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SHORT COMMUNICATION

Formation of a Highly Stable Complex Between BN 50730 [Tetrahydro-4,7,8,10 Methyl-1(Chloro-2 Phenyl)-6 (Methoxy-4 Phenyl-Carbamoyl)-9 Pyrido [4′,3′-4,5] Thieno [3,2-*f*] Triazolo-1,2,4 [4,3-*a*] Diazepine-1,4] and the Platelet-Activating Factor Receptor in Rabbit Platelet Membranes

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ABSTRACT. BN 50730 [tetrahydro-4,7,8,10 methyl-1(chloro-2 phenyl)-6 (methoxy-4 phenyl-carbamoyl)-9 pyrido [4′,3′-4,5] thieno [3,2-*f*] triazolo-1,2,4 [4,3-*a*] diazepine-1,4], a novel platelet-activating factor (PAF) receptor antagonist with a hetrazepine structure, decreased the maximal number of binding sites (B_{max}) of [^{3}H]PAF in rabbit platelet membranes without altering its dissociation constant. Platelet aggregation induced by 1 μM PAF was prevented by preincubation with 1 μM BN 50730. The washing of the platelets preincubated with BN 50730 failed to revert its inhibitory effects. We conclude that BN 50730 acts as a non-competitive antagonist of the PAF receptor, due to the formation of a highly stable drug–receptor complex. BIOCHEM PHARMACOL 51;2: 193–196, 1996.

KEY WORDS. platelet-activating factor; platelets; BN 50730

PAF§ (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) is a potent phospholipid mediator, initially identified as a soluble factor generated by IgE-sensitized basophils. The spectrum of inflammatory cell targets that produce and/or are activated by PAF has been expanded considerably since early studies (for review, see Ref. 1). Therefore, the pharmacological control of its generation and/or effects may be of interest in several pathological conditions. PAF exerts its biological actions by interacting with a high-affinity G protein-coupled receptor, which was first cloned by Honda *et al.* [2]. Many PAF receptor antagonists have been identified, and most of them have been shown to be competitive in nature [3]. BN 50730 [tetrahydro-4,7,8,10 methyl-1(chloro-2 phenyl)-6 (methoxy-4 phenyl-carbamoyl)-9 pyrido [4′,3′-4,5] thieno [3,2-*f*] triazolo-1,2,4 [4,3-*a*] diazepine-1,4] is a new specific PAF receptor antagonist belonging to the family of hetrazepine derivatives [4]. We reported recently that a single local injection of BN 50730 (10 μg/site) inhibits PAF-induced exudation for 4 days [5], which was indicative of high stability of the drug–receptor complex. Since there is no information concerning the mode of action of this antagonist at the receptor level, in the present study we investigated its influence on the binding of [^{3}H]PAF to rabbit platelet membranes.

MATERIALS AND METHODS

Drugs

C_{16}-PAF (1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was purchased from Bachem (Feinchemikalien AG, Switzerland) and [^{3}H]PAF ([16-^{3}H]PAF, sp. act. 60 Ci/mmol) from New England Nuclear (Boston, MA). Iloprost was obtained from Schering (Berlin, Germany). BN 50730 was provided by Dr. P. Braquet, Institut Henri Beaufour (Le Plessis Robinson, France), and WEB 2086 {3-[4-(2-chlorophenyl)-9-

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§ *Abbreviation*: PAF, platelet-activating factor.
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methyl-6*H*-thieno [3,2-f-[1,2,4] triazolo [4,3-*a*] [1,4]-diazepine-2-yl]-1-(4-morpholinyl)]-1-propanone} was provided by Dr. H. Heuer, Boehringer-Ingelheim (Ingelheim am Rhein, Germany). Solutions were prepared daily for all experiments. BN 50730 was first diluted in absolute ethanol. Following dilutions were performed using BSA (0.25%).

Animals

Normal New Zealand White rabbits (2 to 3.5 kg) of either sex, bred at The Oswaldo Cruz Foundation, were anesthetized with ether before use.

