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TRIAZOLOBENZODIAZEPINES COMPETITIVELY INHIBIT THE BINDING OF PLATELET ACTIVATING FACTOR (PAF) TO HUMAN PLATELETS

Carolyn M. Chesney, D. David Pifer, and Lauren M. Cagen

Departments of Medicine, Pharmacology and Pathology, University of Tennessee and Department of Pathology, Baptist Memorial Hospital, Memphis, Tennessee

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PAF causes dose dependent platelet aggregation of human platelet rich plasma or gel filtered platelets (GFP). The benzodiazepines alprazolam and triazolam, but not diazepam (1-10 μM), inhibit PAF induced aggregation but have no effect on aggregation induced by other platelet agonists such as ADP, epinephrine and collagen. The IC_{50} for aggregation by PAF (4nM) in GFP is 1 μM for both alprazolam and triazolam. The mechanism for this inhibition was explored by studying the binding of ^{3}H-PAF(0.08nM) to GFP in Tyrodes buffer containing albumin (0.35%), Mg^{++} (1mM) and Ca^{++} (0.5mM). GFP was incubated with different doses of the drug for 5 min prior to addition of ^{3}H-PAF. Incubation was then carried out for 60 min at 25°C to achieve binding equilibrium, as previously established. Alprazolam and triazolam, but not diazepam, caused competitive displacement of ^{3}H-PAF from specific binding sites of GFP. The IC_{50} of alprazolam was 3.8 μM while that of triazolam was 0.82 μM. Lineweaver-Burk plots of ^{3}H-PAF binding in the presence of inhibitor were also consistent with competitive inhibition. These results are consistent with the interpretation that the specific inhibition of PAF induced platelet aggregation by alprazolam and triazolam, respectively, is due to competitive inhibition of binding of PAF to its receptor.

In 1984 Kornecki et al(1) reported that the triazolobenzodiazepines alprazolam and triazolam inhibited platelet aggregation by platelet activating factor (PAF), an alkyl ether lipid (1-O-alkyl-2 acetyl-sn-glyceryl-3-phosphorylcholine). This inhibition was specific for PAF effects on platelets. These compounds showed no inhibition of platelet aggregation by ADP, thrombin, epinephrine, collagen, arachidonic acid, or the calcium ionophore A23187. Interestingly, the benzodiazepine diazepam did not inhibit PAF induced platelet aggregation.

Several lines of evidence suggest that PAF induces platelet responses through a receptor mediated mechanism. The C_{16}(-)PAF

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enantiomer is approximately 1000-fold less active than natural (+) PAF (2). Biological responses are produced by very low concentrations of PAF (3). Specific desensitization occurs after exposure of platelets to PAF (4). In addition the existence of specific binding sites on human platelets has been shown by several laboratories (5,6,7).

The question is whether the inhibitory effects of these triazolo-benzodiazepines is at the level of PAF binding to its receptor on platelets or whether they block an effector pathway subsequent to binding. To answer this we studied the effects of alprazolam, triazolam and diazepam on PAF induced aggregation and binding to gel filtered human platelets.

MATERIALS AND METHODS

Alprazolam and triazolam were kindly provided by the Upjohn Company, Kalamazoo, MI. Diazepam was purchased from Roche Products, Inc., Manati, Puerto Rico. Pure synthetic PAF was obtained from Bachem Feinchemikalien Bubesdorf, Switzerland. Tritiated PAF came from Amersham, Arlington Heights, IL, (specific activity 120 Curies per mmol). Bovine serum albumin (Fraction V) was a product of Sigma Chemical Co., St. Louis, MO; human fibrinogen (grade L, 95% clottable) came from Kabi, Stockholm, Sweden.

Platelet rich plasma and gel filtered platelets (GFP) were prepared as previously described (8). Studies on GFP were done in Tyrode's buffer containing 0.5 mM $CaCl_2$ and albumin (0.35%). Fibrinogen (final concentration of 1.67 mg/ml) was added for aggregation studies but omitted for binding studies. Platelet aggregation was measured on a Payton Dual Channel aggregometer, Payton Associates, Inc., Buffalo, NY, according to the method of Born (9). Binding studies were carried out on GFP as previously described (6). In brief, gel-filtered platelets in 0.5 ml (platelet count 200,000 $\pm$ 50,000 per ul) were incubated with ^{3}H-PAF (0.08nM) and increasing concentrations of unlabeled PAF in the presence or absence of drug 60 min at 25° to achieve equilibrium

binding. For displacement studies GFP were incubated with different concentrations of the drug for 5 min prior to the addition of 0.08 nM ^{3}H-PAF. Incubation was then carried out for 60 min at 25°. Platelets were separated from unbound PAF by centrifugation at 12,500xg for 2 min in an Eppendorf microcentrifuge. The entire supernatant was carefully transferred to a scintillation vial and counted. The remaining platelet pellet was dissolved in 10% Triton X-100. The bound radioactivity was then assessed in like manner. Non specific binding was assessed by measurement of binding in the presence of 100-fold excess unlabeled PAF.

