saline (NaCl, 0.9%) followed by rapid filtration though Whatman GF/C glass fiber filters. The filters were washed with 3 ml of ice-cold saline buffer five times and dried and their radioactivity was counted by liquid scintillation (model LS 3801; Beckman Instruments, Fullerton, CA). Nonspecific binding was determined with an excess of unlabeled PAF-C_{18} (10 μM final concentration); thus, specific binding is given by the difference between the total binding and that remaining in the presence of PAF-C_{18}.

Saturation experiments were performed by adding to the incubation medium concentrations of [^{3}H]PAF ranging between 0.035 and 2 nM. The number of assays reported in Results refers to membrane preparations obtained from different animals.

Competition curves and kinetics were determined in at least three separate experiments performed in duplicate by using a concentration (0.2 nM) of [^{3}H]PAF, close to the K_d value. The protein content of the membrane preparation was measured by using the method of Lowry *et al.* (17).

***In vitro* aggregation studies in PRP.** Blood obtained from conscious dogs was collected into 20-ml plastic tubes and centrifuged at 15° to prepare PRP. After removal of PRP, the tubes were further centrifuged at 400 × *g* for 5 min to yield platelet-poor plasma, which was used to adjust the PRP to a final platelet concentration of 4×10^8/ml. Platelet aggregation was determined according to the method of Born and Gross (18) in 250-μl aliquots of PRP containing either a PAF antagonist or its solvent (control). These samples were prewarmed for 10 min at 37° in platelet aggregometers (Chrono-log, Model 540; Chrono-log Corporation, Havertown, PA) and stirred at 1000 rpm for 1 min, and then 25 μl of PAF solution was added to achieve a final PAF-C_{16} concentration of 0.15 μM. Each compound was studied in at least three independent experiments.

***In vitro* aggregation studies in washed platelets.** Platelets were prepared by a slight modification of the method described by Ardlie *et al.* (19). Briefly, PRP was centrifuged at 1000 × *g* for 15 min (15°). The platelet pellet was washed once with Tyrode's solution containing $MgCl_2$ (2mM), EGTA (0.2 mM) and then with the same solution deprived of EGTA. The pellet was suspended in the assay buffer solution (NaCl, 140 mM; KCl, 2.7 mM; NaH_2PO_4, 0.4 mM; $MgCl_2$, 1 mM; $NaHCO_3$, 12 mM; $CaCl_2$, 0.9 mM; Tris·HCl, 10 mM; dextrose, 6.2 mM; BSA, 0.25%, w/w, pH 7.4; apyrase, 0.2 mg/ml). The concentration of platelets in the suspension was 4×10^8/ml. Aggregation was performed by using a 340-μl platelet suspension prewarmed for 10 min at 37° in the presence of a PAF antagonist or solvent (control, 30 μl). Samples were stirred at 1000 rpm for 1 min and then PAF-C_{16} (30 μl) was added to give a final PAF concentration of 70 nM.

Analysis of results. The results from equilibrium saturation and kinetic experiments were analyzed by conventional graphical and linear least squares regression techniques. Data from competition experiments were fitted by applying an iterative, nonlinear, least squares regression analysis (the algorithm used was developed by Marquardt).

K_i values were calculated by using the equation of Cheng and Prussoff (20), $K_i = IC_{50}/(1 + C/K_d)$, where C is the concentration of [^{3}H]PAF used and IC_{50} is the concentration of displacer that inhibits 50% of specific binding at equilibrium conditions.

Binding kinetics parameters were calculated as described by Bennett and Yamamura (21). The relationship $\ln (B_0/B_0 - B_t) = k_{obs} \times t$ allowed the determination of the observed association rate constant (k_{obs}), whereas the first order rate dissociation constant (k_{-1}) was derived from the relationship $\ln Bt/B_0 = -k_{-1} \times t$. It is recalled that B_t and B_0 are the amount of specific binding at time t (min) and equilibrium, respectively.

The relation $k_{+1} = (k_{obs} - k_{-1})/L$, where L represents the concentration of free radioligand used, provided the second order rate constant of association. Finally, the equilibrium constant (K_d) was calculated from the equation $K_d = k_{-1}/k_{+1}$.

