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Platelet-Activating Factor–Induced Aggregation of Human Platelets Specifically Inhibited by Triazolobenzodiazepines

Abstract. *Platelet-activating factor (PAF), a naturally occurring phospholipid, is a potent activator of various biological processes, including platelet aggregation. The mechanisms by which PAF acts are largely unknown, partly because of the lack of specific inhibitors for PAF-elicited responses. It was found that in washed human platelets the psychotropic triazolobenzodiazepine drugs alprazolam and triazolam potently inhibited PAF-induced changes in shape, aggregation, and secretion. The effects were specific for PAF activation, since the responses of human platelets to adenosine diphosphate, thrombin, epinephrine, collagen, arachidonate, and the calcium ionophore A23187 were not inhibited by the triazolobenzodiazepines. These psychotropic drugs should be useful in investigating the possibility that PAF or PAF-like phospholipids play a role in neuronal function and in elucidating biochemical mechanisms activated specifically by PAF in a variety of cells.*

Platelet-activating factor (PAF) is a naturally occurring phospholipid (1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) that is released into the blood in vivo during immunoglobulin E–induced anaphylaxis (*1*). It is released from basophils in vitro on immunological challenge (*1*) and from platelets (*2*), neutrophils, and macrophages (*3*) in response to specific stimuli. This phospholipid is a potent mediator of inflammation (*1*), has antihypertensive activity (*4*), induces bronchoconstriction and contraction of smooth muscle (*1, 5*), and is one of the most powerful platelet activators known, inducing platelet shape change, aggregation, and secretion (*6*). The specific mechanisms underlying the responses of cells to PAF are unknown, and specific antagonists of PAF's action have not been identified.

Benzodiazepines are used clinically as anxiolytic and hypnotic agents. A derivative with a triazole ring (triazolobenzodiazepine), alprazolam, is particularly useful in the treatment of panic disorder with or without agoraphobia (*7*). A second triazole derivative, triazolam, is used as an effective, short-acting sleep medication (*8*). Benzodiazepine receptors are present primarily in the brain (*9*), but have also been found in platelets (*10*). We examined the effects of several benzodiazepines on human platelets activated by various agonists. We report that alprazolam and triazolam are potent and specific inhibitors of PAF-induced activation of human platelets.

The effects of alprazolam on PAF—induced aggregation of platelets and shape change (*11*) in human platelet-rich plasma are shown in Fig. 1A. At a concentration of 10 μ*M*, alprazolam inhibited the second wave of aggregation that is associated with granular release. At 40 μ*M* alprazolam completely inhibited PAF-induced shape change as well as the primary wave of aggregation (Fig. 1A). The concentration of alprazolam producing a 50 percent decrease in the initial velocity of PAF–induced aggregation in platelet-rich plasma was about 5 μ*M* (Fig. 1B). Triazolam was even more potent, showing a median inhibitory concentration (IC_{50}) of less than 2 μ*M* (Fig. 1B). Platelets from different individuals varied in their sensitivity to PAF. Accordingly, the IC_{50} values for alprazolam and triazolam inhibition also varied, ranging from 2 to 12 μ*M* and 1 to 7 μ*M*, respectively. A benzodiazepine without the triazole ring, diazepam, did not inhibit PAF-induced platelet activation even at concentrations at which alprazolam and triazolam produced complete inhibition (Fig. 1B).

