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Bakkenolide G, a Natural PAF-receptor Antagonist

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Abstract

Because platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine) participates in many physiopathological responses, including inflammatory reaction, endotoxic shock, allergic diseases and platelet aggregation, PAF-receptor antagonists are important in the treatment of these diseases.

A biologically active compound, bakkenolide G, extracted from the plant *Petasites formosanus* selectively and concentration-dependently inhibited PAF-induced platelet aggregation and ATP release. The IC50 of bakkenolide G for PAF (2 ng mL^{-1})-induced platelet aggregation was $5{\cdot}6 \pm 0{\cdot}9$ μM. Bakkenolide G also concentration-dependently inhibited PAF-induced intracellular signal transductions, including thromboxane B_2 formation, and increased intra-cellular calcium concentration and phosphoinositide breakdown without affecting those caused by thrombin (0·1 units mL^{-1}), collagen (10 μg mL^{-1}), arachidonic acid (100 μM) and U46619 (1 μM). Bakkenolide G shifted the concentration–response curves of PAF-induced platelet aggregation parallel to the right; the Schild plot slope and the pA_2 value were $1{\cdot}31 \pm 0{\cdot}31$ and $6{\cdot}21 \pm 0{\cdot}75$, respectively. Moreover, bakkenolide G concentration-dependently competed with [^{3}H]PAF binding to platelets, with an IC50 value of $2{\cdot}5 \pm 0{\cdot}4$ μM.

These data strongly indicate that bakkenolide G is a specific PAF-receptor antagonist as an antiplatelet aggregatory agent.

In the last decade platelet-activating factor, a phospholipid mediator, has been found to be involved in intracellular signalling in a variety of cells and tissues. In early 1970 it was found that a compound, denoted PAF, released from rabbit leucocytes could induce rabbit platelet aggregation. In the next decade its chemical structure was identified and its biological activities proven. PAF participates in many physiopathological responses, including acute inflammation, stimulation of neutrophils and macrophages, asthma, systemic anaphylaxis, endotoxic shock, acute allergic diseases and platelet aggregation (Henson & Pinckard 1977; Vargaftig et al 1981; Etienne et al 1986; Casals-Stenzel 1987). It also has physiological effects on the reproductive (O'Neill 1992; Baldi et al 1994), cardiovascular and central nervous systems (Bazan & Allan 1996). PAF produced by cells is either released into extracellular fluids or remains associated with the cells. Cell-associated PAF is exposed on the outer plasma membrane, where it can activate other cells (McManus et al 1993). PAF attaches its receptor, a member of the G protein-coupled receptor superfamily characterized by seven trans-membrane domains, and triggers a series of intracellular signals including phosphoinositide breakdown, arachidonic acid metabolism, intracellular calcium mobilization and protein phosphorylation. These signals cause the many pathophysiological phenomena mentioned above. Because most actions of PAF can be prevented by blocking its receptors with antagonists, a PAF-receptor antagonist is necessary to prevent these pathophysiological situations.

In a large scale screening test, we found that bakkenolide G (Fig. 1), isolated from the plant *Petasites formosanus*, inhibited aggregation of rabbit platelets. In this study, we evaluated the antiplatelet mechanism of bakkenolide G and proved it to be a PAF-receptor antagonist in platelets.

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Materials and Methods

Materials

Bakkenolide G (Fig. 1) was dissolved in dimethylsulphoxide (DMSO). Collagen (type 1, bovine Achilles tendon) was homogenized in 25 mM acetic acid and stored at −20°C at a concentration of 1 mg mL^{-1}. Platelet-activating factor (PAF) was dissolved in chloroform and diluted to 0·1% with BSA–saline solution immediately before use. Arachidonic acid, EDTA (disodium salt), luciferin-luciferase, DMSO, Dowex-I (100-200 mesh: X8, chloride) resin, myoinositol, bovine serum albumin (BSA), U46619, trichloroacetic acid and fura-2/AM were purchased from Sigma (St Louis, MO). Thrombin (bovine) was purchased from Parke Davis (Detroit, MI) and dissolved in 50% glycerol to furnish a stock solution of 100 NIH units mL^{-1}. Thromboxane B_2 EIA kit, myo-[2-^{3}H]inositol (10–20 Ci $mmol^{-1}$) and [^{3}H]PAF (182 Ci $mmol^{-1}$) were obtained from Amersham, UK.

