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BINDING OF [^{3}H]SR 27417, A NOVEL PLATELET-ACTIVATING FACTOR (PAF) RECEPTOR ANTAGONIST, TO RABBIT AND HUMAN PLATELETS AND POLYMORPHONUCLEAR LEUKOCYTES

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Abstract—Binding of [^{3}H]SR 27417 to washed rabbit platelets was time-dependent and saturable. [^{3}H]-SR 27417 binding was reversible after short incubation periods but became progressively irreversible when incubated for more than 2 hr. Scatchard analysis of the saturation binding data indicated that [^{3}H]-SR 27417 bound to two populations of specific binding sites with high (K_D = 0.75 ± 0.06 nM; B_{max} = 58.7 ± 4.3 fmol/10^8 cells; N = 3) and low affinity (K_D = 53.8 ± 4.9 nM; B_{max} = 1665 ± 87 fmol/10^8 cells; N = 3). Unlabelled C_{16}-PAF competitively and selectively inhibited the specific binding of [^{3}H]SR 27417 with an IC_{50} value of 1.9 ± 0.04 nM (N = 3). SR 27414 fully and competitively displaced [^{3}H]SR 27417 from its binding sites on rabbit platelets with a K_i value of 260 ± 20 pM (N = 3) therefore demonstrating that SR 27417 was more potent than C_{16}-PAF itself. On washed human platelets, [^{3}H]SR 27417 displayed specific as well as saturable binding to two populations of binding sites (K_D = 0.21 ± 0.01 nM; B_{max} = 13.9 ± 0.9 fmol/10^8 cells and K_D = 4.75 ± 1.9 nM; B_{max} = 82.2 ± 2.9 fmol/10^8 cells; N = 3) for high- and low-affinity binding sites, respectively, whereas [^{3}H]SR 27417 bound to only one single class of binding sites on human polymorphonuclear leukocytes (K_D = 0.31 ± 0.1 nM; B_{max} = 9.36 ± 1.2 fmol/10^6 cells; N = 3). IC_{50} values for C_{16}-PAF, SR 27417 and other PAF receptor antagonists on all three cell types indicated that SR 27417 was at least three times more potent than C_{16}-PAF itself and more than 30-fold as active as the best synthetic PAF receptor antagonist tested (L 659,989). In conclusion, these data indicate that SR 27417 appears to be one of the most potent PAF receptor antagonists yet described, as well as a suitable radioligand for labelling PAF receptors on intact blood cells.

Platelet-activating factor (PAF†) is one of the most potent inducers of platelet aggregation known and has been reported to evoke numerous biological effects such as platelet and neutrophil activation, bronchoconstriction, leukocyte infiltration by enhancing microvasculature permeability, hypotension and anaphylactic reactions (for review see Refs 1 and 2). Moreover, PAF is believed to be involved in allergic responses, asthma, anaphylactic and septic shocks and inflammation [1, 2].

Specific PAF receptor sites have been identified by using radioligand binding techniques in rabbit [3, 4], canine [5] and human platelets [4, 6, 7], human polymorphonuclear leukocytes (PMNs) [8, 9] and human lung [10] and, recently, we described the binding characteristics of [^{3}H]PAF to guinea pig tracheal epithelial cells in culture [11]. Honda *et al.* [12] described the cloning of the PAF receptor from the guinea pig lung. A large number of PAF antagonists have been identified in the past few years (for review see Refs 1, 2 and 13). These compounds appear to compete with specific binding sites present on platelets, lung or brain tissue, neutrophils or eosinophils and their potencies correlated with their respective abilities to antagonize PAF-induced platelet aggregation, bronchospasm and hypotension [14–16].

Recently, we described the biochemical and pharmacological properties of SR 27417, the first compound of a newly developed PAF receptor antagonist series [17]. This compound exhibited high potency against [^{3}H]PAF binding and was characterized by a higher affinity for PAF receptors than PAF itself on rabbit platelets. *In vivo*, SR 27417 exhibited specific, long-acting PAF-antagonist activity on various pharmacological parameters either after intravenous or oral administration [17].

The aim of this study was to determine the binding characteristics of [^{3}H]SR 27417 to rabbit and human platelets and human PMNs.