IC_{50} values (concentrations of antagonist inhibiting PAF-induced aggregation by 50%) from *in vitro* platelet aggregation studies were calculated by linear regression analysis applied to log concentrations of the antagonist versus percentage values for inhibition of PAF aggregation.

All results are reported as means ± standard error unless otherwise stated.

A least squares linear regression model was applied to assess the degree of correlation between the IC_{50} values of PAF antagonists obtained in aggregation studies and the corresponding K_i values determined in binding studies. The computer program used for this analysis was also able to indicate which points did not belong to the calculated regression line for a probability value of 0.01 (Statgraphics; Uniware, Paris, France).

Results

Characterization of [³H]PAF Radioligand Binding to Canine Platelet Membranes

Kinetic analysis. At 25°, the association of [³H]PAF to platelet membranes attained equilibrium in less than 50 min and during the additional 70-min incubation period specific binding remained unchanged (Fig. 1). Four independent determinations provided a k_{obs} value of 0.073 ± 0.009 min^{-1}. At equilibrium conditions, the addition of 10 μM PAF-C_{18} to the incubation medium reversed [³H]PAF binding entirely. The time to obtain 50% dissociation of the ligand-receptor complex ($t_{1/2}$) was 20.5 ± 1.7 min (n = 4) (Fig. 1).

The determination of k_{-1} and k_{+1} yielded 0.035 ± 0.003 min^{-1} and 0.157 ± 0.027 $nM^{-1} \cdot min^{-1}$ (n = 4), respectively. The kinetically derived dissociation constant ($K_d = k_{-1}/k_{+1}$) was 0.23 ± 0.02 nM (n = 4). The latter value was of the same order as that determined in the equilibrium saturation experiments described in the next section.

[³H]PAF binding was stable over a 30-min period when performed at 37° (temperature used in aggregation experiments; see the following section). At the latter temperature, [³H]PAF (0.15 nM) association was relatively rapid and when the equilibrium was reached (in about 15 min) the specific binding remained unchanged for the subsequent 30 min.

Equilibrium saturation analysis. [³H]PAF binding was saturable and displayed concentration dependency, high affinity, and elevated specific binding; the latter attained 75% of total binding at a [³H]PAF concentration of 0.2 nM (Fig. 2). The Scatchard plot was characterized by a straight line that yielded an equilibrium dissociation constant (K_d) of 0.213 ± 0.055 nM and a maximum binding density (B_{max}) of 911 ± 31 fmol/mg of protein (n = 8). The Hill coefficient (n_H) calculated from these binding data was 0.97 ± 0.019 (n = 8).

Inhibition of binding by PAF agonists and antagonists. Specific [³H]PAF binding was totally inhibited by PAF-C_{16} and PAF-C_{18} (K_i values, 0.19 ± 0.03 nM and 0.11 ± 0.02 nM, n = 4, respectively). Some structural analogs of PAF (enantio-PAF-C_{16}, 2-*O*-methyllyso-PAF-C_{18}, lyso-PAF-C_{18}, and 2-*O*-benzyl-lyso-PAF-C_{16}) were studied and found to be weak displacers of PAF-C1 and poor pro-aggregant agents in platelets (Table 1).

Racemic 52770 RP, a new PAF receptor antagonist (7), fully displaced [³H]PAF from its binding sites with a K_i value of 237 ± 28 nM (n = 4). A K_i value of 158 ± 28 nM (n = 4) was found for (+)-52770 RP and 7115 ± 872 nM (n = 4) for the (−)-52770 RP; thus, the latter enantiomer is 45 times less potent than the (+)-form (Fig. 3).

The most potent PAF antagonists in our binding assay were 59227 RP, CV-6209, and Ro 19-3704, characterized by K_i values of 7.9 ± 1.5 nM, 10.2 ± 1.2 nM, and 37 ± 5 nM (n = 4/group), respectively (Fig. 4). They were followed by brotizolam, SRI 63-441, WEB 2086, and L-652,731, which were approximately equipotent (K_i values close to 200 nM). BN 52021 was the least active antagonist studied (K_i = 16840 ± 1279 nM, n = 3) (Table 2).

[³H]PAF binding to washed canine platelets. A binding assay was performed on washed platelets by using a platelet suspension (5 × 10^7 platelets/tube) incubated for 30 min at 25° and exposed to [³H]PAF concentrations ranging from 0.14 to 4 nM. The other experimental conditions were the same as those described for [³H]PAF binding to platelet membranes. Linear transformation of the saturation results gave a K_d value of 0.78 nM and B_{max} of 4100 sites/platelet.

