Characterization of the Activity of a Platelet Activating Factor Antagonist, CV-3988

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Abstract—CV-3988 inhibited the vascular permeability increase induced by C16-PAF and C18-PAF in rat skin in a dose-dependent manner. The inhibition was shown to be specific and competitive with PAF on its receptor by the following observations: 1) Parallel shift of the dose-response curve; 2) Crossing of double reciprocal plots on the intersection of the ordinate; and 3) No inhibition on other autacoids such as bradykinin, histamine, 5-hydroxytryptamine and LTC4. PAF-induced blood pressure fall in rats was also suppressed by pretreatment with CV-3988 selectively.

Platelet-activating factor (Acetyl glyceryl ether phosphorylcholine, AGEPC) has been considered as a possible mediator of various biological phenomena since it has been shown to have various and potent biological activities in small doses (1). As we have been working on the mediators of inflammatory reaction (2, 3), an antagonist of PAF, CV-3988 (rac-3-(N-n-octadecylcarbamoyloxy)2-methoxypropyl-2-thiazolioethyl phosphate) (4), if it is specific, might be a good tool for examining whether PAF may have a role in animal models of inflammation. CV-3988 was previously reported as a specific antagonist for the PAF receptor of rabbit platelets by using a receptor binding assay (5). However, in this report, we intend to examine if CV-3988 is also specific in an in vivo system. Therefore, before using CV-3988 for a "search-tool" of inflammatory mediators, it is necessary to characterize the specificity and selectivity of this antagonist in animal experiments in vivo.

Materials and Methods

PAF, 1-0-hexadecyl-2-0-acetyl-sn-glycerol-3-phosphocholine (C16-PAF), and 1-0-octadecyl-2-0-acetyl-sn-glycerol-3-phosphocholine (C18-PAF) (Bachem Finechemicals, Switzerland, via Funakoshi Pharmaceutical Co., Tokyo) and alprazolam (Japan Upjohn Limited) were kindly donated. CV-3988, rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethyl phosphate, was synthesized (6). Bradykinin (Peptide Institute, Minoh), histamine 2HCl (Wako Pure Chemicals, Tokyo) and 5-hydroxytryptamine creatinine sulfate (5-HT, Sigma, St. Louis) were purchased. The agents were freshly dissolved in Tyrode's solution containing 0.1% bovine serum albumin.

Measurement of vascular permeability increase: The precise method was previously reported (7). In short, male Sprague-Dawley rats, 7–8 weeks old (Shizuoka Laboratory Animal Center, Hamamatsu), and male albino rabbits weighing 2–3 kg (Doken, Ibaragi) were anesthetized with pentobarbital sodium (Tokyo Kasei; 50 mg/kg, intraperitoneally). Pontamine sky blue (Tokyo Kasei, 60 mg/kg in saline) was injected intravenously, and 10 min later, the test agents were injected into the shaved dorsal skin (0.1 ml/site). Animals were exsanguinated from the carotid artery at 40 min after the intradermal injections, and the skin at the injected...
site (about a 1.5 cm disc) was extracted as described previously (7). Dye amount in each site was calculated from the optical density of the extract of each site by subtraction of the value of the skin injected with Tyrode only.

**Blood pressure measurement:** Rats were anesthetized with pentobarbital sodium, 30 mg/kg, and a polyethylene cannula was inserted into the left femoral artery for the measurement of systemic blood pressure via a polygraph (RMP 6008, Nihon Kohden, Tokyo), and a cannula was inserted into the right femoral vein for drug injection. Heart rate was recorded through a tachograph (AT 600G, Nihon Kohden) via an electrocardiograph (AC 601G, Nihon Kohden).

**Results**

The relative potencies of PAF-induced vascular permeability increase was compared in rat and rabbit skin as assessed by the amount of the dye extracted from the injected sites of the skin. As illustrated in Fig. 1, C16- and C18-PAF showed a dose-dependent effect in the dose range of 5-500 pmol/site in rats. Stronger activity was observed with C16-PAF than C18-PAF at the dose of approximately 200 pmol. In rabbits, both C16- and C18-PAF showed only slight responses as shown in Fig. 1. The average concentration of dye in the serum of the injected animal 40 min later was about 120 and 140 μg/ml in rats and rabbits, respectively. The amount of dye extracted from the site of skin injected with vehicle alone was approximately 15 μg/site, in both rats and rabbits. Thus the value for each agonist was corrected by subtraction of the value of vehicle only.

The inhibitory effect of CV-3988 on PAF-induced vascular permeability increase was examined when C16-PAF, 40 pmol/site, and C18-PAF, 150 pmol/site, were injected into rats. The simultaneous injections of CV-3988 at the site inhibited dose-dependently the activities of both PAFs. As shown in Fig. 2A, CV-3988 itself showed a slight agonistic activity of increasing vascular permeability at the dose of 77 nmol/site. However, CV-3988 suppressed PAF-induced action at doses less than those that induced agonistic action as shown in Fig. 2A and 2B. In comparison with the inhibitory activity of CV-3988 on PAF actions, the inhibition on C18-PAF was more prominent than that on C16-PAF (Fig. 2A and 2B).

