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INHIBITION BY CV-3988 OF THE BINDING OF [^{3}H]-PLATELET ACTIVATING FACTOR (PAF) TO THE PLATELET

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Abstract—The inhibitory effects of CV-3988, a specific antagonist of PAF, on the binding of [^{3}H]-PAF to washed platelets of various species including human were examined. The dissociation constant (K_d), binding capacity (B_{max}), and the number of receptor/platelet for the specific binding site of rabbit platelets were 2.2 ± 0.2 nM, 93.7 ± 8.3 fmoles/10^8 platelets, and 568 ± 50, respectively. CV-3988 selectively inhibited the specific binding of [^{3}H]-PAF to rabbit platelets with an IC_{50} of 7.9×10^{-8} M, and it slightly increased the K_d value (2.5 ± 0.8 nM) and decreased the binding capacity for PAF (B_{max}: 54.3 ± 16.3 fmoles/10^8 platelets). The K_i value of CV-3988 for the specific binding of [^{3}H]-PAF to rabbit platelets was 1.2×10^{-7} M. CV-3988 had no effects on the binding of [^{3}H]-5-hydroxytryptamine (5-HT) to rabbit platelets and on the shape change of the platelet induced by 5-HT. CV-3988 also inhibited the specific binding of [^{3}H]-PAF to human and guinea-pig platelets with IC_{50} values of 1.6×10^{-7} and 1.8×10^{-7} M, respectively. CV-3988 inhibited the PAF-induced aggregation in rabbit, guinea-pig, and human platelets. These findings show that CV-3988 is a specific antagonist of PAF at the receptor site(s) of platelets and, in these species, inhibits PAF-induced platelet aggregation by inhibiting the binding of PAF to the "PAF receptor". No specific binding of [^{3}H]-PAF to the platelet of rats and mice was observed, indicating that these species lack a PAF receptor.

Platelet activating factor (PAF), released from the rabbit basophil [1] through an IgE-dependent mechanism, has recently been identified as 1-*O*-alkyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine [2]. It is not only a potent activator of platelets [1–3] and leukocytes [4–6], but also causes hypotension [7–9], depression of cardiac function [7], and bronchoconstriction [9]. Rabbit [10, 11] and human [12] platelets have specific binding sites for PAF, suggesting that platelet aggregation induced by PAF may be induced via a receptor-mediated mechanism. Recently, we have reported that CV-3988, an analogue of PAF, is a specific PAF antagonist, which inhibits PAF-induced hypotension and platelet aggregation [13].

In this paper, we describe the direct evidence that CV-3988 specifically inhibits the binding of [^{3}H]-PAF to the site of PAF receptor in platelets; various species including human were used.

MATERIALS AND METHODS

Preparation of washed platelets. A syringe containing citrate anticoagulant (final citrate, 0.315%) was used to collect blood by cardiac puncture from conscious male rabbits (New Zealand White rabbit, 3–4 kg), male guinea-pigs (350–500 g) and male mice (Jcl:ICR, 30–40 g) and from rats (Jcl:Sprague–Dawley, 300–400 g) anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Blood from three mice was pooled to obtain one sample. Human blood was collected by venipuncture from normal healthy volunteers who had not taken any drugs for at least two weeks preceding the study. Platelet rich plasma (PRP) was obtained by centrifuging the blood at 100 *g* for 5 min at room temperature. The platelet was washed by the method of Valone [14] with slight modifications. Five ml (human, rabbit, guinea-pig, and rat) or 1 ml (mouse) of PRP was sedimented at 800 *g* for 6 min onto 1 ml (all species except mouse) or 0.5 ml (mouse) cushions of autologous erythrocytes and washed three times in a standard platelet buffer (SPB) (pH 7.0, 4 mM KH_2PO_4, 6 mM $NaHPO_4$, 100 mM NaCl, 0.1% glucose) containing 20% (v/v) citrate anticoagulant (225 mM, pH 5.2) adjusted to a final pH of 6.5 with NaOH.