In addition, displacement curves with the PAF antagonists listed in Table 2 were determined for washed platelets using 0.9 nM [³H]PAF (concentration for which specific binding attained 80% of total binding). The rank order of potencies found in this binding procedure was of the same order as that reported in Table 2 for platelet membranes. However, the intrinsic potency of the compounds was 1.5 to 6 times higher than for platelet membranes.

The K_i values of the PAF antagonists studied for [³H]PAF binding to platelet membranes (Table 2) were highly correlated with those obtained for [³H]PAF in washed canine platelets (r = 0.96; slope = 1.02; df = 14).

Pharmacological specificity of [³H]PAF binding. Many

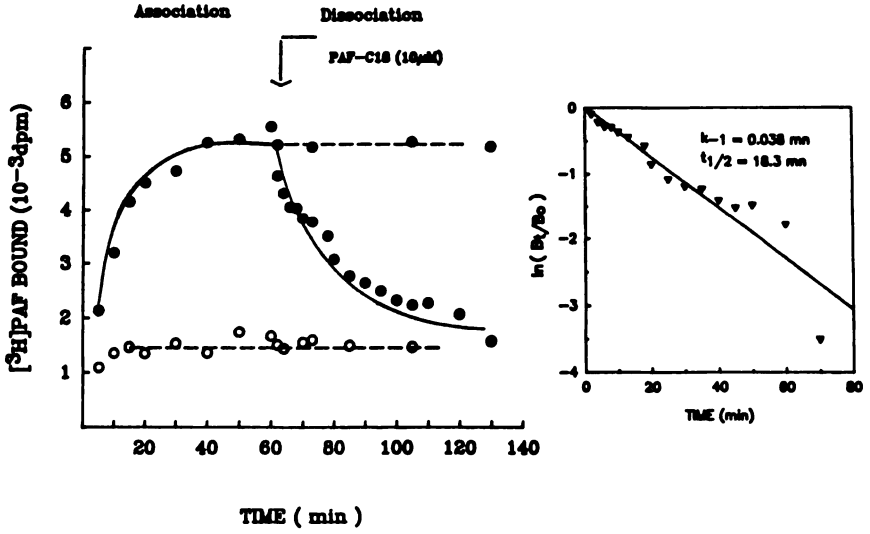

Fig. 1. Time-course of total (●) and nonspecific (○) [³H]PAF binding in dog platelet membranes. [³H]PAF (0.2 nM) was incubated in a suspension of platelet membrane protein (40 μg) at 25°, which was sampled at various time intervals for filtration and then radioactivity counting. Dissociation was started at equilibrium conditions by the addition (↓) of 10 μM PAF-C_{18}. The *curve* shown is from a typical experiment run in duplicate. *Right panel*, the dissociation kinetics are expressed as ln (B_t/B_0) where B_t is the amount of specific binding at time t (min) and B_0 the amount of specific binding at equilibrium.