MATERIALS AND METHODS

Drugs. C_{16}-PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphoryl-choline), C_{18}-PAF, lysoPAF-C_{16} and enantio-PAF-C_{16} were purchased from Bachem (Switzerland). Stock solutions of PAF or analogs were prepared in absolute ethanol and subsequent dilutions were made with Tyrode's solution containing 0.25% bovine serum albumin (BSA). [^{3}H]-SR 27417 (sp. act. 12.7 Ci/mmol), SR 27417 [*N*-(2-dimethylamino ethyl)-*N*-(3-pyridinyl methyl) [4-(2,4,6-triisopropyl phenyl)thiazol-2-yl]amine] [17], L 652,731 (*trans*-2,5-bis(3,4,5-trimethoxyphenyl)-tetrahydrofuran) [18] and WEB-2086 (base, APAFANT) (3-[4-(2 chlorphenyl)-9-methyl-6*H*-thi-

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† Abbreviations: BSA, bovine serum albumin; PAF, platelet-activating factor; PMN, polymorphonuclear leukocyte; PRP, platelet-rich plasma.

eno [3,2-*f*] [1,2,4] triazolo-[4,3-*a*] [1,4]-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) [19] were synthesized in the chemistry laboratories of Sanofi Recherche. The structures of SR 27417, L 652731 and WEB-2086 were determined by ^{1}H- and ^{13}C-NMR mass spectrometry, i.r. spectroscopy and elemental analysis. Their purities were measured by HPLC and TLC and were >99%. 59227 RP, WEB-2170 and L 659989 were kind gifts of Rhône-Poulenc Rorer (Vitry sur Seine, France), Boehringer Ingelheim (Ingelheim am Rhein, F.R.G.) and Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.), respectively. For experiments, fresh solutions of the compounds were prepared daily in dimethyl sulphoxide. All other chemicals were from Prolabo (France).

Preparation of rabbit platelets. Washed rabbit platelets were prepared according to the method described by Ardlie *et al.* [20]. Blood was withdrawn from the marginal ear artery of male New Zealand rabbits (2–2.5 kg; Charles River, France) and collected in tubes containing an aqueous solution of buffer ACD [citric acid (9 mM), sodium dihydrogen citrate (1.75 mM) and dextrose (5.6 mM)]. Blood was then immediately centrifuged (120 *g*, 15°, 25 min) to prepare platelet-rich plasma (PRP) from which a pellet was obtained by further centrifugation (1000 *g*, 15°, 15 min). The pellet was first washed with a Tyrode solution containing BSA (0.35%, w/v), $MgCl_2$ (2 mM), EGTA (0.2 mM) and then with the same Tyrode solution minus EGTA. The platelets were finally suspended in the assay buffer solution of the following composition: NaCl (140 mM), KCl (2.7 mM), NaH_2PO_4 (0.4 mM), $MgCl_2$ (2 mM), $NaHCO_3$ (12 mM), Tris–HCl (10 mM), dextrose (6.2 mM) and BSA (0.25%, w/v), pH 7.4 (buffer A). The final suspension was adjusted to contain 4×10^8 platelets/mL.

Preparation of human platelets. Human blood (25 mL) was collected by venipuncture from normal healthy volunteers who had not taken any medication for at least 10 days and transferred into a tube containing 5 mL ACD as an anticoagulant. PRP was obtained by centrifuging the blood sample at 170 *g* for 20 min at 20°. Centrifugation of the PRP yielded a platelet pellet that was suspended in 30 mL of buffer B: 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 5 mM $MgCl_2$. The platelets were washed twice in buffer B by further centrifugation (2500 *g*, 10 min) and finally suspended in buffer C: 50 mM Tris–HCl (pH 7.4), 100 mM KCl, 5 mM EDTA, 5 mM $MgCl_2$, 0.25% BSA (w/v) to give a final concentration of 3×10^8 cells/mL.

Preparation of human PMN leukocytes. Human PMNs were prepared according to Boyum [21] from freshly drawn venous blood into a 0.1 volume of 3.8% sodium citrate. Blood was then immediately centrifuged (170 *g*, 10 min, 20°) and PRP was discarded. The plasma was aspirated and the cells reconstituted to original volume with normal saline. Dextran (Sigma) (6%) was added at a ratio of 3 mL every 10 mL of cells and incubated at room temperature for 60 min. The top layer containing the PMNs was collected. One millilitre of Ficoll-hypaque (1077) was added to every 4 mL of cells to the bottom of the tubes to set up a cushion gradient. The mixture was centrifuged for 30 min at 180 *g*. The supernatant fluid was discarded. The pellet was dislodged and an excess of red blood cell lysing solution consisting of 150 mM NH_4Cl, 0.1 mM EDTA and 10 mM KCO_3H was added and the sample was stirred for 5 min at 37°. This preparation was centrifuged at 180 *g* for 15 min and the supernatant fluid was discarded. The pelleted PMNs were finally suspended (5×10^6 cells/mL) in the assay buffer (buffer A). The cell population consisted of more than 95% mononuclear cells as judged by morphological observation. At the end of the binding experiment, cell viability was determined by Trypan blue exclusion and was always >97%.