Assay for PAF-receptor binding. The experiments were done according to the method of Valone [12] with slight modifications. The washed platelets were resuspended at a concentration of $2.2 \times 10^5/\mu$l in a binding buffer (10 mM phosphate-buffered saline, pH 7.1, containing 0.1% (w/v) bovine serum albumin and 0.9 mM $CaCl_2$). Platelets (10^8) in 460 μl of the buffer were added to siliconized culture tubes and preincubated with unlabelled PAF or PAF analogues (20 μl) for 6 min at 23°; [^{3}H]-PAF (20 μl, 60,000 dpm, 0.6 nM) was then added to the tubes, which were incubated for 6 min. The binding reaction was stopped by adding 3 ml of ice-cold saline containing 0.1% BSA (washing solution). Platelets were isolated by vacuum filtration on glass filters (Whatman GF/C filters, Fisher Scientific, U.S.A.). The filters were presoaked with the washing solution. Each tube and filter were successfully and rapidly washed 3 times with 3 ml of ice-cold washing solution. The radioactivity on the glass filter was measured in 4 ml of ACS II scintillator (Amersham, U.S.A.) with a scintillation counter (Aloka LSC 903, Nihon Musen, Japan). The experiments were done in triplicate.

The Scatchard plot analysis. To determine the affinity of the binding of [^{3}H]-PAF to rabbit platelets and the binding capacity of the platelet to [^{3}H]-PAF in the presence or absence of CV-3988, Scatchard plot analysis [15] was used. Preliminary binding experiments for this analysis were carried out in the presence of 0.5, 1, 2, 4, 5, 8, 10, 20 and 30 nM of unlabelled PAF. In the presence of higher doses of PAF (10–30 nM), the data were variable. Thus, the experiments for the Scatchard plot analysis were done in the presence of lower doses (less than 8 nM) of unlabelled PAF, in which the Scatchard plot was linear ($P < 0.05$). The apparent binding affinity of the [^{3}H]-PAF was calculated from the slope of the line and the binding capacity expressed as fmole/10^8 platelets was calculated from the intercept with the abscissa.

Assay for 5-HT-receptor binding. The experiments were carried out according to the method of Peters [16]. Rabbit PRP was prepared as described above, using 1% EDTA in 0.7% NaCl as anticoagulant. PRP was diluted by 50% with 0.1% EDTA in 0.9% NaCl (pH = 7.4) and stored on ice. Drugs and reagents all were diluted with 0.9% NaCl. Platelets (10^8) in 460 μl of PRP were added to siliconized culture tubes and preincubated with 0.9% NaCl, unlabelled 5-HT (10^{-9}–10^{-4} M) or CV-3988 (10^{-6} M) (20 μl) for 6 min in the ice cold water; [^{3}H]-5-HT (20 μl, 50,000 dpm, 1.5 nM) was then added to the tubes, which were incubated for 6 min. The stop of the binding reaction, separation of bound [^{3}H]-5-HT and measurement of radioactivity on the glass filter were done as the same same as the PAF-receptor binding.

Platelet aggregation and shape change study. Platelet aggregation and shape change were studied by the method of Born [17], using a 3-channel aggregometer (Rikadenki, Japan). PRP was prepared, as mentioned above, from the blood of rabbits, guinea-pigs and man. The concentration of platelets was adjusted to 450,000/μl with platelet poor plasma. Varying concentrations of CV-3988 (25 μl) were added to the platelet suspension (250 μl) 2 min after PAF (25 μl) was added. The extent of aggregation was expressed by the maximum change of light transmission expressed as a percentage, taking the difference between light transmission for PRP and PPP as 100%. Percent inhibition of aggregation with CV-3988 was calculated by dividing the percentage of aggregation by that observed in the control, and then multiplying by 100. The IC_{50} value of CV-3988 was calculated.