Preparation of Rabbit Platelet Membranes

Plasma membranes were prepared from washed platelets according to Hosford *et al.* [6]. Rabbit whole blood (6 vol.) obtained by cardiac puncture [5] in 1 vol. of sodium citrate (3.8%) was centrifuged at 150 *g* for 15 min. The platelet-rich plasma was centrifuged at 1000 *g* for 15 min, and the resultant pellet was then washed twice in 10 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl, 5 mM $MgCl_2$, and 2 mM EDTA and once in sodium-free Tris-HCl buffer. The pellet was resuspended in the sodium-free buffer, quickly frozen in liquid nitrogen, and slowly thawed at room temperature. The freeze–thaw cycle was repeated five times. Lysed platelets were then centrifuged at 100,000 *g* for 30 min (4°). Platelet membranes were stored at −70°.

[^{3}H]PAF Binding Assay

The incubation was carried out in tubes (total volume: 1 mL) containing 60–100 μg protein, 10 mM Tris-HCl buffer (pH 7.0 at 4°) 10 mM $MgCl_2$, 0.025% (w/v) BSA, 0.2 nM [^{3}H]PAF, and various concentrations of unlabeled PAF, in the absence (control) or presence of 0.3 and 0.7 μM BN 50730 (experimental). The presence of 10 mM Mg^{2+} in the incubation medium was based on the fact that specific binding of [^{3}H]PAF is increased by Mg^{2+} at this final concentration [7]. After a 2-hr incubation at 4° (time period sufficient to reach equilibrium), the bound and free [^{3}H]PAF were separated rapidly by vacuum filtration on Whatman glass fiber filters (GF/C). Tubes were washed twice with 5 mL of a chilled solution (10 mM Tris–HCl, pH 7.0), and filters were washed further with 10 mL of the same buffer. Filters were then desiccated and added to 10 mL of the scintillation liquid. The radioactivity was counted in a liquid scintillation counter (Beckman LS-150) with an efficiency determined by internal standardization (41%). Specific binding was defined as the difference between the binding measured in the absence (total) and presence (non-specific) of 1 μM unlabeled PAF [6].

In another assay, the washed rabbit platelet membranes were preincubated at 4° in 14 mL of incubation medium for 2 hr in the absence (control) or presence of 0.7 μM BN 50730 (experimental). After this period, the platelet membranes were diluted to 34 mL with solution A (5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.0, at 4°), incubated for 30 min at 4°, and then centrifuged for 30 min at 100,000 *g*. The pellets were washed again by resuspension and incubation for 30 min in solution A and centrifuged as described above. The final pellets were used to quantify the binding of 0.2 nM [^{3}H]PAF. Two separate experiments (A and B) were performed in quintuplicate, using the same platelet membranes.

Platelet Aggregation Assay

Blood was obtained through cardiac puncture [5] and mixed with 2% sodium citrate (1 vol. for 9 vol. of blood). A platelet-rich plasma was prepared by centrifugation of the blood at 270 *g* for 15 min. Platelets were further washed twice by centrifugation at 1700 *g* for 10 min in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 2 mM $MgCl_2$, 12 mM $NaHCO_3$, 5.5 mM glucose, and 0.35% BSA, pH 6.5). The buffer was enriched with both 10 nM iloprost and 2 mM EGTA in the first washing, and with only 10 nM iloprost in the second washing. Platelets were resuspended to a final concentration of 6×10^5/μL in an iloprost–EGTA-free Tyrode buffer, pH 7.4. Washed platelets were incubated for 4 min with BN 50730 (1 μM) or WEB 2086 (10 μM) before stimulation with 1 μM PAF. In another set of experiments, platelets treated with each of these antagonists were subjected to two consecutive washings before PAF challenge, as previously stated. The effect of preincubation for 4 min with an excess of WEB 2086 (10 μM) before BN 50730 treatment was also assessed. In this case, platelets were also washed twice before PAF challenge. Platelet aggregation was monitored according to Born [8].

Analysis of the Results

Binding data were represented graphically using the classical Scatchard plot, but parameters (K_d and B_{max}) were calculated using a computerized non-linear regression analysis of the untransformed data (EBDA-Ligand, Elsevier-Biosoft, Cambridge, U.K.) [9], assuming a single class of saturable binding sites, to avoid misinterpretation of binding data that can occur when linearizing transformations are used [10, 11]. The data were statistically analyzed by ANOVA, followed by the Student's-Newman–Keuls test (saturation experiment) and Student's *t*-test (binding after the washing procedure).