[^{3}H]SR 27417 binding to washed platelets and PMN leukocytes. Experiments on the specific binding of [^{3}H]SR 27417 to washed rabbit and human platelets and PMN leukocytes and its inhibition by PAF receptor antagonists were performed as described previously [3, 7, 8] with a filtration technique to separate the free from bound [^{3}H]SR 27417. Incubations were carried out in a total 0.5 mL volume of buffer A (for rabbit and human PMN leukocytes) and C (for human platelets) which contained washed platelets (1.2×10^8 platelets/mL) or PMN leukocytes (2×10^6 cells/mL) and [^{3}H]-SR 27417 (0–30 nM for saturation experiments or 0.5 nM for competition studies). Other substances were added in dimethyl sulphoxide as indicated. Triplicate incubations were carried out at 25° for the indicated period of time and were terminated by addition of a 3 mL ice-cold assay buffer followed by rapid vacuum filtration over Whatman GF/C glass-fiber filters. Filters were then washed twice with 5 mL ice-cold incubation buffer, dried and the radioactivity measured by scintillation counting. Non-specific binding was defined as the total binding measured in the presence of excess unlabelled SR 27417 (1 μM) and specific binding was defined as the difference between total binding and non-specific binding. During the incubation period, the stability of [^{3}H]SR 217417 in the binding medium was assessed by HPLC analysis and the degradation of the ligands, under our standard conditions, represented less than 2% after a 2-hr incubation at 25° (data not shown).

Expression of data. All data are means of at least triplicate experiments. The per cent inhibition was expressed as: $\%I$ = (total binding − total binding with antagonist)/specific binding × 100. The IC_{50} value was defined as the concentration of inhibitor required to obtain 50% inhibition of the specific binding and was determined with an indirect Hill plot [22]. The equilibrium inhibition constant (K_i) was calculated from the Cheng and Prusoff [23] equation: $K_i = IC_{50}/(1 + L/K_D)$ where L was the concentration of labelled ligand and K_D the apparent dissociation constant of radioligand binding to washed rabbit platelets. The apparent dissociation constants (K_D) and the maximal number of binding sites (B_{max}) were calculated by using Scatchard representation of the experimental data [24]. Data from saturation, competition and association studies were analysed using a non-linear regression program [25].

RESULTS

[^{3}H]*SR 27417 binding to washed rabbit platelets*

As shown in Fig. 1, total binding of [^{3}H]SR 27417

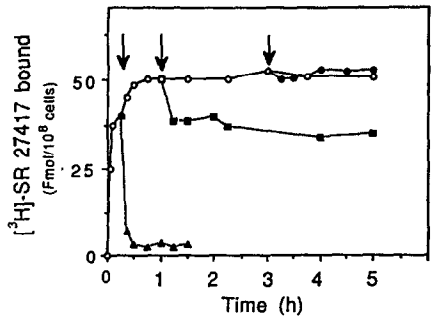

Fig. 1. Time-course of [³H]SR 27417 binding to rabbit platelets. Washed rabbit platelets (1.2×10^8 platelets/mL) were incubated for various periods of time with [³H]-SR 2747 (1 nM) at 25°. The arrows indicate the time at which unlabelled SR 27417 (1 μM) was added to initiate the dissociation process. Each point represents the mean calculated from three experimental determinations.

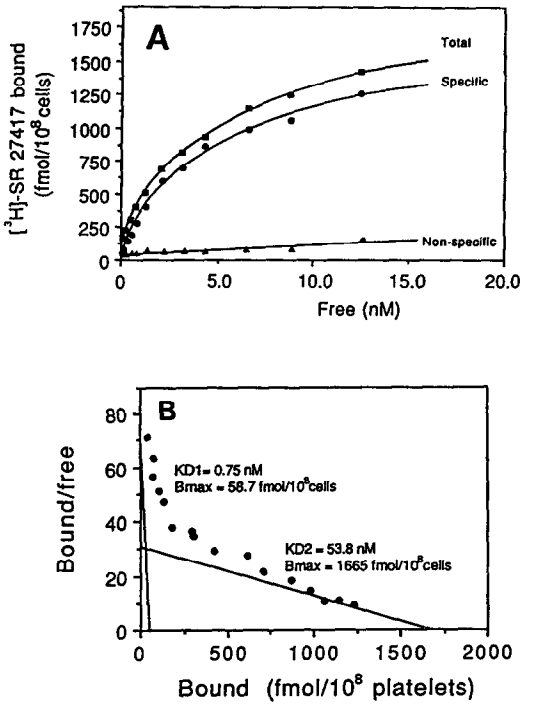

Fig. 2. Scatchard analysis of the binding of [³H]SR 27417 on rabbit platelets. (A) Saturation curve: platelets (1.2×10^8 cells/mL) were incubated for 20 min at 25° with increasing concentrations of [³H]SR 27417 (0.01–30 nM). Specific binding is given by the difference between total and non-specific binding determined in the presence of 1 μM of SR 27417. (B) Scatchard plot of the specific binding of [³H]SR 27417 was calculated from saturation isotherms determined from at least three independent experiments performed in triplicate.

to washed rabbit platelets at 25° was time-dependent and reached an equilibrium 20–30 min after the beginning of the experiment. An apparent equilibrium binding constant (K_{obs}) of $0.031 \pm 0.001\ min^{-1}$ and a time of half-maximum association ($T_{1/2}$) of 8.3 ± 0.6 min. (N = 3) were calculated from this time-course binding study.