Preparation of rabbit washed platelets

Washed platelets were prepared from blood withdrawn with a siliconized syringe from the marginal vein of New Zealand rabbits. Blood from the rabbits was mixed with EDTA (final concentration 6 mM) and centrifuged at 90 *g* at room temperature; platelet-rich plasma (PRP) was obtained from the upper portion. The platelet suspension was obtained from EDTA-PRP according to the washing procedure described previously (Teng et al 1987). Platelet number was counted using a cell counter (Hemalaser 2, Sebia, France) and adjusted to $3{\cdot}0 \times 10^8$ platelets mL^{-1}. The platelet pellets were re-suspended in Tyrode's solution of composition (mM): NaCl (136·8), KCl (2·8), $NaHCO_3$ (11·9), $MgCl_2$ (2·1), NaH_2PO_4

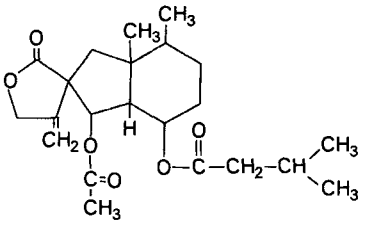

FIG. 1. The chemical structure of bakkenolide G.

(0·33), $CaCl_2$ (1·0) and glucose (11·2) containing BSA (0·35%). All glassware was siliconized.

Platelet aggregation and ATP release

Platelet aggregation was measured by the turbidimetric method (O'Brien 1962; Born & Cross 1963). The absorbance of the platelet suspension was taken as 0% aggregation and that of Tyrode's solution as 100% aggregation. ATP released from platelets was measured by bioluminescence (DeLuca & McElory 1987). Both aggregation and ATP release were measured by means of a Lumi-aggregometer (Model 1020, Payton, Canada) connected to two dual-channel recorders.

Thromboxane B_2 assay

EGTA (2 mM) and indomethacin (50 μM) were added to stop the reaction 6 min after challenge of platelets with various aggregation inducers. After 2 min centrifugation in an Eppendorf (Germany) model 5414C microcentrifuge the supernatant was obtained and thromboxane B_2 was assayed by EIA kits.

Measurement of intracellular calcium ($[Ca^{2+}]_i$) concentration in platelets

The method of Pollock and Rink (1986) was followed. Platelets (3×10^8 mL^{-1}) were incubated with fura-2/AM (5 μM) at 37°C for 40 min, centrifuged at 500 *g* and the resultant pellet was washed with Tyrode's solution containing EDTA (1 mM). After centrifugation the platelets were re-suspended in the Tyrode's solution containing $CaCl_2$ (1 mM). Fluorescence (excitation wavelength 339 nm, emission wavelength 500 nm) was measured with an Hitachi (Japan) model F4000 fluorescence spectrophotometer. At the end of the experiment the cells were treated with 0·1% Triton X-100 then by addition of 10 mM EGTA to give the maximum and minimum fluorescence, respectively. $[Ca^{2+}]_i$ was calculated as described for fura-2 using the Ca^{2+}-dye dissociation constant of 224 nm (Grynkiewicz et al 1985).

Labelling of membrane phospholipids and measurement of the production of [^{3}H]inositol monophosphate

This method was modified from those of Huang & Detwiler (1986) and Neylon & Summers (1987). EDTA-PRP was centrifuged at 500 *g* for 10 min, the platelet pellets were suspended in 1 mL Ca^{2+}-free and BSA-free Tyrode's solution containing 75 μCi mL^{-1} [^{3}H]inositol and 1 mM EDTA. After incubation at 37°C for 2 h the platelet pellets were collected by centrifugation (500 *g*, 4 min) and re-suspended in Tyrode's solution. In the presence of 5 mM LiCl, which inhibits inositol monophosphate phosphatase, the reaction was performed at 37°C for 6 min with a stirring bar driven at 900 rev min^{-1}. An equal volume of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 mL supernatant was pooled and trichloroacetic acid was removed by extracting with 5×2 vols diethyl ether. The aqueous phase, containing the inositol phosphate, was adjusted to pH 7–8 with 1 M NaOH and diluted to 4 mL with distilled water before application to a Dowex-I ion-exchange column for separation of the inositol phosphates as described previously by Neylon & Summers (1987). Because the levels of inositol bisphosphate and inositol trisphosphate were very low, we measured inositol monophosphate as an index of the total inositol phosphate formation. The radioactivity was counted by means of a liquid scintillation counter.