RESULTS

Effect of BN 50730 on [^{3}H]PAF Binding to Rabbit Platelet Membranes

[^{3}H]PAF binding to rabbit platelet membranes was measured in the presence of 10 mM $MgCl_2$, using concentrations of ligand ranging from 0.2 to 20 nM. As shown in Fig. 1, the Scatchard plot was linear (Hill coefficient not different from 1), indicating the presence of a single class of specific binding sites, with a K_d of about 3 nM. Scatchard plots for [^{3}H]PAF saturation experiments performed in the presence of BN 50730 were parallel to the control curve, but shifted to the left in a concentration-dependent manner, indicating a non-competi-

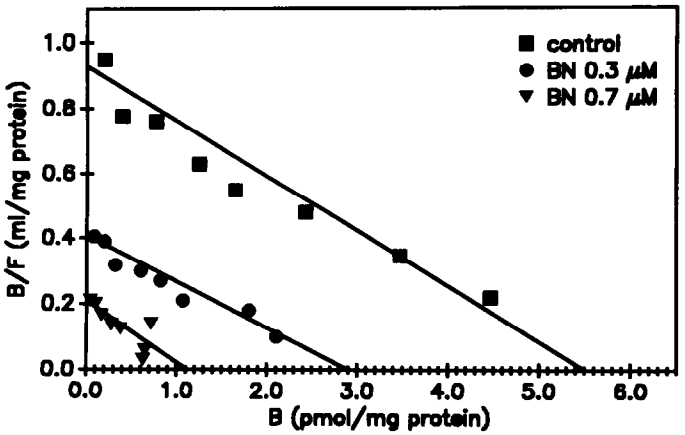

FIG. 1. Scatchard plots for the specific binding of [^{3}H]PAF to rabbit platelet membranes in the presence of BN 50730. About 60–100 μg protein was incubated at 0° for 2 hr in the absence (■) and presence of 0.3 μM (●) or 0.7 μM (▼) BN 50730. The assay medium contained 10 mM $MgCl_2$, 10 mM Tris, 0.025% BSA, 0.2 nM [^{3}H]PAF, and various concentrations of unlabeled PAF, at pH 7.0. Each point is the mean of triplicate determinations in a typical experiment. The fitted curves were obtained by non-linear regression, using the model of a single class of binding sites (see Materials and Methods).

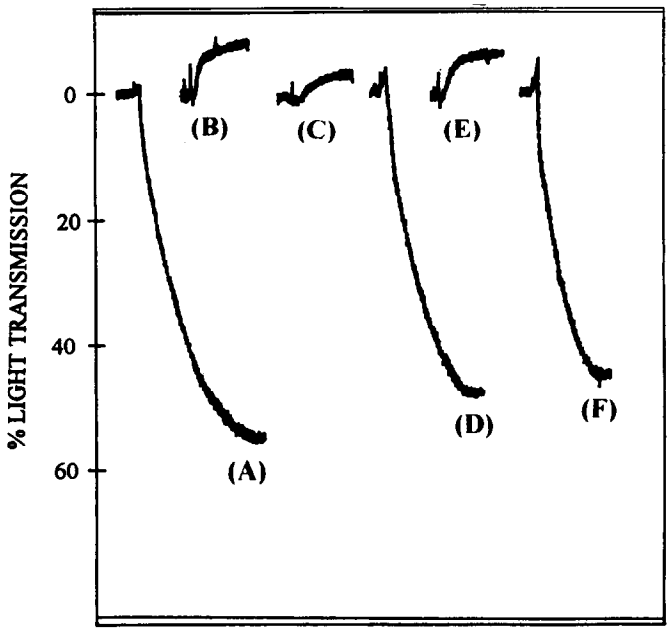

FIG. 2. Effect of BN 50730 on the PAF-induced platelet aggregation (expressed as a percentage of light transmission). Typical traces of rabbit platelet aggregation induced by 1 μM PAF are shown (the same profile has been reported in two other experiments). Platelets were pretreated for 4 min with vehicle (A), 10 μM WEB 2086 (B) or 1 μM BN 50730 (C) prior to PAF challenge. Platelets pretreated for 4 min with 10 μM WEB 2086 (D) or 1 μM BN 50730 (E) were washed twice before PAF challenge. BN 50730 (1 μM) was added to platelets preincubated for 4 min with 10 μM WEB 2086; these platelets were then washed twice prior to the PAF challenge (F).

tive type of antagonism. The quantitative analysis of the results from four independent experiments further confirmed that BN 50730 altered the maximum number of PAF binding sites (B_{max}) without modifying the PAF dissociation constant (K_d): K_d was 3.1 ± 1.0, 3.8 ± 1.3, and 4.3 ± 1.0 nM, while B_{max} decreased from 3.9 ± 0.8 to 2.7 ± 0.9 and 1.9 ± 0.7 pmol/mg protein ($P \leq 0.05$) (mean ± SEM) following pretreatment with 0, 0.3 and 0.7 μM BN 50730, respectively.