In the experiments of shape change platelets, 25 μl of CV-3988 (3×10^{-5} M) or ketanserin (3×10^{-5} M) was added to the suspension (250 μl) of rabbit platelets 2 min after 5-HT (25 μl; 3×10^{-6} M) was added.

Materials. PAF (1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine), lyso-PAF (1-*O*-hexadecyl-*sn*-glyceryl-3-phosphorylcholine), CV-3988 ((RS) - 2 - methoxy - 3 - (octadecyl - carbamoyloxy) propyl 2-(3-thiazolio)ethyl phosphate) were synthesized by Drs. H. Nomura and S. Tsushima in the Chemistry Laboratories, Takeda Chemical Industries. [^{3}H]-PAF (1-*O*-[^{3}H]-octadecyl) (90 Ci/mmole) and [^{3}H]-5-hydroxytryptamine creatinine sulfate (30 Ci/mmole) were purchased from Amersham (U.S.A) and New England Nuclear (U.S.A.), respectively. 5-Hydroxytryptamine creatinine sulfate was purchased from Sigma (U.S.A.). Ketanserin was synthesized in the Chemistry Laboratories, Takeda Chemical Industries. All reagents used were reagent grade. PAF and its analogues were dissolved in ethanol and stored at −20°. For use, the samples were dried with nitrogen gas and then dissolved in 0.9% saline containing 0.25% bovine serum albumin except for 5-HT-receptor binding.

RESULTS

Binding of [^{3}H]-PAF to the platelet

In a preliminary experiment, the binding of [^{3}H]-PAF to rabbit platelets was the lowest in the presence of 10^{-6} and 10^{-5} M of unlabelled PAF. Thus, the nonspecific binding was defined as the amount of binding that was not inhibited with 10^{-6} M unlabelled PAF. The specific binding (total binding minus nonspecific binding) was linear with the number of platelets over the range of 0.5×10^8 to 2×10^8 in 500 μl of incubation medium (data not shown). A 6-min incubation resulted in approximately steady-state binding (Fig. 1). Therefore, in the subsequent studies, we used 10^8 of platelets in 500 μl of incubation medium and an incubation time of 6 min.

Analysis of [^{3}H]-PAF binding

The Scatchard plot analysis of the binding of [^{3}H]-PAF to rabbit platelets indicated that the rabbit platelet possessed a binding site with the K_d 2.2 ± 0.2 nM and B_{max} 93.7 ± 8.3 fmoles/10^8 platelets, which corresponded to 568 ± 50 binding sites/platelet (mean ± S.E.M., N = 10).

Displacement of [^{3}H]-PAF binding with unlabelled PAF, CV-3988 and lyso-PAF

Unlabelled PAF (10^{-11}–10^{-7} M) and CV-3988 (10^{-8}–10^{-6} M) dose-dependently inhibited the specific binding of [^{3}H]-PAF to the platelet with the IC_{50} values of 1.2×10^{-9} and 8.0×10^{-8} M, respectively (Fig. 2). The nonspecific binding was barely inhibited even by the highest dose of CV-3988 (10^{-6} M) (inhibition was −1 ± 5%). The Scatchard plot analysis in the presence of CV-3988 (10^{-7} M) demonstrated that CV-3988 reduced the binding

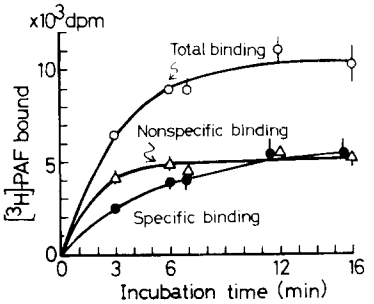

Fig. 1. Time course of total, nonspecific and specific binding of [^{3}H]-PAF to washed rabbit platelets. The platelet was incubated with [^{3}H]-PAF (0.6 nM, 60,000 dpm) for 3 to 16 min at 23°. Each point represents the mean of 2 experiments or the mean ± S.E.M. of 3 to 4 experiments.