[^{3}H]PAF binding

This method was according to Inarrea et al (1984). Briefly, washed platelets were prepared as described above but re-suspended at a concentration of 10^8 cells mL^{-1} in phosphate-buffered medium (pH 6·9) containing 0·35% BSA and 1 mM $CaCl_2$. Binding studies were performed by adding 2 ng mL^{-1} [^{3}H]PAF to washed platelet suspension (10^8 mL^{-1}) at room temperature; agitation was omitted to minimize platelet aggregation. After incubation for 30 min, 400 μL platelet suspension was layered on the top of sucrose solution (20%, w/v) and centrifuged in an Eppendorf microcentrifuge (model 5414 C; 14 000 rev min^{-1} for 5 min). Tube bases containing the centrifuged pellet were placed in a vial containing Triton X-100 (0·1%) in 5% HNO_3 (0·3 mL) for scintillation counting. Radioactivity was determined by adding scintillation cocktail. Non-specific binding was defined in the presence of 2 μg mL^{-1} unlabelled PAF, and was subtracted from total binding to give specific binding.

Data analysis

In each experiment, agonist concentration–response curves in the presence of bakkenolide G were related to the control concentration–response curve, of which the maximum response was taken as 100%. In most experiments four concentrations of bakkenolide G were tested and the slopes of the resulting Schild plots were used to assess competitive antagonism. The pA_2 values were calculated for each concentration of bakkenolide G according to $pA_2 = -\log([\text{antagonist}]/[\text{dose-ration} - 1])$ (Mackay 1978).

Results

Effects of bakkenolide G on rabbit platelet aggregation and ATP release

Thrombin (0·1 units mL^{-1}), collagen (10 μg mL^{-1}), arachidonic acid (100 μM), U46619 (1 μM) and PAF (2 ng mL^{-1}) induced 80–90% aggregation and ATP release in rabbit washed platelets. Bakkenolide G specifically and concentration-dependently inhibited PAF-induced platelet aggregation and ATP release (Fig. 2); its IC50 value for PAF-induced platelet aggregation was calculated to be $5{\cdot}6 \pm 0{\cdot}9$ μM. However, bakkenolide G (100 μM) did not affect thrombin-, collagen-, arachidonic acid- and U46619-induced platelet aggregation and ATP release (data not shown). Bakkenolide G (1–20 μM) also shifted the concentration–response curves of PAF-induced platelet aggregation parallel to the right (Fig. 3a).