RESULTS

Effect of triazolobenzodiazepines on PAF induced aggregation of GFP (Figures 1 and 2). Gel filtered platelets were exposed to increasing concentrations of alprazolam (Fig. 1) or triazolam (Fig. 2) for 1 min prior to addition of PAF (4nM). Dose dependent inhibition of PAF induced aggregation can be seen with each drug. Analysis of tracings from 4 subjects revealed an IC_{50} for aggregation by 4nM PAF to be 1 μM for both alprazolam and triazolam. Concentrations of diazepam (up to 10

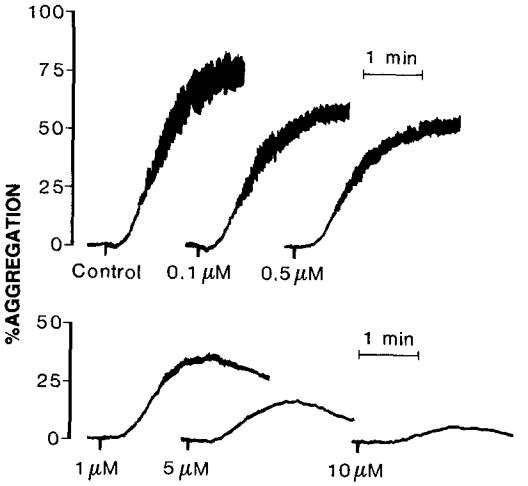

FIGURE 1. Effect of alprazolam on platelet aggregation. Gel filtered platelets were incubated with the specified concentration of alprazolam for 1 min at 25°C prior to the addition of PAF (4 nM, final concentration). The figure depicts a representative tracing of four experiments.

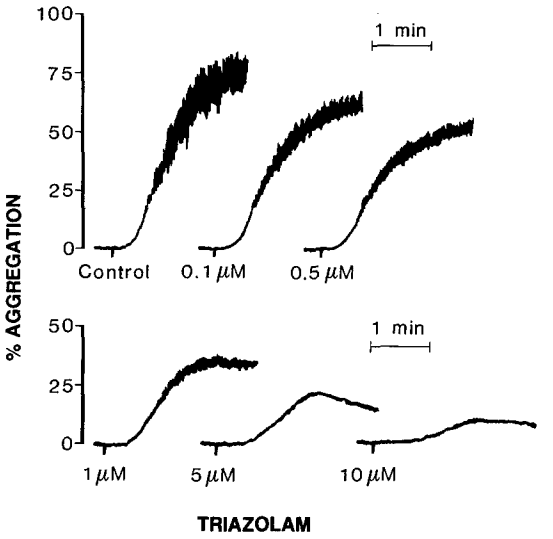

FIGURE 2. Effect of triazolam on platelet aggregation. Gel filtered platelets were incubated with the specified concentration of triazolam for 1 min at 25°C prior to the addition of PAF (4 nM final concentration). The figure depicts a representative tracing from four experiments.

μM) showed no inhibition of PAF induced aggregation. Neither alprazolam nor triazolam (10 μM) showed inhibition of platelet aggregation by ADP (10 μM), epinephrine (55 μM) or collagen (8 μg/ml).

Studies

For displacement studies GFP were incubated with different concentrations of drug for 5 min prior to the addition of 0.08nM