After 20 min of incubation, the binding of [³H]-SR 27417 was totally reversible since the addition of excess unlabelled ligand (1 μM) dissociated [³H]-SR 27417 from the cells. From these kinetics, the apparent dissociation constant was found to be 0.81 ± 0.09 nM (N = 3) which is in good agreement with the K_D determined in the saturation experiment.

After a 60-min incubation period, only a minor part of the [³H]SR 27417 binding could be displaced by an excess of unlabelled SR 27417 and after 3 hr, [³H]SR 27417 binding was almost irreversible. It is of interest to note that [³H]PAF behaved in a similar way, its binding becoming progressively irreversible when incubated with the cells for more than 2 hr (not shown).

On the basis of this finding, equilibrium binding experiments were performed by setting the incubation time at 30 min.

Binding of [³H]SR 27417 to washed rabbit platelets was dose-dependent and the non-specific binding as measured in the presence of an excess of unlabelled C_{16}-PAF (1 μM) was linearly dependent on the concentration of [³H]SR 27417 (Fig. 2A). The specific binding, defined as the total amount of [³H]-SR 27417 bound minus the non-specific binding, was saturable, reaching a maximum around 20 nM (Fig. 2A). At 0.5 nM of [³H]SR 27417, the non-specific binding to rabbit platelets varied between 5 and 10% of the total binding.

A Scatchard analysis of the bound/free ratio of the radiolabelled [³H]SR 27417 versus bound [³H]-SR 27417 revealed the presence of two classes of binding sites (Fig. 2B). One population of sites exhibited high affinity with an apparent equilibrium dissociation constant (K_D) value of 0.75 ± 0.06 nM (N = 3) and a total number of receptor sites (B_{max}) of 58.7 ± 4.3 fmol/10^8 platelets (360 ± 26 sites/cell, N = 3). The other site demonstrated a very high binding capacity (B_{max} = 1665 ± 87 fmol/10^8 platelets; $10{,}110 \pm 530$ sites/cells; N = 3) and low affinity (K_D = 53.8 ± 4.9 nM; N = 3).

Inhibition of [³H]*SR 27417 binding to rabbit platelets by PAF receptor antagonists*

As shown in Fig. 3, unlabelled C_{16}-PAF and C_{18}-PAF displaced [³H]SR 27417 specifically bound to its high-affinity receptor sites in rabbit platelets in a dose-dependent manner. The concentrations required to inhibit 50% of the specific binding (IC_{50}) were 1.9 ± 0.04 and 5.0 ± 0.8 nM, respectively (K_i = 0.81 ± 0.02 and 2.14 ± 0.34 nM, respectively; N = 3). Under the same experimental conditions, lysoPAF and enantioPAF (100 μM), two close derivatives of PAF devoid of biological activity via PAF receptors, inhibited the specific [³H]SR 27417 binding by only 1 and 4%, respectively, whereas SR 27417 dose-dependently inhibited the specific binding of [³H]SR 27417 to platelets with the IC_{50} value of 0.62 ± 0.05 nM (N = 3). When calculated from the Cheng and Prusoff equation, the K_i value for the specific binding of [³H]SR 27417 was 0.26 $\pm$ 0.02 nM. A Shild analysis of inhibition of [³H]-

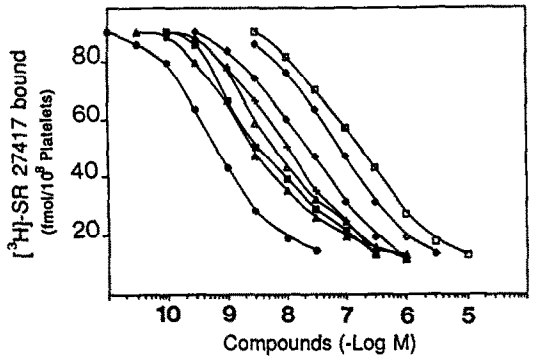

Fig. 3. Competition of [^{3}H]SR 27417 binding by agonists and antagonists of PAF receptors on washed rabbit platelets. Rabbit platelets (1.2×10^8 cell/mL) were incubated with 1 μM of [^{3}H]SR 27417 for 20 min at 25° with increasing concentrations of SR 27417 (●) C_{16}-PAF (▲) C_{18}-PAF (△) WEB-2086 (◆) WEB-2170 (◇) 59227 RP (+) L 652731 (□) or L 659989 (■). Each data point is the average of results from at least three independent determinations performed in triplicate.

