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INHIBITION OF THE METABOLISM OF PLATELET ACTIVATING FACTOR (PAF-acether) BY THREE SPECIFIC ANTAGONISTS FROM *GINKGO BILOBA*

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Abstract—Washed rabbit platelet suspensions were incubated in the presence of 1-[^{3}H]*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine ([^{3}H] PAF-acether), which was metabolized into 1-[^{3}H]*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) through the sequential action of cytosolic acetylhydrolase and membrane transacylase. Within 60 min at 37°, percentage of [^{3}H] PAF-acether metabolized was 50.3 ± 5.2% (9 experiments). This conversion was inhibited in a dose-dependent manner by various concentrations of ginkgolides A, B and C (BN 52020, 52021, 52022) known as specific antagonists of PAF-acether. The three compounds displayed the following order of potency: BN 52021 (IC_{50} = 3.6×10^{-6} M) > BN 52020 (IC_{50} = 9.7×10^{-6} M) > BN 52022 (IC_{50} = 37.6×10^{-6} M). As this order is the same as that previously defined for inhibition of platelet aggregation to PAF-acether or for inhibition of PAF-acether binding to platelets, our data bring further support to the view that PAF-acether metabolism in platelets involves in some way its binding to its membrane receptor.

Platelet activating factor (PAF-acether or 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine)§ is a lipid mediator derived from numerous cells and able to promote various specific responses such as platelet secretion and aggregation, neutrophil activation, bronchoconstriction, hypotension [1–3]. Owing to this large panel of biological effects, PAF-acether is thought to be possibly involved in various pathophysiological states, including inflammation and thrombosis [1–3]. This stimulated research for specific antagonists of the mediator, a number of which have been shown to compete for binding of PAF-acether to its putative membrane receptor [4, 5]. Among them, ginkgolides A,B and C (BN 52020, 52021, 52022, see structure in Fig. 1) represent an interesting group of PAF-acether antagonists from natural origin, displaying no evident structural analogy with the lipid mediator [6].

Besides its well known biological effects, PAF-acether is actively metabolized in platelets through the sequential action of acetylhydrolase and transacylase, allowing its conversion into membrane 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) [7–12]. In a previous work [13], we reported that BN 52021 strongly reduced PAF-acether metabolism in rabbit platelets. Instead of a direct inhibition of any of the two herefore mentioned enzymes, this effect involved an inhibition of PAF-acether transport through the membrane, which represents the limiting step of its metabolism [13]. Since BN 52021 is able to displace PAF-acether from its membrane receptor [14, 15], to inhibit the various events (Ca^{2+} movements, phosphoinositide metabolism) involved in the mechanism of signal transduction [16], and to inhibit platelet physiological responses [5, 6, 14, 15], we proposed that transmembrane movement of PAF-acether through the platelet plasma membrane was related to one of these effects [13].

In the present study, we have compared three ginkgolides for their ability to inhibit the metabolic conversion of PAF-acether in rabbit platelets. Our data show that the three compounds display the same order of potency as that previously defined for their

	R_1	R_2	R_3
BN 52020	OH	H	H
BN 52021	OH	OH	H
BN 52022	OH	OH	OH

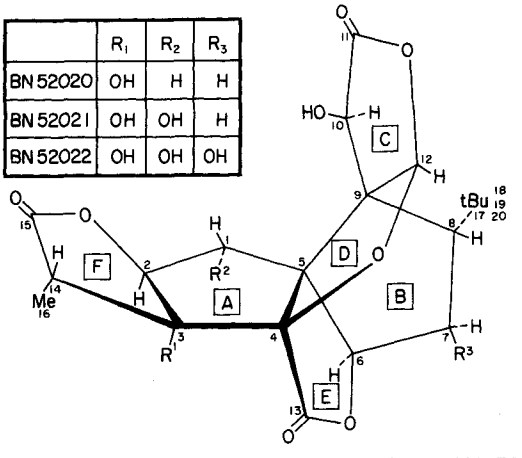

Fig. 1. Structure of ginkgolides A, B and C (BN 52020, BN 52021 and BN 52022, respectively).