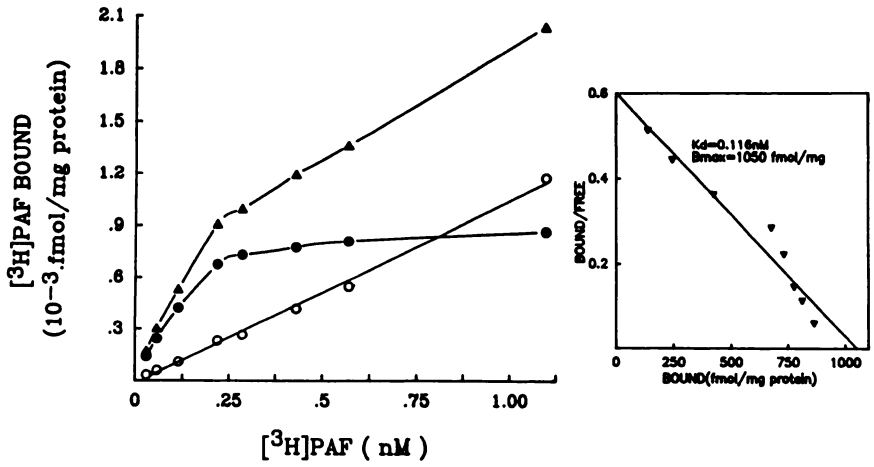

Fig. 2. Saturation experiment of [^{3}H]PAF binding in dog platelet membranes and Scatchard plot analysis (*right panel*) of specific [^{3}H]PAF binding. Membrane proteins (40 μg) were incubated at 25° for 60 min with increasing concentrations of [^{3}H]PAF. Specific binding (●) is defined as the difference between the total (▲) and nonspecific (○) binding. Each *point* is the mean of duplicate determinations. The saturation isotherm shown is from a representative experiment. The calculated values for K_d and B_{max} are 0.213 ± 0.055 nM and 911 ± 31 fmol/mg of protein (n = 8), respectively.

TABLE 1

Inhibition of [^{3}H]PAF specific binding (expressed as K_i) to canine platelet membranes (n = 3 or 4 preparations/compound) by PAF and some of its analogs. The potency of these compounds (ED_{50}) in inducing aggregation in dog platelets is also shown.

Compounds	Binding, K_i	Aggregation, ED_{50}
	nM	
PAF-C_{18}	0.111 ± 0.019	250 ± 70
PAF-C_{16}	0.19 ± 0.02	140 ± 20
2-*O*-Methyl-lyso-PAF-C_{18}	56.7 ± 4.9	>100,000
Enantio-PAF-C_{16}	1,268 ± 65	>100,000
Lyso-PAF-C_{18}	5,804 ± 646	>100,000
2-*O*-Benzyl-lyso-PAF-C_{16}	8,313 ± 989	>100,000

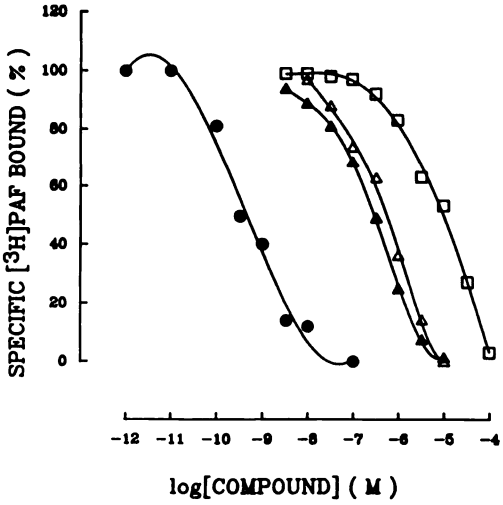

Fig. 3. Inhibition of specific [^{3}H]PAF binding to dog platelet membranes by PAF-C_{18} (●), (+)-52770 RP (▲), (±)-52770 RP (△), and (−)-52770 RP (□). [^{3}H]PAF was incubated in a platelet membrane protein (40 μg) suspension in the presence of increasing concentrations of various PAF antagonists. Competition results were obtained from four separate assays. The data represented are from a typical experiment in which each *point* is the mean of duplicate determinations.

classical pharmacological agents at concentrations up to 100 μM (atropine, diazepam, dopamine, dipyridamole, forskolin, haloperidol, histamine, imipramine, indomethacin, isoproterenol, mepyramine, naloxone, phentolamine, prazosin, propranolol, quinidine, 5-hydroxytryptamine, sulfinpyrazone, and yohimbine) failed to displace [^{3}H]PAF from its binding sites. The calcium antagonists diltiazem (K_i = 22.8 ± 4.7 μM, n = 3) and

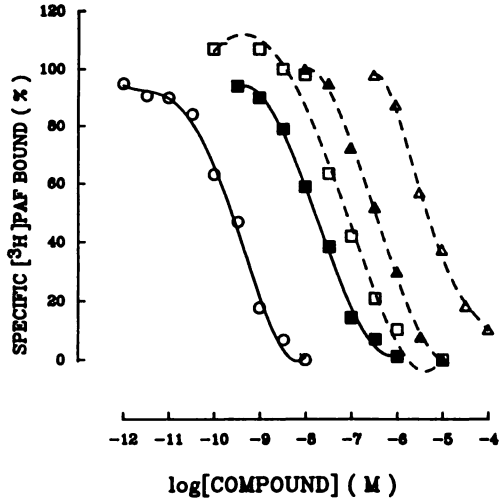

Fig. 4. Inhibition of specific [^{3}H]PAF binding to dog platelet membranes. Experiments were carried out as described in Fig. 3, using PAF-C_{16} (○), CV 6209 (■), Ro 19-3704 (□), SRI 63-441 (▲), and alprazolam (△). The reported results are from a typical experiment run in duplicate.

verapamil (K_i = 16.4 ± 2.4 μM, n = 2) exhibited low affinity for [^{3}H]PAF binding sites.