Table 1. Comparative abilities of various PAF analogs and antagonists to inhibit [^{3}H]SR 27417 binding to washed rabbit and human platelets and human PMNs.

Compounds	IC_{50} (nM)		
	Rabbit platelets	Human Platelets	Human PMNs
C_{16}-PAF	1.9 ± 0.04	6.0 ± 1.3	1.1 ± 0.05
C_{18}-PAF	5.0 ± 0.8	8.9 ± 0.4	2.1 ± 1.4
LysoPAF	(1%)	(0%)	(8%)
EnantioPAF	(4%)	(3%)	(12%)
SR 27417	0.62 ± 0.05	0.22 ± 0.05	0.12 ± 0.01
L 652731	367 ± 18	190 ± 24	287 ± 15
L 659989	20.6 ± 1.4	17 ± 4.8	12.2 ± 2.5
59227 RP	28.1 ± 4.5	31 ± 5.4	25.1 ± 4.6
WEB-2170	75.2 ± 6.0	98 ± 25	65 ± 7
WEB-2086	166 ± 19	131 ± 11	74 ± 4.2

Values are means ± SD obtained from at least three independent experiments performed in triplicate. Values in parentheses are % inhibition at 100 μM.

SR 27417 binding by SR 27417 to rabbit platelets was linear and of unit slope, indicative of competitive binding (not shown). PAF receptor antagonists, including WEB-2086, WEB-2170, 59227 RP, L 652731 and L 659989 could fully displace the [^{3}H]SR 27417-specific binding. For comparative purposes, IC_{50} values of SR 27417 and of these various PAF receptor antagonists are provided in Table 1. Despite minor differences in experimental conditions, Our results confirmed those already published concerning the ability of these compounds to displace [^{3}H]PAF from its high affinity binding sites on washed rabbit platelets [3, 13, 19, 26–28]. For all compounds, the Hill slope factors (n_H) were near unity, suggesting a bimolecular reaction (data not shown). This also indicated that the inhibition of [^{3}H]SR 27417 binding by these compounds was due to an interaction with the receptor itself.

Nature of the antagonism of SR 27417 on [^{3}H]-SR 27417 binding to its high-affinity binding sites on rabbit platelets

Rabbit platelets were incubated for 20 min at 25° with increasing concentrations of [^{3}H]SR 27417 (0–3 nM) in the absence and in the presence of three concentrations of SR 27417 (0.1, 0.25 and 0.5 nM) (Fig. 4). The Scatchard analysis indicated that this compound was a competitive PAF receptor antagonist with respect to the high-affinity receptor site of [^{3}H]SR 27417 (Fig. 4A). The maximal number of receptor sites (B_{max}) was not affected whereas the apparent dissociation constant (K_D) values increased with respect to the increasing concentrations of SR 27417. Under the same experimental conditions, Scatchard analysis of binding data revealed that C_{16}-PAF was also a competitive antagonist of [^{3}H]-SR 27417 binding to rabbit platelets (Fig. 4B).

In a Shild analysis, by plotting log ($K'_D/K_D - 1$ where K'_D and K_D are apparent and equilibrium dissociation constants of [^{3}H]SR 27417 in the presence and absence of antagonist, respectively, versus log (antagonist), a unity slope was found for both competition experiments (not shown). Therefore, the blockade of [^{3}H]SR 27417 binding by unlabelled C_{16}-PAF and SR 27417 on rabbit platelets is consistent with simple competitive antagonism. The equilibrium dissociation constants (K_B) of SR 27417 can be obtained from the inhibition of [^{3}H] SR 27417 binding by SR 27417 and C_{16}-PAF and were found to be 0.23 ± 0.6 and 1.81 ± 0.7 nM (N = 3), respectively.

On the contrary, after prolonged incubation of rabbit platelets with [^{3}H]SR 27417 (180 min, 25°), the Scatchard analysis revealed that SR 27417 altered the maximal number of binding sites (B_{max}) but not the apparent dissociation constant of [^{3}H]SR 27417 binding, indicative of a non-competitive-type inhibitor (Fig. 4C). Under the same experimental conditions, C_{16}-PAF behaved like SR 27417, its type of inhibition varying according to the incubation time (Fig. 4D).

Binding characteristics of [^{3}H]SR 27417 to washed human platelets.

[^{3}H]SR 27417 specific binding to washed human platelets occurred in a dose-dependent manner which was saturable at higher ligand concentrations. Non-specific binding represented less than 10% of total binding. Analysis of the binding data from saturation isotherms revealed two apparent binding sites. [^{3}H]-SR 27417 binding to the high-affinity site possessed a K_D value of 0.21 ± 0.01 nM and a B_{max} of 13.9 ± 0.9 fmol/10^8 platelets (83 ± 5 sites/cell; N = 3). Low-affinity binding sites exhibited a K_D value of 4.75 ± 1.9 nM and a B_{max} of 82.2 ± 2.9 fmol/10^8 platelets (500 ± 18 sites/platelet; N = 3).