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§ Abbreviations used: PAF-acether, platelet activating factor or 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; alkylacyl-GPC, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine.

inhibition of platelet function or PAF-acether binding.

MATERIALS AND METHODS

Chemicals. 1-*O*-[^{3}H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine ([^{3}H] PAF-acether, 90 Ci/mmole) was from the Radiochemical Center, Amersham, U.K. Ginkgolides A, B and C (BN 52020, 52021 and 52022, respectively, see structure in Fig. 1) were from IHB-IPSEN, Institute for Therapeutic Research, Le Plessis Robinson, France. Bovine serum albumin, essentially fatty acid-free, was from Sigma, St. Louis, MO).

Platelet suspensions. Blood was obtained from male albino rabbits by ear vein puncture and withdrawn onto anticoagulant (1 vol. of 0.2 M EDTA, pH 7.0 for 30 vol. of blood). Platelet-rich plasma was prepared by centrifugation of anticoagulated blood at 340 *g* for 20 min. Platelets were sedimented at 1000 *g* for 10 min and washed once in Tyrode buffer (pH 6.5) lacking calcium and containing 1 mM $MgCl_2$, 1 mM EGTA, 1 g/l glucose and 0.25 g/l bovine serum albumin as described by Ardlie *et al.* [17]. After one wash in this medium, platelets were finally suspended in Tyrode buffer (pH 7.35) containing 1 mM $MgCl_2$, 1 g/l glucose and 0.25 g/l bovine serum albumin [17]. The final volume was adjusted to give a platelet concentration ranging around 1.5×10^9 cells/ml. The whole procedure was performed at room temperature.

Incubation of platelet suspensions. Platelets (0.25 ml) were mixed with various concentrations of ginkgolides (10^{-6} to 10^{-3} M) solubilized in dimethylsulfoxide. The solvent (0.002 ml) was also added to control samples. After addition of [^{3}H] PAF-acether (31500 dpm, 0.6 nM, final concentration), incubation was performed at 37° for 60 min and terminated by adding 0.025 ml of EDTA (0.2 M, pH. 7.4) and 1 ml of a chloroform/methanol mixture (1/2, v/v).

Lipid analysis. Lipids were immediately extracted according to Bligh and Dyer [18], following acidification with 0.016 ml of formic acid [19]. The radioactive phospholipids were separated by thin layer chromatography on silica gel plates, 0.25 μm thick (Merck, Darmstadt, F.R.G.) according to Skipski *et al.* [20]. The radioactive spots, essentially PAF-acether, alkylacyl-GPC and 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF-acether) were detected using a Berthold LC 283 thin layer radioactivity monitor (Munich, F.R.G.) and identified by comparison to authentic pure standards. Although this procedure allowed a direct quantitation of the different radioactive compounds, each radioactive spot was directly scraped into scintillation vials containing 10 ml Instafluor (Packard). Radioactivity was determined using a Packard Tricarb spectrometer (model 4530) equipped for automatic quenching correction.

RESULTS

Upon incubation with rabbit platelet suspensions, [^{3}H] PAF-acether was actively metabolized into [^{3}H] alkylacyl-GPC. Within 60 min at 37°, the percentage of conversion attained 50.3 ± 5.2% (mean ± SEM, nine experiments). When the same incubation was performed in the presence of increasing concentrations of ginkgolides, [^{3}H] PAF-acether progressively accumulated and this paralleled a concomitant decrease in the radioactivity of [^{3}H] alkylacyl-GPC (Fig. 2). There was no evidence for accumulation of [^{3}H] lyso-PAF-acether, whose radioactivity always remained below 10%. Some discrete increase of [^{3}H] lyso-PAF-acether could be observed at 10^{-3} M of BN 52021 (Fig. 2B) and 10^{-3} M BN 52022 (Fig. 2C), but this was never significant.