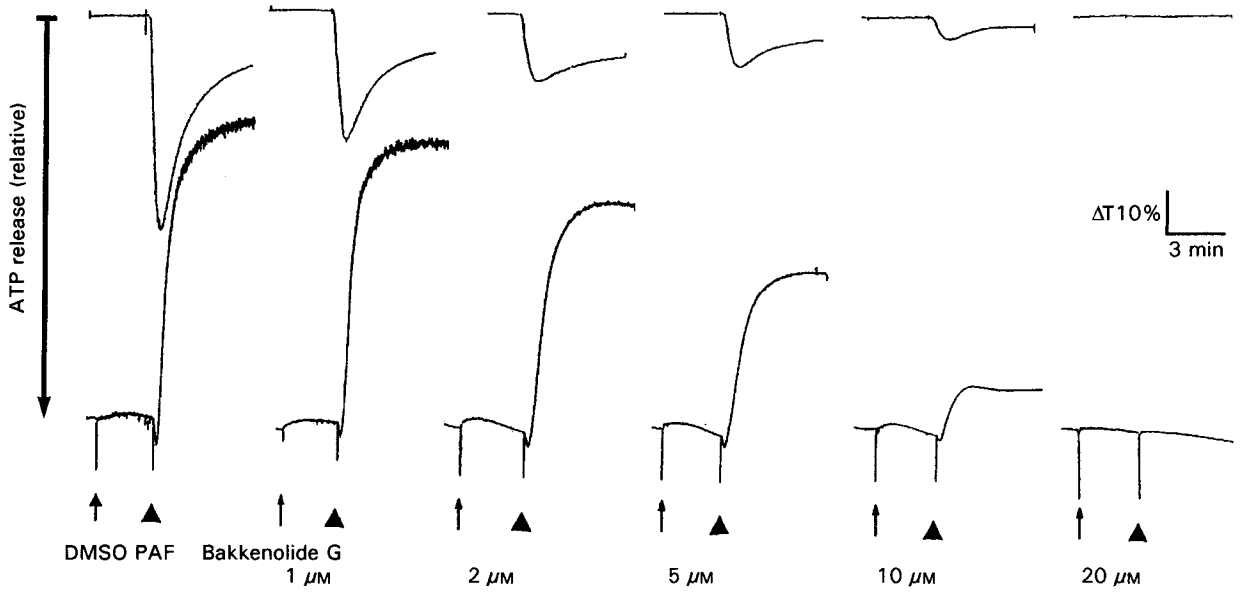

FIG. 2. Inhibitory effects of bakkenolide G on PAF-induced platelet aggregation and ATP release. Rabbit washed platelets were pre-incubated with different concentrations of bakkenolide G or DMSO (0·5%, control) at 37°C for 3 min, then PAF (2 ng mL^{-1}) was added to trigger the aggregation (upper traces) and ATP release (lower traces).

Schild plots were thus constructed (Fig. 3b). The pA_2 value of bakkenolide G was calculated to be 6·21 ± 0·75 (n = 6). The inhibitory effect of bakkenolide G on PAF-induced platelet aggregation and ATP release were easily reversible, full restoration of platelet aggregability was obtained in platelets after washout (data not shown).

Effects of bakkenolide G on thromboxane B_2 formation

As shown in Table 1, the resting level of thromboxane B_2 in rabbit platelets was 0·2 ± 0·1 ng mL^{-1}. Arachidonic acid (100 μM), collagen (10 μg mL^{-1}), thrombin (0·1 units mL^{-1}) and PAF (2 ng mL^{-1}) markedly increased thromboxane B_2 formation to 313·7 ± 38·3, 193·5 ± 25·0, 132·4 ± 28·3 and 114·7 ± 19·6 ng mL^{-1}, respectively. Bakkenolide G inhibited PAF-induced thromboxane B_2 formation concentration-dependently. In contrast, bakkenolide G (100 μM) did not affect arachidonic acid-, collagen- and thrombin-induced thromboxane B_2 formation. U46619 (1 μM) did not markedly increase thromboxane B_2 formation after 6 min incubation with rabbit washed platelets (3·2 ± 1·0 ng mL^{-1}) and bakkenolide G did not affect U46619-induced thromboxane B_2 formation.

Effects of bakkenolide G on intracellular calcium concentration

In fura-2/AM-loaded platelets, thrombin (0·1 units mL^{-1}), PAF (2 ng mL^{-1}), collagen (10 μg mL^{-1}), arachidonic acid (100 μM) and U46619 (1 μM) increased intracellular calcium concentrations to 215·2 ± 18·5, 202·3 ± 12·6, 152·5 ± 19·2, 162·3 ± 11·9 and 183·2 ± 15·4 nM, respectively. Bakkenolide G (20 μM) almost completely inhibited the PAF-induced increase in intracellular calcium concentration (45·2 ± 9·3 nM), but did not affect those caused by the other four stimulators, even at a concentration of 100 μM (data not shown).