Studies on PAF-Induced Aggregation of Canine Platelets

Determination of the potency of PAF agonists in PRP. PAF-C_{18} was almost twice as potent as PAF-C_{16} as an aggregant agent. Enantio-PAF-C_{16}, lyso-PAF-C_{18}, 2-*O*-methyl-lyso-PAF-C_{18}, and 2-*O*-benzyl-lyso-PAF-C_{16} did not induce aggregation at concentrations up to 0.1 mM (Table 1).

Determination of the potency of PAF antagonists in PRP. CV-6209, WEB 2086, and 59227 RP were found to be the most potent inhibitors (IC_{50} values, 99 ± 9 nM, 208 ± 19 nM, and 146 ± 15 nM, n = 4 or 5/group, respectively) of PAF-induced platelet aggregation. The IC_{50} values for Ro 19-3704, 52770 RP, brotizolam, and SRI 63-441 against PAF aggregation were approximately 2 μM (Table 2).

For the other compounds listed in Table 2, concentrations ranging from 10 to 60 μM were necessary to inhibit PAF aggregation by 50%. Triazolam (IC_{50} = 59.3 ± 6.8 μM, n = 3) was the least potent of the antagonists studied.

From Table 2, it appears that 13 out of 16 PAF receptor antagonists investigated require a concentration 5 to 67 times greater to inhibit PAF-induced platelet aggregation than to

TABLE 2

Comparison of inhibitory potency for several PAF antagonists against [³H]PAF binding in platelet membranes (K_i) and against PAF-induced aggregation in PRP or washed platelets (WP) (IC_{50}). Each compound was studied in at least four independent preparations.

Antagonist	K_i	IC_{50}, PRP	IC_{50}, WP	Ratio IC_{50}/K_i	
				PRP	WP
	nM				
59227 RP	7.9 ± 1.5	208 ± 19	52 ± 2.6	26.3	6.5
CV-6209	10 ± 1.2	99 ± 9	8.6 ± 0.8	9.9	0.9
Ro 19-3704	36 ± 5	2430 ± 427	357 ± 26	67.5	9.9
(+)-52770 RP	158 ± 29	1685 ± 214	137 ± 6	10.7	0.9
Brotizolam	158 ± 26	2412 ± 212	387 ± 74	15.3	2.4
SRI 63-441	162 ± 26	1708 ± 113	736 ± 11	10.6	4.5
WEB 2086	203 ± 27	148 ± 16	21.4 ± 0.7	0.7	0.1
(±)-52770 RP	237 ± 28	2390 ± 140	72.5 ± 3.1	10.0	0.3
L-652,731	374 ± 20	16040 ± 1400	193 ± 20	43.0	0.5
Kadsurenone	863 ± 126	1050 ± 95	828 ± 98	1.2	0.9
CV-3988	1397 ± 142	17146 ± 2853	3922 ± 112	12.3	2.8
RU 45703	2893 ± 237	9894 ± 940	4004 ± 345	3.4	1.4
Alprazolam	3301 ± 555	46967 ± 3243	2872 ± 121	14.2	0.9
(−)-52770 RP	7115 ± 872	34743 ± 2856	12219 ± 831	4.9	1.7
Triazolam	7126 ± 1085	59244 ± 6850	28585 ± 2251	8.4	4
BN 52021	16840 ± 1475	4300 ± 123	1190 ± 63	0.25	0.07