The effect of CV-3988 was further tested by measuring the dye concentration in the serum of rats and rabbits. Fig. 1 shows the dose-response curves of the vascular permeability increase induced by PAF in rat and rabbit skins. Indicated concentrations of C16-PAF (Δ) and C18-PAF (○) in 0.1 ml Tyrode’s solution were injected into the dorsal skin of rats (n=6) (—) and rabbits (n=4) (——). The dye in each injected site was extracted as described in the text and expressed as the mean with standard error.

![Fig. 1. Dose-response curves of the vascular permeability increase induced by PAF in rat and rabbit skins.](image_url)
Fig. 2. Dose-related inhibition curves of CV-3988 on the C_{16}\textsuperscript{-} and C_{18}\textsuperscript{-}PAF induced vascular permeability increase in rat skin. Indicated varied doses of CV-3988 and a fixed dose of C_{16}\textsuperscript{-}PAF (40 pmol) (A) or C_{18}\textsuperscript{-}PAF (150 pmol) (B) were simultaneously injected into the dorsal skin of anesthetized rats. Precise experimental conditions are described in the text. The values represent the means with standard errors as vertical bars. Figures indicate the numbers of rats used. (A) ■ CV-3988 alone (0.46–77 nmol/site), △ C_{16}\textsuperscript{-}PAF (40 pmol/site), ● C_{16}\textsuperscript{-}PAF+CV-3988 (0.46–46 nmol/site). (B) ○ C_{18}\textsuperscript{-}PAF (150 nmol/site). ● C_{18}\textsuperscript{-}PAF+CV-3988 (0.46–46 nmol/site).

examined in dose-response curves of C_{16}\textsuperscript{-} and C_{18}\textsuperscript{-}PAF. The dose-response curves of vascular permeability increase induced by C_{16}\textsuperscript{-} and C_{18}\textsuperscript{-}PAF shifted to the right by 3.6- and 4.5-fold, respectively, when 15.4 nmol of CV-3988 was simultaneously injected at each site. These results are plotted in Fig. 3A and 3B in a double reciprocal manner. Lines of the data in the presence of CV-3988 intersect the ordinates at the points which intersect the lines of the data obtained by PAF alone. These results indicate that the inhibition of CV-3988 on the vascular permeability increase induced by C_{16}\textsuperscript{-} and C_{18}\textsuperscript{-}PAF is competitive.

The selectivity of the antagonist activity of CV-3988 on PAF was examined in a series of experiments on the vascular permeability increase induced by bradykinin, histamine and 5-HT. As shown in Fig. 4, bradykinin (0.3–3 nmol/site), histamine (9–90 nmol/site) and 5-HT (0.46–4.6 nmol/site) expressed the dose-dependent increase in vascular permeability in rat skin. Simultaneous injection of CV-3988, at the dose of 15.4 nmol/site, which clearly inhibited the effect of PAF as demonstrated above, had no significant effect on these dose-response curves.

Furthermore, the intravenous injection of CV-3988 at 10 mg/kg was also effective on the PAF-induced vascular permeability increase, and the inhibitions were 60 and 75% on the activities of C_{16}\textsuperscript{-} and C_{18}\textsuperscript{-}PAF, respectively (Fig 5). A shift of the dose-response curve similar to that in the simultaneous case was also observed (data not shown). As shown in Fig. 5, both treatments, local and intravenous injection of CV-3988, showed no significant effect on the bradykinin, histamine, 5-HT and leukotriene C_{4}\textsuperscript{-} induced vascular permeability increase.

Alprazolam in a dose of 10 mg/kg, i.v., and 32 nmol or 97 nmol/site did not show any significant effect on the activities of C_{16}\textsuperscript{-}PAF, C_{18}\textsuperscript{-}PAF, histamine and bradykinin (Fig. 6). Alprazolam itself did not show any significant increase in vascular permeability.

Figure 7 shows the effect of CV-3988 on the hypotension induced by PAF in rats. In
Fig. 3. Double reciprocal plots of PAF induced vascular permeability increase in the presence and absence of CV-3988. CV-3988 (15.4 nmol/site) was injected simultaneously with varied doses of C_{16}-PAF (A) or those of C_{18}-PAF (B). The values indicate the means with standard errors (n=5). (A) △ C_{16}-PAF alone (50-500 pmol/site). ▲ C_{16}-PAF+CV-3988. (B) ○ C_{18}-PAF alone (50-500 pmol/site). ● C_{18}-PAF+CV-3988.

Fig. 4. Effect of CV-3988 on the dose-response curves of vascular permeability increase induced by bradykinin, histamine and 5-hydroxytryptamine. CV-3988 (15.4 nmol/site) was injected simultaneously with each dose of the agonists into the dorsal skin of rats. Experimental conditions are the same as those described in previous figures, and details are in the text. ◯ bradykinin (0.3-3 nmol/site), ■ bradykinin+ CV-3988, ○ 5-hydroxytryptamine (0.46-4.6 nmol/site), ● 5-hydroxytryptamine+CV-3988, △ histamine (9-90 nmol/site), ▲ histamine+CV-3988.