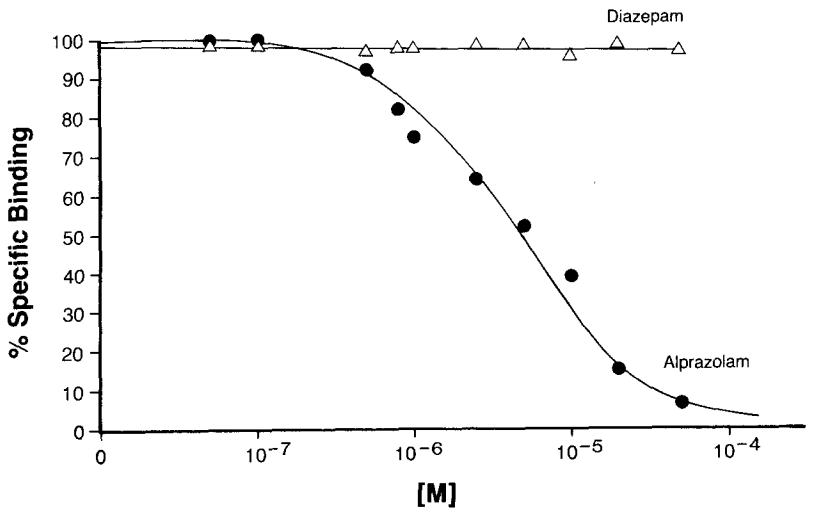

FIGURE 3. Inhibition of ^{3}H-PAF binding to GFP by alprazolam and diazepam. The GFP was incubated with drug for 5 min at 25° prior to addition of 0.08 nM ^{3}H-PAF.

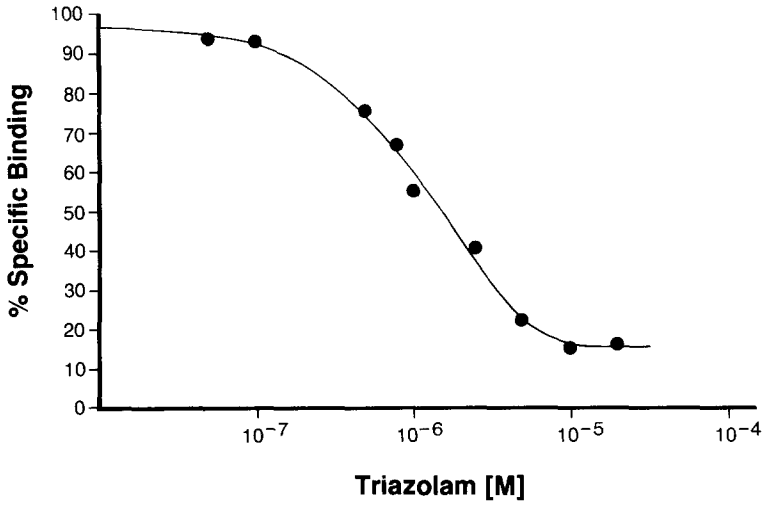

FIGURE 4. Inhibition of ^{3}H-PAF binding to GFP by triazolam. The GFP was incubated with drug for 5 min at 25° prior to addition of 0.08 nM ^{3}H-PAF.

^{3}H-PAF. Incubation was then carried out for 60 min at 25°. Alprazolam showed competitive displacement of ^{3}H-PAF from specific binding sites of GFP (Fig. 3). The slope of the Hill Plot of these data is 1.039 $\pm$ 0.062 (mean $\pm$ SD) and the IC_{50} is 3.8 μM (data not shown). Diazepam did not displace ^{3}H-PAF. Figure 4 shows the displacement curve for triazolam. The slope of the Hill Plot of these data is 0.89 $\pm$ 0.11 and the IC_{50} is 0.82 μM.

Binding Studies

Binding studies were carried out in the presence of inhibitor at concentration of 5 μM and 10 μM in the presence of 0.08 nM ^{3}H-PAF and increasing concentrations of unlabeled PAF. Double reciprocal plots of the data for alprazolam (Fig. 5) and triazolam (Fig. 6) are shown. The data are plotted as the average of four or five different experiments at each point for each inhibitor concentration. The control is the average of 10 individual experiments. In the case of alprazolam the lines cross at a point not quite on the ordinate. In fact when the data are compared by non-paired Students - t test there is no evidence for a difference in the intercepts of each line on the Y axis ($p > 0.05$). The