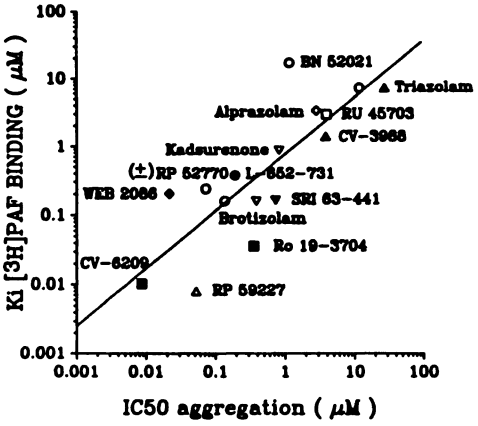

Fig. 5. Least squares regression line fitting the values of potencies of 16 PAF antagonists (reported in Table 2) as displacers of [³H]PAF binding (K_i) and as antagonists of PAF-induced aggregation in washed dog platelets (IC_{50}). The equation of the line is $\hat{y} = -0.101 \pm 0.82\,\hat{x}$ and the correlation coefficient, 0.82 ($df = 14$, $p < 0.001$).

displace [³H]PAF from its binding sites, whereas the reverse phenomenon was observed for BN 52021. On the other hand, WEB 2086 and kadsurenone were equipotent as [³H]PAF displacers and as antagonists of PAF-induced aggregation.

Determination of the potency of PAF antagonists in washed platelets. CV-6209, WEB 2086, and 59227 RP were found to be the most potent inhibitors (IC_{50} values, 8.6 ± 0.8 nM, 21.4 ± 0.7 nM, and 52 ± 6 nM, $n = 4$ or 5/group, respectively) of PAF-induced platelet aggregation.

The IC_{50} of the other compounds studied ranged between 0.2 and 4 μM. Finally (−)-52770 RP and triazolam were the least potent of the drugs tested (IC_{50} = 12.2 ± 0.8 μM and 28.6 ± 0.2 μM, $n = 4$, respectively).

Correlation analysis. The potencies of the studied PAF antagonists as [³H]PAF displacers and as antagonists of PAF-induced aggregation in washed canine platelets were of the same order of magnitude except Ro-193704 and BN 42021, which were, respectively, 10-fold more potent and 14-fold less potent as inhibitors of PAF-induced aggregation than as displacers of [³H]PAF binding (Table 2).

A linear correlation analysis applied to these results demonstrated that a strong ($p < 0.001$) correlation ($r = 0.82$; slope 0.82; $df = 14$) exists between the potencies of these compounds in displacing [³H]PAF from its binding sites and in inhibiting PAF-induced platelet aggregation (Fig. 5). When the probability level for belonging to the calculated regression line was set to 0.01, BN 52021 and Ro 19-3704 were found to be outliers. When these latter compounds were excluded from the analysis, the correlation coefficient became 0.89 (slope = 0.77; $df = 12$).

Binding experiments with the compounds of Table 2 were also performed on washed platelets. A linear correlation analysis using these binding results and the washed platelet aggregation data demonstrated that a significant ($p < 0.001$) correlation ($r = 0.80$; slope = 0.87; $df = 14$) also exists between the potencies of the compounds in displacing [³H]PAF from its binding sites and in inhibiting PAF-induced aggregation in washed platelets.[1]

Discussion

The present study demonstrates that canine platelet membranes possess binding sites for [³H]PAF that display most of the desirable properties (high affinity, lack of cooperative phenomena, saturability, reversibility, stereoselectivity, specificity, and correlation with functional responses) for a meaningful binding technique. The high affinity of [³H]PAF is indicated by the low value (0.23 nM) of the K_d as determined by kinetics and equilibrium saturation analysis. Furthermore, [³H]PAF would appear to label a single class of noninteracting sites as suggested by a Hill coefficient close to unity ($n_H = 0.97$).

The maximal number of binding sites for [³H]PAF (B_{max} = 911 fmol/mg of protein) was relatively high although it is 3-fold smaller than that in rabbit platelet membranes (B_{max} = 2900 fmol/mg of protein) (7). This finding would appear to parallel functional studies, which have demonstrated that in the latter species PAF is a more potent platelet aggregant than in dogs (9).

[³H]PAF binding was fully reversible, as it was completely displaced in approximately 70 min by a large concentration (10 μM) of unlabeled PAF.

[1] L. Tahraoui, A. Floch, S. Mondot, and I. Cavero, unpublished data.

Stereoselective discrimination was also displayed by this binding. In fact, enantio-PAF-C_{16} (the enantiomer of PAF that does not occur naturally) exhibited poor affinity for the [^{3}H]PAF receptor. Furthermore, the (−)-enantiomer of 52770 RP, a PAF antagonist with an asymmetric carbon center, was 45 times less potent than its (+)-enantiomer in competing for [^{3}H]PAF binding. Recently, [^{3}H]52770 RP was reported to label PAF receptor sites in rabbit platelets (7) and human neutrophils (22).