Inhibition of [^{3}H]SR 27417 binding to human platelets

Unlabelled PAF displaced [^{3}H]SR 27417 specifically bound to high-affinity receptor sites in human

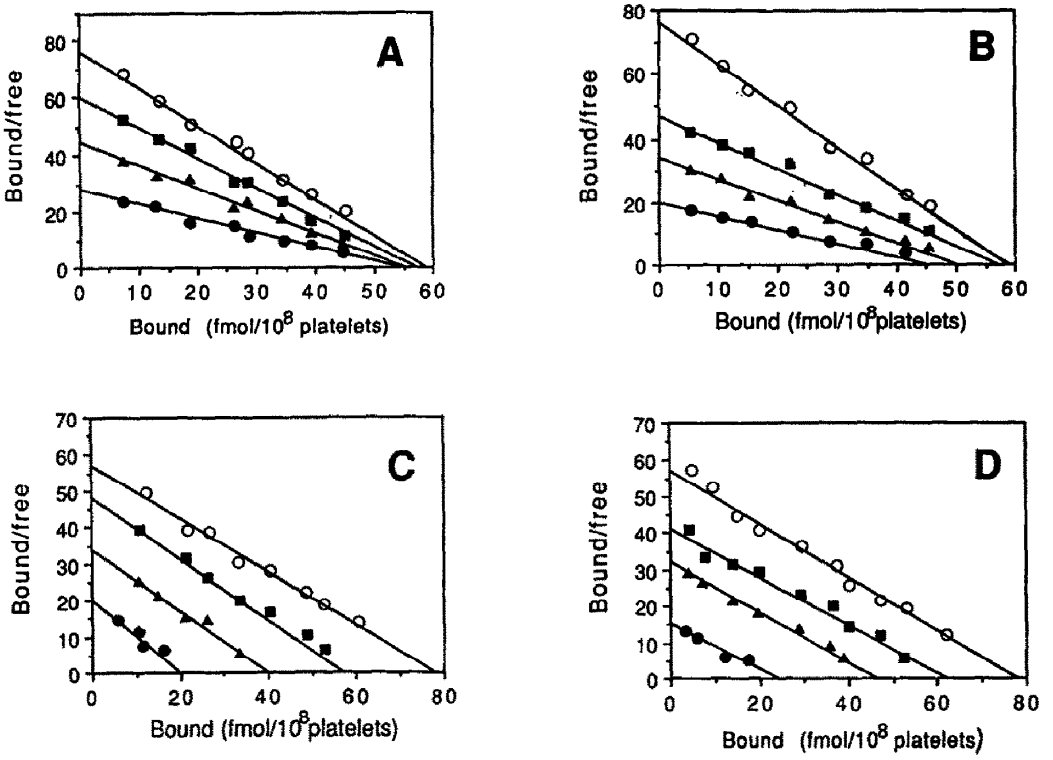

Fig. 4. Inhibition of SR 27417 and C_{16}-PAF on the binding of [³H]SR 27417 to washed rabbit platelets. Washed rabbit platelets (1.2×10^8 cells/mL) were incubated for 20 min (A, B) or 180 min (C, D) at 25° with increasing concentrations of [³H]SR 27417 (0.01–3 nM) in the absence (○) or presence of SR 27417 [0.1 (■); 0.25 (▲) or 0.5 nM (●)] (A, C) or C_{16}-PAF [0.5 (■); 1.0 (▲) or 2.5 nM (●)] (B, D).

platelets in a dose-dependent manner. The IC_{50} value was 6.0 ± 1.3 nM (N = 3) (Table 1). When calculated from the Cheng and Prusoff equation, the K_i value for the specific binding of [³H]SR 27417 was 1.77 ± 0.38 nM. Under the same experimental conditions, lysoPAF and enantioPAF (100 μM) inhibited the specific [³H]SR 27417 binding by only 0 and 3%, respectively. However, SR 27417 dose-dependently inhibited the specific binding of [³H]-SR 27417 to platelets with the IC_{50} value of 220 ± 50 pM (K_i = 65 ± 15 pM; N = 3). Other PAF receptor antagonists, including WEB-2086, WEB-2170, 59227 RP, L 652731 and L 659989 could fully displace the [³H]SR 27417 specific binding (Table 1). For all compounds, the Hill slope factors (n_H) were near unity (data not shown). Scatchard analysis revealed that SR 27417 competitively inhibited [³H]-SR 27417 binding to washed human platelets with a K_i value of 0.11 ± 0.02 nM (N = 3) (not shown).