Subsequent experiments were performed to determine whether the effects

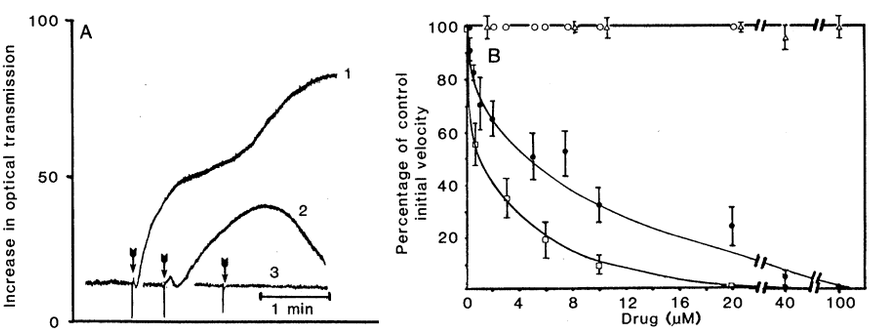

Fig. 1. (A) Inhibition of PAF-induced aggregation of human platelets in plasma by alprazolam. Blood was taken from normal individuals after obtaining their informed consent and the approval of the Institutional Human Experimentation Committee of the University of Vermont. The donors had fasted for at least 10 hours to prolong platelet responses to PAF (*12*) and claimed not to have taken any medication during the preceding 2 weeks. Blood was collected into 3.8 percent trisodium citrate (1:9), layered over a Ficoll Hypaque gradient, and centrifuged at 150*g* for 15 minutes at 22°C to obtain platelet-rich plasma. The platelet-rich plasma was removed and 0.45-ml portions (2×10^8 to 4×10^8 platelets per milliliter) were incubated in a Chronolog-Lumi aggregometer for 1 minute at 37°C with stirring under the following conditions: (trace 1) no drugs added [addition of ethanol at 0.034 and 0.12 percent (final concentrations when added with alprazolam) had no effect on this activity]; (trace 2) 10 μ*M* alprazolam; and (trace 3) 40 μ*M* alprazolam (both concentrations were final). Alprazolam (8-chloro-1-methyl-6-phenyl-4H-*s*-triazolo[4,3a][1,4]benzodiazepine) (provided by R. Purpura, Upjohn) was dissolved as a 1 m*M* stock solution in 3 percent ethanol. Platelet aggregation was initiated by adding PAF at a final concentration of 51 n*M* (arrows). PAF (L-α-lecithin,β-acetyl,γ-*O*-alkyl; lot 386021) was obtained from Calbiochem-Behring as a pure lyophilized preparation, with the length of the alkyl chain consisting mainly of C_{16} and C_{18}. A 10 m*M* stock solution dissolved in a solution of 0.35 percent bovine serum albumin (BSA) and 0.15*M* NaCl was stored frozen at −20°C. (B) Dose-response curves of inhibition of PAF-induced aggregation of platelets by alprazolam and triazolam. Platelet-rich plasma was stirred for 1 minute at 37°C with one of the following: (○) Tyrode's buffer or ethanol (controls); (●) alprazolam; (□) triazolam[8-chloro-6-(*o*-chlorophenyl)-1-methyl-4H-*s*-triazolo[4,3a][1,4]benzodiazepine]; or (△) diazepam. PAF (51 n*M*) was used to initiate platelet aggregation. Triazolam, prepared as a 1 m*M* stock solution in 30 percent ethanol, was provided by Upjohn. Diazepam, freshly prepared as a 1 m*M* stock solution in 3 percent ethanol, was provided by H. Sheppard and S. Spector (Hoffmann–La Roche). Each point is the mean ± standard deviation of the initial velocity of aggregation (*14*) for three separate experiments, each involving platelet-rich plasma from a different donor.

of alprazolam and triazolam on platelet function are mediated by a plasma factor or involve direct interaction with platelets. Washed platelets were obtained from platelet-rich plasma by gel filtration (*12*). As shown in Fig. 2A, the aggregation and shape change induced by 25 n*M* PAF were completely inhibited by 20 μ*M* alprazolam. We also found that secretion from platelet granules, monitored by measuring release of adenosine triphosphate from gel-filtered platelets with a chronolog-Lumi aggregometer, was completely inhibited by this concentration of alprazolam. Fifty percent inhibition of the initial velocity of PAF-induced aggregation occurred at an alprazolam concentration of 500 n*M*; the IC_{50} for inhibition of the initial velocity of PAF-induced shape change was approximately 3 μ*M* (Fig. 2B). The IC_{50} for inhibition by triazolam of the initial velocities of aggregation and shape change were 300 n*M* and 2 μ*M*, respectively. Diazepam, chlordiazepoxide, and the benzodiazepine Ro5-4864 had no significant effects on PAF–induced aggregation of gel-filtered platelets when tested at concentrations in which both alprazolam and triazolam produced complete inhibition.

Inhibition of PAF-induced platelet aggregation by alprazolam and triazolam was reversed by increasing the concentration of PAF (Fig. 2C). The concentration of PAF required to induce 50 percent of the maximum initial velocity of aggregation of the gel-filtered platelets used in this experiment was approximately 7 n*M*. This value shifted to 35, 100, and 400 n*M* in the presence of 10, 20, and 40 μ*M* alprazolam, respectively (Fig. 2C). Such parallel shifts of the dose-response curve to the right generally indicate competitive antagonism.