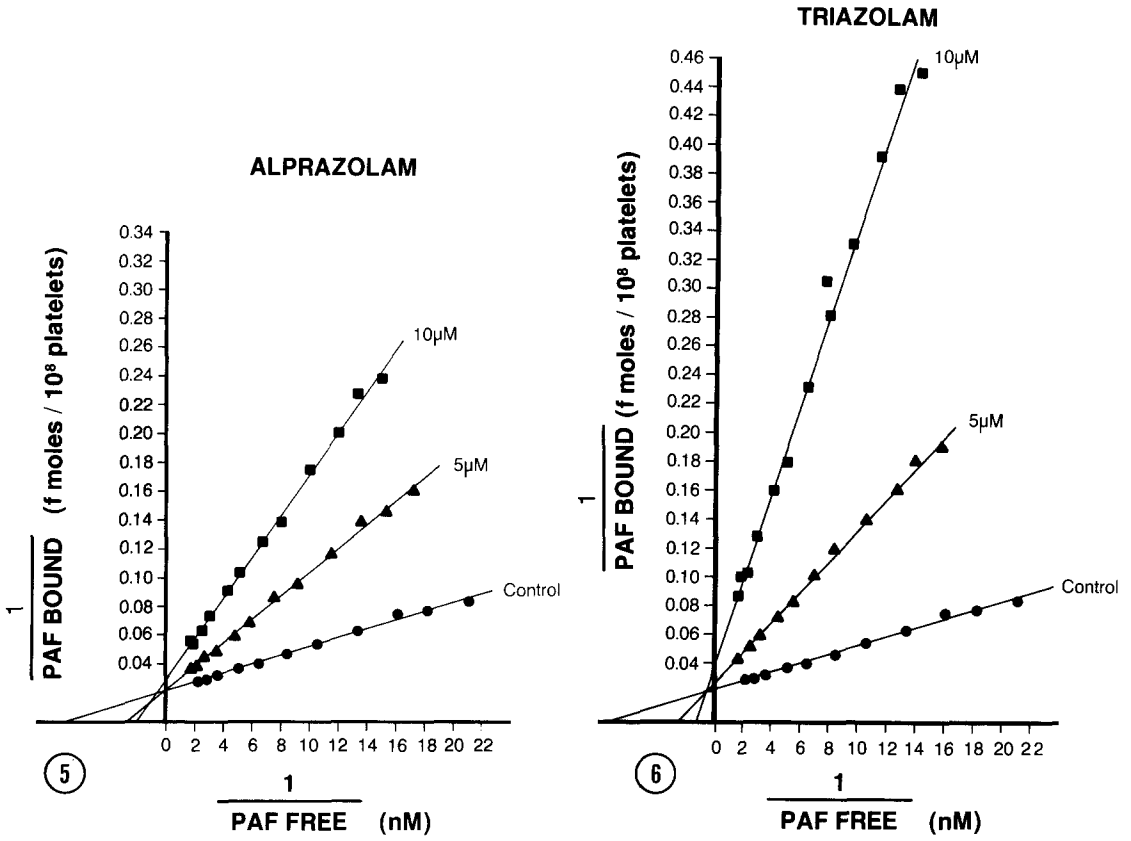

FIGURE 5. Double reciprocal plots of PAF binding to GFP in the presence or absence of alprazolam.

FIGURE 6. Double reciprocal plots of PAF binding to GFP in the presence or absence of triazolam.

K_i predicted from the effect of 5 μM alprazolam on the intercept of the reciprocal plot is 2.81 μM while that predicted from the effect of 10 μM is 2.28 μM. From the intercept effect of 5 μM triazolam a K_i of 3.22 μM is predicted while the effect of 10 μM corresponds to a K_i of 1.89 μM. There is no significant difference in number of binding sites at any drug concentration tested when compared with control. The dissociation constants increase with increasing drug concentration, as would be expected with competitive inhibition (Table I).

DISCUSSION

Our data confirm the finding of Konecki et al showing that the triazolobenzodiazepines alprazolam and triazolam inhibit PAF induced

TABLE I

Effect of triazolobenzodiazepines on the apparent dissociation constant (K_D) of ^{3}H-PAF receptor binding and the maximum number of high affinity receptor sites (B max).

Inhibitor	Number (n)	Concentration μM	K_D nM	B max fmoles per 10^8 platelets
Control	(13)	0	0.129 ± 0.008	42.2 ± 3.31
Alprazolam	(5)	5	0.427 ± 0.083*	53.7 ± 11.6**
	(4)	10	0.687 ± 0.015*	47.0 ± 14.4**
Triazolam	(4)	5	0.373 ± 0.080*	37.4 ± 0.85**
	(4)	10	0.903 ± 0.352*	35.4 ± 16.7**

Results are expressed as mean ± SEM,
*Result compared to control value $p < 0.001$
**Result not significantly different from control

aggregation of human GFP in a dose dependent manner while diazepam has no effect. Displacement curves as well as binding studies carried out in the presence of the alprazolam or triazolam and increasing concentrations of unlabeled PAF are consistent with competitive inhibition at the concentrations of inhibitor employed. As would be expected from functional studies, diazepam did not show any inhibitory effect on ^{3}H-PAF binding to GFP. Therefore the inhibitory effects of alprazolam and triazolam may be exerted by the triazolo moiety which is common to these compounds but is lacking in the benzodiazepine diazepam. Further studies of structure activity relationships will be required to define the site at which these triazolobenzodiazepines effect competitive inhibition of binding of PAF to platelets.

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