Effects of bakkenolide G on [^{3}H]inositol monophosphate formation

In [^{3}H]myo-inositol-labelled platelets, thrombin (0·1 units mL^{-1}), PAF (2 ng mL^{-1}), collagen (10 μg mL^{-1}), arachidonic acid (100 μM) and U46619 (1 μM) increased [^{3}H]inositol monophosphate formation 4·1 ± 0·3-, 3·7 ± 0·4-,

Table 1. Effects of bakkenolide G on arachidonic acid-, collagen-, U46619-, thrombin- and PAF-induced thromboxane B_2 formation in rabbit washed platelets.

	Thromboxane B_2 formation (ng mL^{-1})				
	Arachidonic acid	Collagen	U46619	Thrombin	PAF
Control	313·7 ± 38·3	193·5 ± 25·0	3·2 ± 1·0	132·4 ± 28·3	114·7 ± 19·6
Bakkenolide G 100 μM	166·6 ± 37·9	159·5 ± 26·7	1·9 ± 0·8	114·7 ± 19·6	–
Bakkenolide G 20 μM	–	–	–	–	2·1 ± 0·3*
Bakkenolide G 10 μM	–	–	–	–	2·7 ± 0·5*
Bakkenolide G 5 μM	–	–	–	–	5·4 ± 2·3*
Bakkenolide G 2 μM	–	–	–	–	6·6 ± 2·2*
Bakkenolide G 1 μM	–	–	–	–	11·2 ± 2·1*
Bakkenolide G 0·5 μM	–	–	–	–	14·9 ± 3·0*

The thromboxane B_2 level of resting platelets was 0·2 ± 0·1 ng mL^{-1}. Values are presented as means ± s.e.m. (n = 8). *P < 0·001 compared with the respective control. Final concentrations: arachidonic acid, 100 μM; collagen, 10 μg mL^{-1}; U46619, 1 μM; PAF, 2 ng mL^{-1}; thrombin, 0·1 units mL^{-1}.

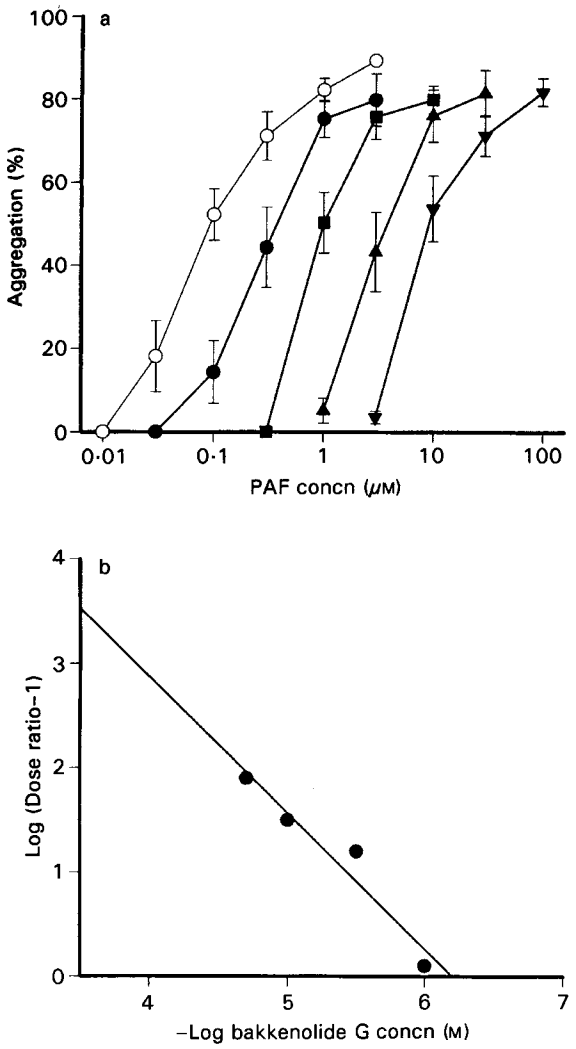

FIG. 3. a. Concentration-dependent shifting by bakkenolide G of the platelet aggregation induced by PAF. Rabbit washed platelets were incubated with DMSO (0·5%, ○) or different concentrations of bakkenolide G (1 μM, ●; 3 μM, ■; 10 μM, ▲; 20 μM, ▼) at 37°C for 3 min, then various concentrations of PAF were added to trigger aggregation. The values are expressed as means ± s.e.m. (n = 6). b. Schild plot of the inhibition by bakkenolide G of PAF-induced platelet aggregation. The data were obtained from a. The equation of the regression line was $y = (-1{\cdot}31 \pm 0{\cdot}31)x + (8{\cdot}1 \pm 1{\cdot}2)$.