Our [^{3}H]PAF binding sites exhibited high specificity for the ligand used. In competition experiments, close analogs of PAF such as enantio-PAF, lyso-PAF-C_{18} (= deacetylated PAF, the main catabolic product of PAF in biological systems), and 2-*O*-benzyl-lyso-PAF-C_{18} were poor displacers of [^{3}H]PAF binding. Similarly, a variety of classical pharmacological agents (e.g., dopamine, histamine, isoproterenol, 5-hydroxytryptamine, atropine, imipramine, prazosin, and naloxone, etc.) including the pro-aggregant cyclooxygenase inhibitors failed to exhibit affinity for [^{3}H]PAF sites. In contrast, recognized PAF antagonists such as CV-6209 (23), Ro 19-3704 (24), brotizolam (25), WEB 2086 (26), 52770 RP (7), SRI 63-441, and L-652,731 (27) and the new PAF antagonist, 59227 RP, were full and potent displacers of [^{3}H]PAF binding.

A membrane binding site of biological significance is expected to be the counterpart of a membrane receptor mediating upon stimulation an appropriate physiological or pharmacological response (28). Currently, this issue is generally answered by investigating, whenever possible, whether a correlation exists, within a relatively large series of agonists and antagonists of variable potencies, between their ability to displace the ligand from its binding sites and their ability to mimic (agonists) or inhibit (antagonists) the functional effects of a recognized agonist of the investigated binding site. For 16 PAF antagonists studied herein, a significant ($r = 0.82$; $p < 0.001$) correlation existed between their potencies as displacers of [^{3}H]PAF binding in canine platelet membranes and as antagonists of PAF-induced aggregation in washed platelets.

Our initial comparison between [^{3}H]PAF binding results and aggregation responses to PAF studied in PRP revealed large differences between the potency exhibited by these compounds in binding as compared with the functional test. Thus, an additional study was performed in washed platelets to avoid possible binding of the more lipophilic PAF receptor antagonists to the various proteins in PRP, which could decrease effective biophase concentrations of the studied antagonists. The results with PAF-evoked aggregation in washed platelets reduced the differences in potency for the various compounds as inhibitors of platelet aggregation as opposed to [^{3}H]PAF displacers. Ideally, comparative studies of biochemical and functional tests should be performed in the same preparation and under the same experimental conditions. We attempted this approach in a few preliminary studies and found that [^{3}H]PAF binding to washed platelets[1] was slightly more sensitive than in platelet membranes. However, the potency rank for the studied antagonists was the same for both types of binding. Thus, it is reasonable to infer that the studied membrane binding sites labeled by [^{3}H]PAF have the same pharmacological characteristics as those present in intact platelets, which, when activated by PAF, induce the aggregation response.

As mentioned in Results, two PAF antagonists did not lie within the 99% confidence limits of the regression line incorporating the results of all studied PAF antagonists. In fact, Ro 19-3704 was about 10-fold less potent as an antagonist of PAF-induced platelet aggregation than as a [^{3}H]PAF-binding displacer, and BN 52021 exhibited the reverse relationship. Why these compounds are outliers of our correlation model is not evident at the present time. It is possible that the higher potency of Ro 19-3704 as a [^{3}H]PAF displacer than as an antiaggregant agent may result from the partial agonist activity of this compound (suggested by its chemical structure being closely related to PAF); however, CV-3988, which is also a partial agonist at PAF receptors (29), did not behave like Ro 19-3704.

BN 52021 was more potent as an antagonist of PAF-induced platelet aggregation than as a displacer of [^{3}H]PAF. It cannot be excluded that other pharmacological properties of BN 52021 may intervene in its ability to antagonize PAF-induced platelet aggregation better than [^{3}H]PAF binding.

In conclusion, this investigation provides convincing evidence that the determined properties of the [^{3}H]PAF binding sites characterized on canine platelet membranes are those generally considered desirable for valid and valuable binding procedures. Furthermore, these sites would appear to be the counterparts of the PAF receptors that mediate platelet aggregation. Thus, this binding technique may be of use in advancing our understanding of the role played by PAF in certain pathophysiological processes.

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