Specific binding of [³H]*SR* 27417 *to human PMN leukocytes*

To assess the affinity and number of recognition sites of SR 27417 on isolated PMN leukocytes, cells (2×10^6 cells/mL) were incubated with 0–30 nM [³H]SR 27417. The Scatchard analysis of the bound/free ratio of the [³H]PAF versus bound [³H]PAF revealed the presence of one class of binding sites. This population of sites exhibited high affinity with a K_D value of 0.31 ± 0.1 nM (N = 3) and a B_{max} of 9.36 ± 1.2 fmol/10^6 cells (5680 ± 728 sites/cell; N = 3).

Unlabelled PAF displaced [³H]SR 27417 specifically bound to its high-affinity receptor sites on human PMN leukocytes in a dose-dependent manner. The IC_{50} value was 1.07 ± 0.05 nM (K_i = 0.4 ± 0.02 nM; N = 3) (Table 1). Under the same experimental conditions, SR 27417 dose-dependently inhibited the specific binding of [³H]SR 27417 to its high-affinity binding sites on PMNs with the IC_{50} value of 0.12 ± 0.01 nM (N = 3). The K_i value was 46 ± 4 pM (N = 3). A Shild analysis of inhibition of [³H]PAF binding by SR 27417 to PMN was linear and of unit slope, indicative of competitive binding (not shown). Other PAF receptor antagonists fully displaced the [³H]SR 27417-specific binding whose IC_{50} values are shown in Table 1.

As already shown for rabbit platelets, the association of [³H]SR 27417 to human platelets and PMNs was rapid inasmuch as an equilibrium was reached within the initial 30 min of the incubation time at 25° (not shown). Shortly after the beginning of the incubation period (i.e. less than 30 min), the specifically bound [³H]SR 27417 could be totally displaced by 1 μM unlabelled SR 27417, indicating that the binding of [³H]SR 27417 was fully reversible.

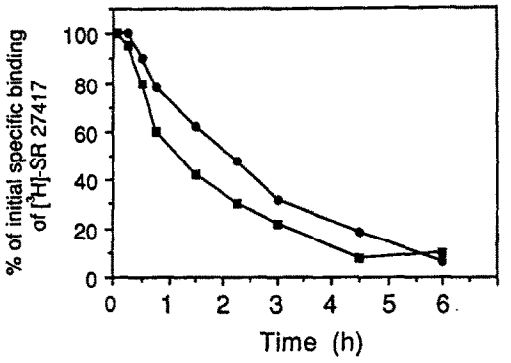

Fig. 5. Specific binding of [^{3}H]SR 27417 to human platelets and PMNs as a function of the incubation time. Human platelets (1.2×10^8 platelets/mL) (■) and PMNs (2×10^6 cells/mL) (●) were incubated at 25° with 0.5 nM of [^{3}H]SR 27417. At the indicated periods of time, unlabelled SR 27417 (1 μM) was added and specific binding was determined after an additional 1 hr of incubation. Results, expressed as a percentage of the initial specific binding of [^{3}H]SR 27417, are averages of results from three determinations performed in triplicate.

However, as indicated in Fig. 5, when [^{3}H]SR 27417 was incubated for more than 180 min with both cell types, [^{3}H]SR 27417 was irreversibly associated with the cells and could not be displaced by an excess of unlabelled SR 27417.

DISCUSSION

Along with several other mediators, it has been proposed that PAF is intimately involved in the etiology of a variety of disorders and it is anticipated that the development of potent and specific PAF receptor antagonists may offer a rational and novel therapeutic approach to these diseases [1, 2] as well as provide invaluable experimental tools with which to elucidate the real involvement of PAF in the pathophysiology of various diseases.

In the past few years, specific PAF receptor sites have been characterized by using radioligand techniques in platelets from various origins, human PMNs, human lung and brain. A large number of PAF receptor antagonists have been identified (for review see Refs 1, 2 and 13).

Among these drugs, SR 27417, the first member of a newly developed PAF antagonist series, has been shown to be a highly potent, specific and competitive PAF receptor antagonist by its inhibitory results on the PAF-induced effect in various systems either *in vitro* or *in vivo* [17]. This compound competed with [^{3}H]PAF for its binding sites with a K_i value in the subnanomolar range, exhibiting a K_i several-fold lower than that of C_{16}-PAF on washed rabbit platelets [11, 17, 28].