2·1 ± 1·0-, 2·2 ± 0·9- and 1·6 ± 0·2-fold, respectively. Bakkenolide G selectively inhibited PAF-induced [^{3}H]inositol monophosphate formation without affecting its formation by the other four stimulators (Table 2).

Effect of bakkenolide G on [^{3}H]PAF binding

According to a previous study PAF receptor binding was maximum at pH 7·0 and decreased at pH > 7·4 (Hwang et al 1986). Therefore, the [^{3}H]PAF binding study was performed at pH 6·9. The total binding of 2 ng mL^{-1} [^{3}H]PAF to intact rabbit platelets in the presence of 0·35% BSA was 11 366·8 ± 1412·4 counts 7 min^{-1} (n = 5), whereas non-specific-binding assay in the presence of 2 μg mL^{-1} unlabelled PAF was 2142·6 ± 410·2 counts min^{-1}. As shown in Fig. 4, bakkenolide G concentration-dependently antagonized [^{3}H]PAF binding to intact rabbit platelets. The IC50 value of bakkenolide G for [^{3}H]PAF binding was 2·5 ± 0·4 μM.

Discussion

Specific receptors for PAF have been identified in numerous tissues and cells. The first experiment utilizing [^{3}H]PAF was conducted with human platelets in 1982 (Valone et al 1982). A PAF receptor has also been identified on rabbit platelets (Inarrea et al 1984). After binding by PAF, PAF receptor elicited diverse biochemical signals, including activation of phospholipase C and A_2, leading to hydrolysis of phosphoinositide, release of arachidonic acid, intracellular calcium mobilization and activation of protein kinase C and protein tyrosine kinase (Chao & Olson 1993; Takash & Takao 1995), all of which play a pivotal role in platelet aggregation. Therefore, a selective PAF-receptor antagonist could antagonize PAF-induced intracellular signals and platelet activation.

Bakkenolide G selectively inhibited PAF-induced platelet aggregation and ATP release without affecting similar thrombin-, collagen-, arachidonic acid- and U46619-induced behaviour. This implied that bakkenolide G might be a selective PAF-receptor antagonist in washed rabbit platelets. Indeed, bakkenolide G concentration-dependently shifted the concentration–response curves of PAF-induced aggregation to the right but did not affect the maximum response. Moreover, the Schild plot slope for bakkenolide G was near −1·0. These data imply that bakkenolide G is a competitive PAF-receptor antagonist. Furthermore, bakkenolide G inhibited the specific binding of [^{3}H]PAF to washed rabbit platelets; the potency was similar to its inhibiting effect on PAF-induced platelet aggregation. This result strongly suggested that bakkenolide G inhibited PAF-induced platelet aggregation by antagonizing PAF binding to its receptor. Also, the inhibitory effect of bakkenolide G on PAF-induced platelet aggregation was easily washed out (data not shown), which implies that bakkenolide G is a reversible PAF antagonist.

Table 2. Effects of bakkenolide G on thrombin-, PAF-, collagen-, arachidonic acid- and U46619-induced inositol monophosphate formation by rabbit washed platelets.

	[^{3}H]Inositol monophosphate formation				
	Thrombin	PAF	Collagen	Arachidonic acid	U46619
Control	4·1 ± 0·3	3·7 ± 0·4	2·1 ± 1·0	2·2 ± 0·9	1·6 ± 0·2
Bakkenolide G 100 μM	4·3 ± 0·6		−2·0 ± 0·2	1·8 ± 0·1	1·5 ± 0·1
20 μM	–	1·4 + 0·2*	–	–	–

Values (as x-fold increase in level of inositol monophosphate) are presented as means ± s.e.m. (n = 6). *$P < 0{\cdot}01$ compared with PAF control. Final concentrations: thrombin, 0·1 units mL^{-1}; PAF, 2 ng mL^{-1}; collagen, 10 μg mL^{-1}; arachidonic acid, 100 μM; U46619, 1 μM.