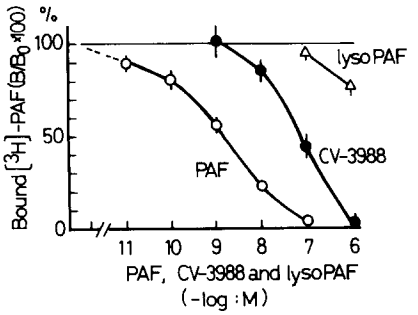

Fig. 2. Inhibition of the specific binding of [^{3}H]-PAF to washed rabbit platelets with unlabelled PAF, CV-3988 and lyso-PAF. The platelet was preincubated with unlabelled PAF, CV-3988 or lyso-PAF for 6 min at 23°, and then incubated with [^{3}H]-PAF (0.6 nM, 60,000 dpm) for 6 min at 23°. Each point represents the mean ± S.E.M. of 5 experiments. IC_{50}: unlabelled PAF, 1.2×10^{-9} M and CV-3988, 8.0×10^{-8} M.

capacity from B_{max} 93.7 ± 8.3 to 54.3 ± 16.3 fmoles/10^8 platelets (N = 5) and slightly increased the K_d value from 2.2 ± 0.2 to 2.5 ± 0.8 nM (N = 5). The K_i value of CV-3988 for the specific binding of [^{3}H]-PAF was 1.2×10^{-7} M. CV-3988 inhibited the specific binding of [^{3}H]-PAF competitively in lower concentration ($<10^{-7}$ M), but noncompetitively in higher concentrations ($>10^{-7}$ M). The inhibitory effect of lyso-PAF on the specific binding of [^{3}H]-PAF was very small; % inhibition was less than 20% at the highest concentration of 10^{-6} M.

Reversibility of [^{3}H]-PAF binding

As shown in Fig. 3, the specific binding of [^{3}H]-PAF to rabbit platelets was reversible. Unlabelled PAF (10^{-7} M) and CV-3988 (10^{-6} M) reversed the [^{3}H]-PAF binding in a time-dependent manner. Ten minutes after these drugs were added, the specific binding of [^{3}H]-PAF was reduced to 2% (unlabelled PAF) and 14% (CV-3988) of the control value. Only slight (about 7%) dissociation of the nonspecific binding of [^{3}H]-PAF was observed when the same concentration of CV-3988 was added.

Species differences in [^{3}H]-PAF binding and PAF-induced platelet aggregation and their inhibition with CV-3988

Table 1 shows the binding of [^{3}H]-PAF to the platelet of various species and its inhibition with CV-3988. No specific binding of [^{3}H]-PAF to the platelets of rats and mice was observed. In the other three species the specific [^{3}H]-PAF binding was very similar and in the order, guinea-pig, rabbit and man. CV-3988 (10^{-7} and 10^{-6} M) inhibited [^{3}H]-PAF binding to these platelets with the IC_{50} values of 1.6×10^{-7}, 7.9×10^{-8} and 1.8×10^{-7} M for man, rabbit and guinea-pig, respectively. Again, the inhibitory effects of CV-3988 on the nonspecific binding in these platelets were negligible in a concentration of 10^{-6} M (data not shown).

Table 1 also shows the PAF-induced platelet aggregation in three species and its inhibition with CV-3988. The concentrations of PAF and % aggregation for human, rabbit and guinea-pig platelets were as follows: 10^{-7} to 10^{-6} and 79 ± 2, 3×10^{-8} and 68 ± 2, and 3×10^{-9} M and 81 ± 2%, respectively. The inhibitory potency (IC_{50}: M) of CV-3988 on the PAF-induced aggregation was 1.4×10^{-6}, 7.8×10^{-6} and 1.6×10^{-5} M for guinea-pig, rabbit and human platelets, respectively.