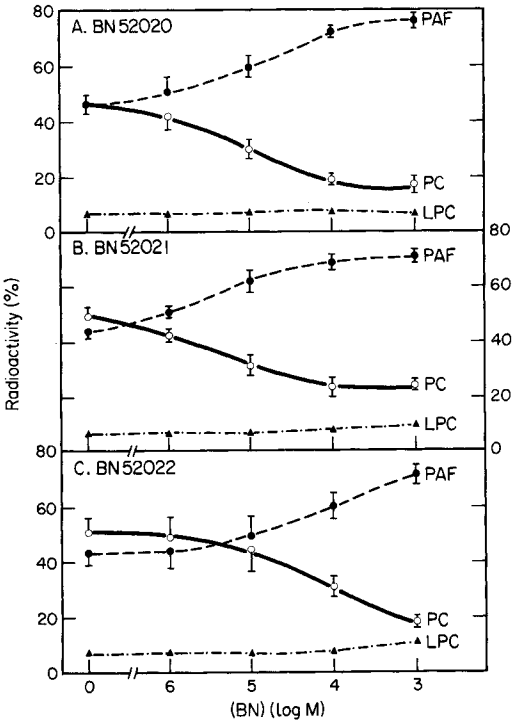

Fig. 2. Effects of BN 52020 (A), BN 52021 (B) and BN 52022 (C) on [^{3}H] PAF-acether conversion into [^{3}H] alkylacyl-GPC by rabbit platelet suspensions. Data are expressed as percentages of the total lipid radioactivity and are means ± SEM of 4–13 determinations. Abbreviations: PAF, platelet activating factor or PAF-acether; PC, phosphatidylcholine (actually 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine); LPC, lysophosphatidylcholine (actually 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine or lyso-PAF-acether).

A careful examination of the curves depicted in Figs 2A, 2B and 2C revealed some differences in the potency of the three ginkgolides. A more quantitative comparison of the effects of the three ginkgolides is presented in Fig. 3. At the highest concentrations used, BN 52020, BN 52021 and BN 52022 inhibited [^{3}H] alkylacyl-GPC formation by 57.2 ± 11.4% (four experiments), 52.6 ± 4.7% (six experiments) and 62.8 ± 3.1% (six experiments), respectively. These differences were not significant. However, the data in Fig. 3 also revealed a progressive shift to the right of the dose–response curves established for BN 52021, BN 52020 and BN 52022, respectively. Confirming this fact, Table 1 indicates that the IC_{50} determined for the three ginkgolides were in the same increasing order. Table 1 also

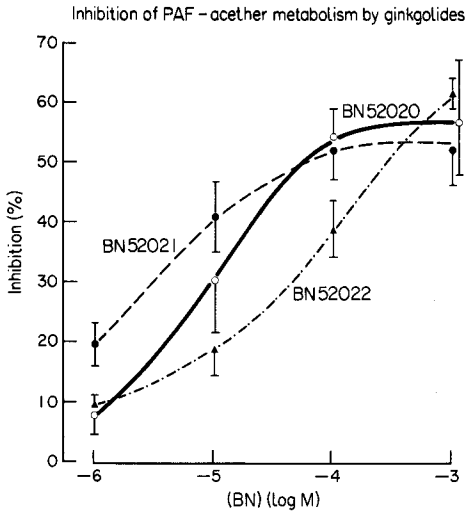

Fig. 3. Dose-dependence of the inhibition by BN 52020, BN 52021 and BN 52022 of [^{3}H] PAF-acether metabolism in rabbit platelet suspensions. Data are the means ± SEM of 4–13 determinations.

compares these data to those previously obtained for rabbit platelet aggregation induced by PAF-acether and for [^{3}H] PAF-acether binding to rabbit platelet membranes [13]. It is interesting to note that the same order of potency was also previously found between the three ginkgolides.

DISCUSSION

One interest of the use of ginkgolides for the purpose of the present study resides in their lack of structural analogy with PAF-acether. This probably explains their failure to inhibit acetylhydrolase and transacylase [13] and enables us to propose a relationship between their inhibition of PAF-acether metabolism and their effects on PAF-acether receptor. Our present data further document such a relationship, since the same order of potency was observed for the three compounds when comparing their ability to reduce metabolism of the precursor with their inhibition of platelet aggregation and of [^{3}H] PAF-acether binding to its receptor (Table 1). In other words, the more potent a compound is in displacing [^{3}H] PAF-acether from its receptor, the more potent it is in inhibiting aggregation or in reducing [^{3}H] PAF-acether metabolism.