Stability of the BN 50730–Receptor Complex

To evaluate the stability of the drug–receptor complex, platelet membranes pretreated (experimental) or not (control) with 0.7 μM BN 50730 for 2 hr were washed twice, for 30 min, prior to incubation with 0.2 nM [^{3}H]PAF (see Materials and Methods). The washing procedure failed to alter the inhibitory effect of BN 50730, since the specific binding of [^{3}H]PAF was reduced significantly ($P \leq 0.001$) from 39.6 ± 1.4 to 11.7 ± 2.5 fmol/mg protein (experiment A), and from 36.4 ± 0.9 to 9.8 ± 1.3 fmol/mg protein (experiment B) (means ± SEM, N = 5) when platelet membranes were pretreated with 0.7 μM BN 50730.

Effect of BN 50730 on PAF-Induced Platelet Aggregation

Incubation of washed platelets with BN 50730 (1 μM) or WEB 2086 (10 μM) for 4 min abolished the platelet aggregation triggered by PAF (1 μM) (Fig. 2, B and C). In the case of BN 50730, but not WEB 2086, this inhibition was maintained even after two consecutive platelet washings (Fig. 2, D and E). Pretreatment with the competitive antagonist WEB 2086 (10 μM) prevented the inhibitory effect of BN 50730 (1 μM) (Fig. 2F).

DISCUSSION

BN 50730, a PAF antagonist with a hetrazepine structure [4], has been shown to be very unusual, as its local inhibitory effect on PAF-induced pleural exudation is long-lasting, remaining effective for at least 4 days [5]. We show here that BN 50730 acts as a non-competitive antagonist of PAF at the receptor level, since it decreased the maximal binding of PAF to rabbit platelet membranes without altering its dissociation constant. In the platelet aggregation assays, BN 50730 (0.5 to 1.0 μM) also reduced the maximal effect of PAF tested in a large range of concentrations (10^{-9}–10^{-5} M) [5]. As such an effect could be due to an irreversible binding of BN 50730 to the PAF receptor, platelet membranes pretreated with BN 50730 were washed twice, by dilution and incubation in a drug-free medium for 30 min, prior to incubation with [^{3}H]PAF, in order to promote the dissociation of BN 50730 from the receptor. Since the washing procedure failed to alter the inhibitory effect of BN 50730, we suggest that BN 50730 is a PAF receptor antagonist that differs from the others, mainly by its non-competitive nature, due to an almost irreversible binding to the PAF receptor. Note that we used a relatively high concentration of PAF (1 μM) to demonstrate that, even when using the same concentration as the antagonist, PAF was unable to reverse the inhibition exerted by BN 50730. This profile of [^{3}H]PAF binding inhibition can explain at the molec-

ular level the apparent irreversibility of the antagonism exerted by BN 50730 on the PAF-induced rabbit platelet aggregation observed *in vitro*. It is of interest to note that prior pretreatment of the platelets with an excess of a competitive PAF antagonist (WEB 2086) prevented the inhibitory effect of BN 50730, as expected for this type of irreversible antagonism [12]. These results indicate that BN 50730 and WEB 2086 have a marked difference in their dissociation kinetics despite the fact that both share the same PAF binding sites on rabbit platelets. In some way, this type of antagonism has already been observed in relation to SR 27417, since this drug selectively abolished the *ex vivo* rabbit platelet aggregation induced by PAF for at least 2–3 days after oral administration [13]. SR 27417 also presented a non-competitive profile of antagonism in binding to rabbit platelets [14]. Another example of this behavior of antagonism was reported recently by Parry *et al.* [15] in relation to a novel 1,4-dihydropyridine PAF antagonist (UK 74505). We conclude that the hetrazepine BN 50730 is a non-competitive antagonist of PAF in rabbit platelets probably as a result of the formation of a highly stable drug–receptor complex.

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