The inhibition by alprazolam and triazolam of platelet aggregation and shape change was specific for PAF. Alprazolam (40 μ*M*) had no significant inhibitory effects on gel-filtered platelets activated by adenosine diphosphate (ADP), thrombin, epinephrine, the Ca^{2+} ionophore A32187, collagen, or arachidonic acid, whereas complete inhibition of PAF-induced aggregation was exerted by alprazolam (Table 1). Similar results were obtained with triazolam. The same agonists were also tested in platelet-rich plasma. At concentrations of alprazolam and triazolam up to 200 μ*M*, only PAF-induced activation was inhibited.

Exposure of fibrinogen receptors and fibrinogen binding are necessary for a platelet aggregation response to physiological agonists (*13*). We found that binding of [^{125}I]fibrinogen to PAF–stimulated platelets (*14*) was inhibited by alprazo-

Table 1. Effect of alprazolam on the aggregation response of gel-filtered platelets to various agonists. Gel-filtered platelets (0.45 ml; 3.6×10^8 per milliliter) in Tyrode's buffer containing 0.35 percent BSA and 0.5 m*M* Ca^{2+} were incubated with 10 μl of alprazolam (final concentration, 40 μ*M*) for 1 minute at 37°C. Aggregation was initiated by adding 25 μl of each agonist at the final concentration shown. Fibrinogen (200 μg/ml, Kabi human) was added to samples tested with ADP, epinephrine, arachidonic acid, and PAF. Each value is the mean ± standard error for a representative experiment performed the number of times shown in parentheses. Similar results were seen in three separate experiments. ADP, epinephrine, collagen, and arachidonic acid were obtained from Sigma, α-thrombin from J. Fenton II, and A23187 from Eli Lilly; N.S., no significant difference.

Agonist	Initial velocity of aggregation (light transmission units per minute)		Inhibition (percent)
	Without alprazolam	With alprazolam	
ADP (5 μ*M*)	26.6 ± 1.6 (6)	27.3 ± 1.3 (6)	0
Thrombin (2 unit/ml)	26.7 ± 0.9 (5)	24.3 ± 1.2 (5)	9 (N.S.)
Epinephrine (10 μ*M*)	6.9 ± 0.3 (7)	6.8 ± 0.7 (7)	0
A23187 (10 μ*M*)	22.0 ± 2.5 (5)	23.0 ± 0.8 (5)	0
Collagen (200 μg/ml)	23.8 ± 2.7 (3)	23.0 ± 2.8 (3)	0
Arachidonic acid (170 μ*M*)	22.2 ± 1.5 (5)	23.5 ± 2.2 (5)	0
PAF (0.051 μ*M*)	35.3 ± 0.8 (4)	0.0 (4)	100