Effects of CV-3988 on the binding of [^{3}H]-5-HT to rabbit platelets

The binding of [^{3}H]-5-HT to rabbit platelets was lowest in the presence of 10^{-5} and 10^{-4} M of unlabelled 5-HT. Thus, nonspecific binding was defined as the amount of the binding that was not inhibited with 10^{-5} M of unlabelled 5-HT. Specific and nonspecific binding reach plateau after 6 min incubation. Amounts of specific binding and nonspecific binding of [^{3}H]-5-HT to rabbit platelets during 6 min incubation were 31.1 ± 9.2 and 1.5 ± 1.0 fmole/10^8 platelets, respectively. Unlabelled 5-HT dose-dependently inhibited the specific binding of [^{3}H]-5-HT to the platelet with the IC_{50} value of 1.1×10^8 M (N = 4). The specific binding of [^{3}H]-5-HT was hardly inhibited even by the highest dose of CV-3988 (10^{-6} M); percent inhibition was 1 ± 4% (N = 4).

Effects of CV-3988 on the platelet activation induced by 5-HT

By the addition of 5-HT (3×10^{-6} M), the light absorbance of each PRP sample (N = 3) increased consequent to platelet shape change. A specific antagonist of 5-HT_2 receptor, ketanserin

Table 1. Binding of [^{3}H]-PAF to the platelet, PAF-induced platelet aggregation and their inhibition with CV-3988

	Binding of [^{3}H]-PAF (N = 4)		Platelet aggregation (N = 4)	
Species	Specific binding (fmole/10^8 platelets)	Inhibition of specific binding with CV-3988 (IC_{50}: M)	Concentration of PAF (M)*	Inhibition of aggregation with CV-3988 (IC_{50}: M)
Human	24.6 ± 1.2	1.6×10^{-7}	$10^{-7} - 10^{-6}$	1.6×10^{-5}
Rabbit	28.3 ± 1.4	7.9×10^{-8}	3×10^{-8}	7.8×10^{-6}
Guinea-pig	32.5 ± 1.5	1.8×10^{-7}	3×10^{-9}	1.4×10^{-6}
Rat	−0.6 ± 0.6	—	—	—
Mouse	0.3 ± 0.4	—	—	—

* The extents of platelet aggregation induced by the concentrations of PAF used were 79 ± 2% for human, 68 ± 2% for rabbit and 81 ± 2% for guinea-pig.

(3×10^{-6} M) completely inhibited the shape change, but CV-3988 (3×10^{-5} M) did not (data not shown).

DISCUSSION

Rabbit platelets had a binding site for PAF: the K_d value and the number of receptor/platelet were 2.2 ± 0.2 nM and 568 ± 50, respectively. Despite differences in experimental conditions, these values resemble those reported by Hwang *et al.* [11], who used rabbit platelet plasma membrane: a K_d value of 1.36 nM and 150–300 receptors/platelets. The K_d value of a binding site in the present experiments with rabbit platelets was approximately one-tenth that with human platelets [12]. These results may explain why the sensitivity of human platelets to PAF is lower than that of rabbit platelets [18].