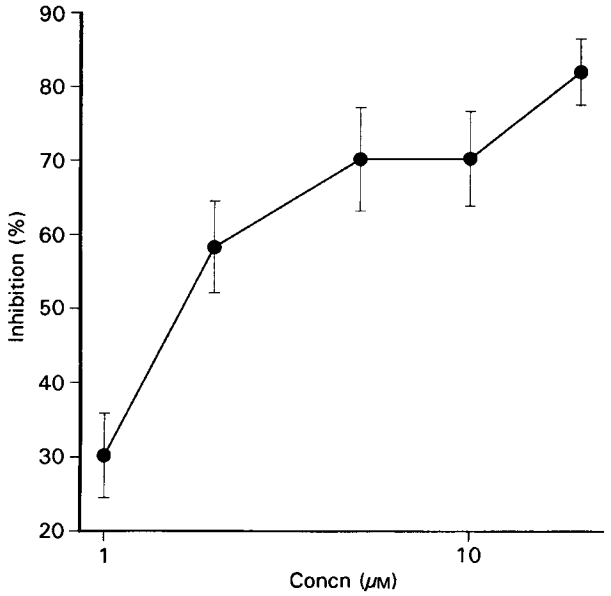

FIG. 4. Inhibitory effect of bakkenolide G on [^{3}H]PAF binding to PAF receptor on rabbit washed platelets. Different concentrations of bakkenolide G were incubated with rabbit washed platelets at room temperature for 3 min, then 2 ng mL^{-1} [^{3}H]PAF was added to compete with bakkenolide G for binding of PAF receptors. Non-specific binding was determined in the presence of 2 μg mL^{-1} cold PAF. Values are expressed as means ± s.e.m. (n = 5).

Thromboxane A_2 is an important molecule in platelet aggregation and the release reaction (Hornby & Skidmore 1982). By activation of phospholipase A_2, many platelet stimulators release free arachidonic acid from platelet membranes and this is metabolized by cyclooxygenase to form thromboxane A_2. Furthermore, thromboxane A_2 is the major mediator in externally applied arachidonic acid-induced platelet aggregation. Formation of thromboxane B_2, a stable metabolite of thromboxane A_2, by externally applied arachidonic acid was not affected by bakkenolide G, which implies that bakkenolide G did not affect the activity of cyclooxygenase. Thus, inhibition of PAF-induced thromboxane B_2 formation by bakkenolide G is mediated via a step before cyclooxygenase.

Phosphoinositide breakdown is a primary signal for many platelet stimulators. Mauco et al (1983) found that phosphoinositide breakdown is also the primary biochemical signal in PAF-induced platelet aggregation. In the current study, bakkenolide G inhibited PAF-induced inositol monophosphate formation and increase in intracellular calcium concentration but not those responses caused by thrombin, collagen, arachidonic acid and U46619. Increase in intracellular calcium concentration also plays an important role in PAF-induced platelet aggregation. The increased intracellular calcium concentration is also very important for activation of phospholipase A_2, which is a rate-limiting enzyme for the generation of arachidonic acid. Many platelet activators induced intracellular calcium mobilization by generating inositol trisphosphate, which releases calcium from intracellular components, or by inducing calcium influx from extracellular space. PAF has been proved to increase intracellular calcium concentrations by both mechanisms (Lee et al 1981, 1983). This also explains why bakkenolide G selectively inhibits PAF-induced thromboxane B_2 formation. These data further support the notion that bakkenolide G is a PAF antagonist.

In conclusion, bakkenolide G selectively inhibited PAF-induced platelet activation, including platelet aggregation, ATP release, thromboxane B, formation, phosphatidylinositol breakdown and increase in intracellular calcium concentration. Furthermore, bakkenolide G competed with [^{3}H]PAF binding to rabbit washed platelets. These data indicate that bakkenolide G is a selective PAF antagonist. Its structural novelty might provide an original chemical basis for the development of new PAF antagonists.

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