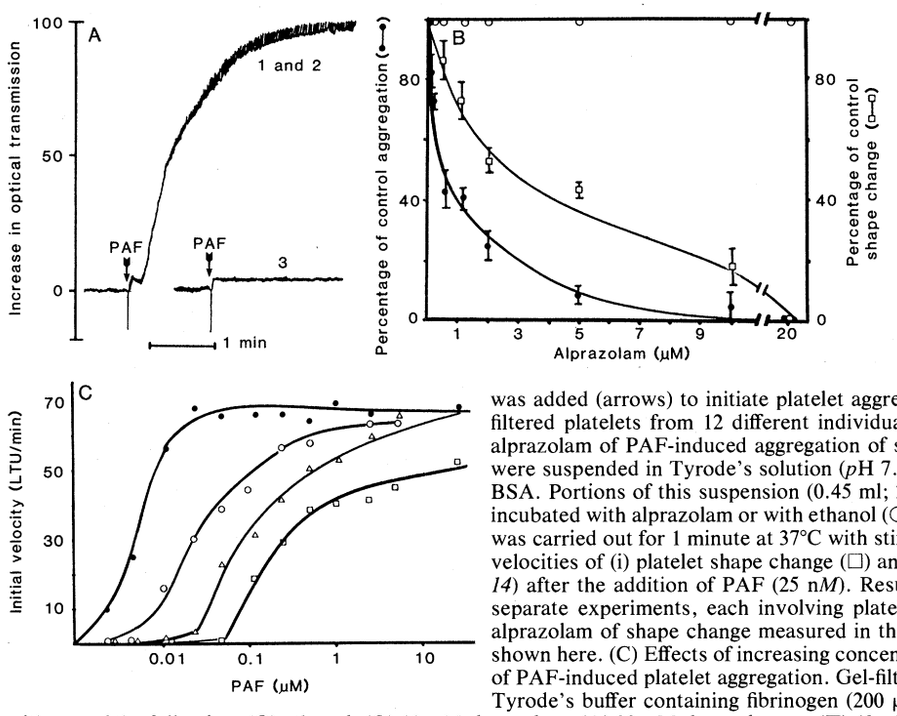

Fig. 2. (A) Inhibition by alprazolam of PAF—induced aggregation in gel-filtered platelets. Platelet-rich plasma was applied over a Sepharose 2B column equilibrated with Ca^{2+}-free Tyrode's solution (*p*H 7.35) containing 0.35 percent BSA and 5 m*M* glucose (*12*). The fraction containing gel-filtered platelets was immediately used for platelet function studies. Gel-filtered platelets (0.45 ml; 3.5×10^8 platelets per milliliter) were incubated for 1 minute at 37°C with stirring in the presence of fibrinogen (200 μg/ml), Ca^{2+} (0.5 m*M*), and one of the following: Tyrode's buffer (trace 1), ethanol (trace 2)—which gave a tracing that overlapped with trace 1—or alprazolam (final concentration, 20 μ*M*) (trace 3). PAF (25 n*M*) was added (arrows) to initiate platelet aggregation. Similar results were obtained with gel-filtered platelets from 12 different individuals. (B) Dose-response curve for inhibition by alprazolam of PAF-induced aggregation of shape change by gel-filtered platelets. Platelets were suspended in Tyrode's solution (*p*H 7.35) containing Ca^{2+} (0.5 m*M*) and 0.35 percent BSA. Portions of this suspension (0.45 ml; 2×10^8 to 4×10^8 platelets per milliliter) were incubated with alprazolam or with ethanol (○) at the final concentrations shown. Incubation was carried out for 1 minute at 37°C with stirring. Simultaneous measurements of the initial velocities of (i) platelet shape change (□) and (ii) aggregation (●) were then performed (*13, 14*) after the addition of PAF (25 n*M*). Results are means ± standard deviations for three separate experiments, each involving platelets from a different donor. The inhibition by alprazolam of shape change measured in the presence of 1 m*M* EGTA was similar to that shown here. (C) Effects of increasing concentrations of PAF on the inhibition by alprazolam of PAF-induced platelet aggregation. Gel-filtered platelets (0.45 ml; 4×10^8 per milliliter) in Tyrode's buffer containing fibrinogen (200 μg/ml) and Ca^{2+} (0.5 m*M*) were stirred at 37°C with one of the following: (●) ethanol, (○) 10 μ*M* alprazolam, (△) 20 μ*M* alprazolam, or (□) 40 μ*M* alprazolam. Platelet aggregation was initiated by adding PAF at the final concentrations shown. Similar results were obtained in two separate experiments.

lam (200 ng of labeled fibrinogen was bound per 10^8 platelets in the absence of alprazolam compared to 39 ng in the presence of 20 μM alprazolam). This inhibition was probably not due to direct blockade of fibrinogen-binding sites, since fibrinogen-dependent aggregation of platelets to agonists other than PAF (Table 1) was not blocked by alprazolam or by triazolam.

Platelet shape change and secretion induced by PAF have been associated with increased breakdown of phosphatidyl inositol, formation of phosphatidic acid, and phosphorylation of endogenous platelet proteins (*15*). However, these events are also activated by other agonists. Investigation of the process by which alprazolam and triazolam selectively inhibit PAF-mediated activation of platelets may shed light on biochemical mechanisms specific for the action of PAF and on the mechanism by which these triazolobenzodiazepines act.

Our results suggest that treatment of patients with alprazolam may reduce PAF-mediated platelet activation in vivo. Indeed, alprazolam treatment of patients with severe agoraphobia and spontaneous panic attacks was reported to reduce the patient's raised plasma concentrations of platelet factor 4 and β-thromboglobulin (*7*)—commonly used measures of increased platelet activation in vivo. The specific effects of psychotropic drugs on PAF-mediated responses also suggest involvement of PAF in the regulation of neuronal function. Consistent with this suggestion, we have observed that treatment of neuroblastoma × glioma hybrid cells [clone NG108-15 (*16*); cells grown in a serum-free medium] for 4 to 6 days with 0.1 to 1 μM PAF resulted in morphological differentiation (neurite extension). Brief exposure of NG108-15 cells to PAF at these concentrations produced changes in the phosphorylation of specific proteins (*17*). PAF-acetylhydrolase (*18*), protein kinase C [an enzyme implicated in PAF's mechanism of action in platelets (*15*)], and a benzodiazepine-sensitive calmodulin-dependent protein kinase (*19*) were all found to be present in high concentrations in brain tissue. Together with the results reported here, these findings suggest that investigators should attempt to ascertain whether some effects of triazolobenzodiazepine treatment of certain neuropsychiatric disorders (*7, 8*) involve drug interaction with processes mediated by PAF or PAF-like phospholipids in the central nervous system.