CV-3988 (10^{-8}–10^{-6} M) inhibited the specific binding of [^{3}H]-PAF to rabbit platelets with an IC_{50} value for binding of 7.9×10^{-8} M and a K_i value of 1.2×10^{-7} M. Lyso-PAF (10^{-6} M) slightly inhibited [^{3}H]-PAF binding. In the presence of CV-3988 (10^{-7} M), the B_{max} was decreased and the K_d value was slightly increased, suggesting that CV-3988 (10^{-7} M) may noncompetitively inhibit the PAF-binding to rabbit platelets. The specific binding of [^{3}H]-PAF to the platelet was reversible (Fig. 3). The reason why CV-3988 did not act as a true competitive antagonist at PAF receptor remains to be clarified. Lipophilic action of CV-3988 might contribute to the noncompetitive nature of the inhibitory action of CV-3988 on the [^{3}H]-PAF binding. However, the inhibitory effects of CV-3988 on the [^{3}H]-PAF binding is not due to the nonspecific lipophilic action of CV-3988, since even the highest dose of CV-3988 (10^{-6} M) did not inhibit the specific binding of [^{3}H]-5-HT and the nonspecific binding of [^{3}H]-PAF to the rabbit platelet. In addition, CV-3988 at a dose of 3×10^{-5} M, which completely inhibits PAF-induced platelet aggregation [13], had no effects on the platelet shape change induced by 5-HT. Ketanserin abolished this shape change. Furthermore, as previously reported [13], the inhibitory action of CV-3988 on PAF-induced platelet aggregation and hypotension was specific; CV-3988 had no inhibitory effects on the platelet aggregation induced by ADP, arachidonic acid, collagen and A-23187, and on the hypotension induced by arachidonic acid, acetylcholine, bradykinin, isoproterenol and histamine. Masugi *et al.* [19] confirmed our findings on the hypotension and also reported that CV-3988 did not inhibit the hypotension induced by protaglanding E_2 and prostacyclin. CV-3988 also inhibited both the specific binding of PAF to human and guinea-pig platelets and the PAF-induced platelet aggregation. These findings suggest that CV-3988 may exhibit the PAF antagonistic action by inhibiting the binding of PAF to platelet PAF receptor in these three species.

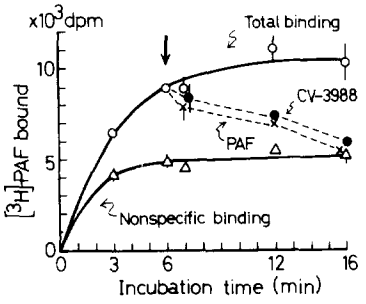

Fig. 3. Reversibility of the specific binding of [^{3}H]-PAF to washed rabbit platelets. The platelet was incubated with [^{3}H]-PAF (0.6 nM, 60,000 dpm) for 6 min at 23°, and then unlabelled PAF (10^{-7} M) or CV-3988 (10^{-6} M) was added to the incubation medium (at the point of the arrow). Each point represents the mean of 2 experiments or the mean ± S.E.M. of 3 to 4 experiments.

Species differences in the response of the platelet to PAF have been reported [18, 20, 21]. The K_d value of the PAF binding in guinea-pig and human platelets was not determined in the present study, although the amount of PAF binding to the platelet was in the decreasing order, guinea-pig, rabbit, and man. Therefore, we could not refer the sensitivity to PAF of the platelet of these three species. Whether PAF could be metabolized during the incubation was not examined, but PAF is reported not to be metabolized in washed platelets of rats and mice [22] and in human platelets [12] which show the lowest PAF binding capacity when compared to guinea-pig and rabbit platelets. [^{3}H]-PAF can bind to leukocytes [23], but, no leukocytes were detected (less than 100 cells/μl) in the platelet suspension prepared from all the species used in the present experiments. There were 10–100 times difference between the IC_{50} of CV-3988 for the PAF binding to the platelet and that for platelet aggregation (Table 1). These differences might depend upon the concentration of PAF used: in the binding experiment, [^{3}H]-PAF was 0.6×10^{-9} M, whereas in the platelet aggregation studies, PAF was 10^{-7}–10^{-6} M for man, 3×10^{-8} M for rabbit and 3×10^{-9} M for guinea pig.

The platelet of rats and mice had no capacity for specific binding to PAF (Table 1), suggesting that their platelets lack PAF receptor. The finding could explain the insensitivity of their platelets to PAF in inducing aggregation [13, 20, 21, 24].

A stereospecific inhibition with unlabeled PAF on the binding of [^{3}H]-PAF to the platelet has been demonstrated by Wykle *et al.* [6]. In the present study, the inhibitory effects of optical isomers of CV-3988 on PAF binding and PAF-induced platelet aggregation were not examined, since the optical isomers of CV-3988 were not available.

In summary, CV-3988 is a specific PAF antagonist that inhibits the specific binding of PAF to the PAF receptor of human, rabbit and guinea-pig platelets. The platelet of rats and mice appears to be devoid of the PAF receptor.

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