Although intact cell preparations appear to be more suited for the physiological characterization of radiolabelled PAF binding sites, PAF can activate the cells, undergo internalization or degradation to inactive metabolites such as lysoPAF [29] or be taken up in the outer leaflet of the plasmalemma [29, 30]. [^{3}H]PAF binding procedure is therefore characterized by high non-specific binding and intersubject variability. For these various reasons, specific radiolabelled PAF receptor antagonists such as [^{3}H]WEB-2086 and [^{3}H]52770 RP have been described as useful markers of PAF receptor sites in intact human platelets [31, 32] and neutrophils [8, 33] and in guinea pig eosinophils [33] and tracheal epithelial cells [11]. Indeed, the use of radiolabelled PAF-specific receptor antagonists may be a better means of studying PAF receptors on cells since excessive levels of non-specific binding of [^{3}H]PAF may interfere with specific [^{3}H]PAF binding measurement. Hence, although experiments were performed at low temperature preventing [^{3}H]PAF from being degraded into lysoPAF, and in order to study more accurately the interactions of SR 27417 with PAF binding sites on intact platelets and PMNs, we used the radiolabelled PAF antagonist, [^{3}H]-SR 27417.

On washed rabbit platelets, [^{3}H]SR 27417 bound in a dose-dependent manner to two populations of binding sites of high and low affinities. The maximum number of PAF receptor sites of both high and low affinity determined by using [^{3}H]PAF in matched parallel studies was virtually identical to that obtained for [^{3}H]SR 27417 [17, 28].

On these cells, SR 27417 competed with [^{3}H]-SR 27417 for its high-affinity binding sites with a very low K_i, SR 27417 being a more potent inhibitor of [^{3}H]SR 27417 binding than C_{16}-PAF itself. SR 27417 exhibited a K_i 3-fold lower than that of C_{16}-PAF and more than 30-fold lower than that of one of the best synthetic PAF antagonists yet described (Table 1). These results were comparable with those concerning the effect of SR 27417 on [^{3}H]PAF binding to these same cells [17, 28].

After short incubation periods, the specific binding of [^{3}H]SR 27417 was totally reversible since the addition of an excess of unlabelled ligand dissociated [^{3}H]SR 27417 from the cells. However, after prolonged incubation, part of the [^{3}H]SR 27417 binding could not be displaced by an excess of unlabelled SR 27417 and after 3 hr, [^{3}H]SR 27417 binding was almost irreversible. Parallel studies demonstrated that [^{3}H]PAF behaved in a similar way, its binding becoming progressively irreversible when incubated with the cells for more than 2 hr. Similar results have been described elsewhere for PAF [30, 34] but, up to now, no PAF receptor antagonist has been reported to behave in such a way. This effect cannot be described as a down-regulation of the PAF receptor, resulting from an antagonist response of SR 27417, since it did not show any agonist effect either *in vitro* or *in vivo* in various animal models [17] but recent observations from our laboratory already described an irreversible association of SR 27417 with rabbit platelets *in vivo* [35]. Indeed, whereas 5 min after i.v. administration of SR 27417 to rabbits, Scatchard analysis revealed a competitive type of association of [^{3}H]PAF, 3 or 24 hr after a single administration SR 27417 behaved as an irreversible inhibitor. Such a difference in SR 27417's effects remains unclear but could be due to irreversibly active metabolite(s), as already shown

for other anti-platelet agents such as ticlopidine or aspirin [36, 37].

As already shown for rabbit platelets, [^{3}H]-SR 27417 bound to two distinct classes of saturable binding sites on human platelets and a strong correlation was found between the ability of SR 27417, C_{16}-PAF and other PAF receptor antagonists to inhibit [^{3}H]SR 27417 binding to its high-affinity sites on rabbit and human platelets. SR 27417 was, respectively, 30- and 80-fold more potent than PAF and the best PAF receptor antagonist tested.

[^{3}H]SR 27417 bound in a specific and saturable manner to a single class of high-affinity binding sites on intact human PMNs. On this population of receptors, SR 27417 displaced the specific [^{3}H]-SR 27417 binding in a competitive manner and exhibited an excellent affinity since the value of its inhibitory constant was in the subnanomolar range. As for platelets, SR 27417 was more potent than C_{16}-PAF or the best synthetic PAF antagonist tested.

As already shown for rabbit platelets, the association of [^{3}H]SR 27417 to human platelets and PMNs was rapid and fully reversible when [^{3}H]-SR 27417 was incubated for less than 60 min, but became progressively irreversible with time.

In conclusion, SR 27417, the first member of a newly developed PAF antagonist series can be considered as one of the most potent antagonists of PAF in platelets and PMNs yet described. Its unique features, consisting of exhibiting a higher affinity for PAF receptor(s) than unlabelled PAF itself and of irreversibly labelling PAF receptors on cells, make it a very interesting tool for furthering our understanding of the mediating role of PAF in certain physiological and pathological situations. Furthermore, [^{3}H]SR 27417 appears to be a more suitable routine research tool for labelling PAF binding sites than [^{3}H]PAF, since its binding is characterized by an extremely low level of non-specific binding.

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