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References and Notes

1. B. B. Vargaftig, M. Chignard, J. Benveniste, J. Lefort, F. Wal, *Ann. N.Y. Acad. Sci.* **370**, 119 (1981); R. N. Pinckard, R. S. Farr, D. J. Hanahan, *J. Immunol.* **123**, 1847 (1979).
2. A. Marcus *et al.*, *Blood* **58**, 1027 (1981); I. Alam, J. B. Smith, M. J. Silver, *Thromb. Res.* **30**, 71 (1983); M. Chignard, J. P. LeCouedic, M. Tence, B. B. Vargaftig, J. Benveniste, *Nature (London)* **279**, 799 (1979).
3. J. M. Lynch, G. Z. Lotner, S. J. Betz, P. M. Henson, *J. Immunol.* **123**, 1219 (1979); J. M. Mencia-Huerta and J. Benveniste, *Eur. J. Immunol.* **9**, 409 (1979).
4. M. L. Blank, F. Snyder, L. W. Byers, B. Brooks, E. E. Muirhead, *Biochem. Biophys. Res. Commun.* **90**, 1194 (1979).
5. S. R. Findlay, L. M. Lichtenstein, D. J. Hanahan, R. N. Pinckard, *Am. J. Physiol.* **241**, C130 (1981)
6. L. M. McManus, D. J. Hanahan, R. N. Pinckard, *J. Clin. Invest.* **67**, 903 (1981); C. M. Chesney, D. D. Pifer, L. W. Byers, E. E. Muirhead, *Blood* **59**, 583 (1982).
7. J. B. Cohn, *J. Clin. Psychiatry* **42**, 347 (1981); D. V. Sheehan *et al.*, *J. Clin. Psychopharmacol.* **4**, 66 (1984).
8. G. E. Pakes, R. N. Brogden, R. C. Heel, T. M. Speight, G. S. Avery, *Drugs* **22**, 81 (1981).
9. R. F. Squires and C. Braestrup, *Nature (London)* **266**, 732 (1977); H. Möhler and T. Okada, *Science* **198**, 849 (1977); J. F. Tallman, S. M. Paul, P. Skolnick, D. W. Gallager, *ibid.* **207**, 274 (1980).
10. J. K. T. Wang, T. Taniguchi, S. Spector, *Life Sci.* **27**, 1881 (1980).
11. Change in platelet shape is shown in Fig. 1 as the decrease in optical transmission after addition of PAF. Platelet aggregation is shown as an increase in optical transmission. The initial velocity of aggregation was measured from the initial slope of the aggregation tracing from the primary wave in light transmission units per minute (*14*).
12. E. Kloprogge, G. H. Haas, G. Gorter, J. W. N. Akkerman, *Thromb. Res.* **29**, 595 (1983).
13. G. A. Marguerie, E. F. Plow, T. S. Edgington, *J. Biol. Chem.* **254**, 5357 (1979); E. I. Peerschke, M. B. Zucker, R. A. Grant, J. J. Egan, M. M. Johnson, *Blood* **55**, 841 (1980); E. Kornecki, S. Niewiarowski, T. A. Morinelli, M. Kloczewiak, *J. Biol. Chem.* **256**, 5696 (1981).
14. Fibrinogen binding was performed as described by E. Kornecki, G. P. Tuszynski, and G. P. Niewiarowski [*J. Biol. Chem.* **258**, 9349 (1983)] using gel-filtered platelets in Tyrode's buffer containing 0.5 mM Ca^{2+}.
15. E. G. Lapetina, *ibid.* **257**, 7314 (1982); H. Ieyasu, Y. Takai, K. Kaibuchi, M. Sawamura, Y. Nishizuka, *Biochem. Biophys. Res. Commun.* **108**, 1701 (1982).
16. M. Nirenberg *et al.*, *Science* **222**, 794 (1983).
17. T. Davis, E. Kornecki, R. Lenox, Y. H. Ehrlich, *Soc. Neurosci. Abstr.* **10**, 196 (1984).
18. M. L. Blank, T. Lee, V. Fitzgerald, F. Snyder, *J. Biol. Chem.* **256**, 175 (1981).
19. R. J. DeLorenzo, S. Burdette, J. Holderness, *Science* **213**, 546 (1981).
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