One could argue against this that the IC_{50} values presented in Table 1 are not identical, for each compound, when considering various platelet responses. One reason for this could come from differences in the experimental conditions used for the different tests: for instance, PAF-acether concentration was 0.6 nM for metabolic studies, 5 mM for aggregation and 1 nM for binding experiments; the latter ones were performed using isolated membranes at 0°, instead of intact platelets at 37° in the other cases; data on platelet aggregation were obtained on platelet-rich plasma and not with washed platelet suspensions. Another possibility to explain the differences observed in the absolute IC_{50} values determined for each ginkgolide depending on the parameter studied might be that each platelet response to PAF-acether requires its own receptor occupancy. For instance, we previously reported, using human platelets, that half-maximal effect of PAF-acether on Ca^{2+} movements occurred at around 1 nM, whereas phosphatidic acid synthesis displayed a shift to higher PAF-acether concentrations of one order of magnitude [16]. Consistent with this, higher concentrations of BN 52021 were required to inhibit Ca^{2+} movements than to depress phosphatidic acid synthesis [16].

Thus our data strongly suggest that PAF-acether metabolism by rabbit platelets is one of the consequences of PAF-acether binding to its receptor. Of course, one cannot definitely exclude that, besides the specific membrane receptor evidenced from binding experiments [4, 5], platelets might possess other binding sites related to a putative transporter, since transmembrane movement of PAF-acether appears to be the limiting step of its metabolism [13]. However, further indication that the effects of ginkgolides described in our previous [13] and present studies involve PAF-acether receptor comes from a recent work of Pieroni and Hanahan [21]. These workers have shown that PAF-acether (also called acetylglyceryl ether phosphocholine or AGEPC) strongly stimulates platelet metabolism of various lysophospholipids. These include lyso-PAF-acether, which is normally converted at a low rate by intact platelets [7–9, 13], owing probably to a slow transmembrane movement, compared to PAF-acether [13]. It thus appears that, besides the various effects occurring after binding to its receptor (Ca^{2+} influx, phosphoinositide metabolism and Ca^{2+} mobilization, stimulation of protein kinase C, shape change, aggregation, secretion), PAF-acether promotes a membrane modification allowing a faster

Table 1. IC_{50} values of the inhibition by BN 52020, BN 52021 and BN 52022 of [^{3}H] PAF-acether metabolism by rabbit platelets, of rabbit platelet aggregation and of [^{3}H] PAF-acether binding to rabbit platelet membranes

	IC_{50} (μM)		
	[^{3}H] PAF-acether metabolism*	Aggregation‡	[^{3}H] PAF-acether binding‡
BN 52021	3.6 ± 1.3 (3)†	0.32	0.25
BN 52020	9.7 ± 2.8 (5)	1.32	0.94
BN 52022	37.6 ± 7.5 (5)	25.4	17.0

*Mean ± SEM; † Number of determinations; ‡ taken from ref. 14.

translocation of various lipid compounds from the external side to the inner leaflet of the membrane. This would explain why PAF-acether is converted much faster than lyso-PAF-acether in intact platelets, why such a difference does not appear with lysed platelets [13] and why ginkgolides specifically inhibit this metabolism as long as whole platelets are used (this study and ref. [13]).

However, such a membrane effect does not seem to be specific for PAF-acether, since the same membrane change also occurs in response to thrombin [21]. There are some examples reported in the literature where platelet activation by A23187, thrombin + collagen or phospholipase C induces dramatic changes in the organization of the lipid bilayer forming the platelet plasma membrane [22–25]. These involve a transfer to the external face of the platelet membrane, by a flip-flop process, of phosphatidylserine, which is normally confined to the internal half of the plasma membrane from resting platelets [26, 27]. In this context, one could recall a preliminary report from Bevers *et al.* [28] showing that PAF-acether promotes a similar, although more discrete, change in rabbit platelets, whose mechanism would merit further investigations. So, the model of rabbit platelet activation by PAF-acether and its inhibition by specific antagonists like ginkgolides could reveal very helpful to determine such a relationship.

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