Discussion

As previously reported (7, 8), a mixture of PAFs (containing 90% C_{18}-PAF) caused a marked vascular permeability increase in rats and guinea pigs, but had only a slight activity in rabbits. In Fig. 1, both PAFs, C_{16}- and C_{18}-PAF, caused a potent vascular permeability increase in rat skin, but had only a slight effect on rabbit skin. Recent reports have described the different potency of C_{16}-PAF and C_{18}-PAF on the responses of rabbit platelet serotonin secretion (9), vasoconstriction in rabbits (10), human neutrophil activation (11) and guinea pig vascular permeability increase (12). On our experiment, C_{16}-PAF is relatively more potent than C_{18}-PAF at the dose range of 50-200 pmol/site in causing vascular permeability increase.
increase of rat skin. In the hypotensive effect of PAF on the rat systemic blood pressure, C16-PAF was also more potent than C18-PAF as shown in Fig. 7. These results suggest

Fig. 5. Effect of the route of administration of CV-3988 on the vascular permeability increase induced by several autacoids in rat skin. C16-PAF (57 pmol/site), C18-PAF (54 pmol/site), bradykinin (3 nmol/site), histamine (27 nmol/site), 5-hydroxytryptamine (5-HT, 1.4 nmol/site) and LTC4 (1.4 nmol/site) were injected with (□) or without (●) simultaneous injection of CV-3988, 15.4 nmol/site, or with the pretreatment of CV-3988, 10 mg/kg, intravenously (M), 20 min before the intradermal injection. Columns express the means of the indicated number of rats with standard errors as vertical bars.

Fig. 6. Effect of alprazolam on the vascular permeability increase induced by C16-PAF, C18-PAF, histamine and bradykinin in rat skin. C16-PAF (54 nmol/site), C18-PAF (19 pmol/site), histamine (90 nmol/site) and bradykinin (3 nmol/site) were injected with or without simultaneous injection of alprazolam, 32 or 97 nmol/site into rat skin, or with pretreatment with intravenous injection of alprazolam, 10 mg/kg, 20 min before. Alprazolam, 32 or 97 nmol/site, alone was also examined. Columns indicate means of the data from the indicated number of rats with standard errors as vertical bars. □ control; □ pretreatment with alprazolam, 10 mg/kg, i.v.; □ simultaneous alprazolam, 32 nmol/site; □ simultaneous alprazolam, 97 nmol/site.
that the receptor site in the vasculature could have different sensitivity to PAF-species or the degradation of each PAF in the blood might be different. For the investigation of this problem, a further extensive study is necessary.

The hypotension induced by PAF in rats was so strong that the simultaneous intradermal multiple injections of PAF in the skin might cause hypotension. Therefore we should be cautious to that the vascular permeability effect of PAF should be examined at the condition of total additive dose of PAF per rat to be less than 1 μg.

The hypotensive effect of PAF in rabbit was 100–1000 times less effective than that in rats. The result is controversial of the fact that rabbit platelets aggregate but rat platelets do not in the presence of PAF (5). The similar species difference of the reactivity of vascular permeability increase was reported (13) in a case of leukotrienes (LTs), in that rabbit skin showed low reactivity to LTC₄ and LTD₄. The reason is not known.

The antagonistic effect of CV-3988 appears to be specific to activities of PAF in rat skin and blood pressure, since CV-3988 in the same dose did not show any effect on the activities of bradykinin, histamine, 5-HT and LTC₄. Furthermore, the effect of CV-3988 on the rat skin vascular permeability increase was shown as competitive, as shown in Fig. 3, where double reciprocal plots cross exactly the same point at the ordinate. The values of the cross points in the ordinates were approximately 0.24 in both C₁₆- and C₁₈-PAF. By the intersections of the abscissa the shift ratios by the antagonist (15.4 nmol/site) are calculated as 3.44 and 4.25 for C₁₆- and C₁₈-PAF, respectively. The data indicates that the antagonistic action of CV-3988 is relatively stronger on C₁₈-PAF in the rat skin model. The above result indicates that CV-3988 is a specific antagonist to PAF in an in vivo system as well as an in vitro system (5).

Alprazolam, a benzodiazepine derivative and used for the treatment of psychic disorders, was reported to inhibit platelet-aggregation induced by PAF (14). However, on the PAF-induced vascular permeability increase in rat skin, the compound did not show inhibition when administered intravenously or when simultaneously injected with PAF, as shown in Fig. 6. Since alprazolam, 10 mg/kg by intravenous in-
jection, showed a slight sedative effect on rats, we did not further increase the dose. Recently, many PAF-antagonists have been reported (15–17). However, there are not many highly specific antagonists and little extensive work has been done to characterize the antagonistic activity. For instance kadsurenone, a compound extracted from a Chinese plant, was reported as a PAF-antagonist, but it is also reported that it partly suppresses the vascular permeability increase induced by bradykinin and histamine in rat skin (18). The fact indicates that kadsurenone may be relatively less specific than CV-3988.

Therefore our results clearly indicate that CV-3988 is a specific, competitive and selective antagonist to PAF in these animal models, in vivo systems, and thus is useful for predicting the involvement of PAF.

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