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Sensitization of human neutrophil defense activities through activation of platelet-activating factor receptors by ginkgolide B, a bioactive component of the *Ginkgo biloba* extract EGB 761

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Abstract

Ginkgolide B (GKB, BN 52021) was described as a platelet-activating factor (Paf) receptor antagonist. However, it is not known whether all GKB biological effects are mediated through Paf receptor antagonism only. To gain insight into the drug mode of action, we investigated here the effects of GKB *per se* on functional and signaling activities in human polymorphonuclear leukocytes (PMN). Treatment of PMN with GKB (0.5–12 μM) stimulates a rapid and weak production of reactive oxygen species determined by chemiluminescence. ROS production required the activation of protein kinase C (PKC), tyrosine kinases and p38 mitogen-activated protein kinase as indicated by inhibitory effects of, respectively, GF 109203X (IC_{50} of 0.5 μM), genistein (IC_{50} of 0.5 μM) and SB 203580 (IC_{50} of 0.2 μM) or SB 202190 (IC_{50} of 1.1 μM). GKB stimulated a *Pertussis* toxin-sensitive PLD activity assessed by the formation of tritiated phosphatidic acid and choline. By contrast, GKB did prevent the Paf-mediated PLD activity and CL response (IC_{50} of 2 μM). Interestingly, both GKB and Paf-induced CL response were prevented by selective Paf antagonists such as CV 6209 or WEB 2086 indicating that GKB may directly activate Paf receptors. Finally, GKB potentiated the CL response induced by fMet-Leu-Phe and zymosan. These results show that GKB is the first partial agonist of the Paf receptor described so far capable of priming the polymorphonuclear leukocyte function.

Keywords: Paf antagonist; Phospholipase D; Chemiluminescence; Partial agonist

1. Introduction

Paf, one of the most potent membrane-derived mediators [1,2], has been implicated in numerous physiological and pathological processes (reviewed in [3–5]). Paf exerts its biological action through specific receptors that are expressed by various cell types including PMN. Stimulation of PMN by Paf or other classical chemoattractants such as formyl Met-Leu-Phe (fMLP) or complement-derived C5a, triggers various cellular responses such as directed locomotion (chemotaxis), generation of superoxide anion and related reactive oxygen species (respiratory burst) and release of granules content (exocytosis), which all contribute to the microbicidal functions of PMN [6]. Defense activities of PMN may be potentiated by other agonists or by drugs that alter intracellular signaling events. This phenomenon, termed ''priming'' [7,8] causes either beneficial or adverse effects depending on the extent of PMN stimulation. A moderate stimulation may improve defense activities of PMN whereas excessive oxidant production may cause tissue damage [9,10].

GKB, a natural triterpene, was shown to be a competitive Paf receptor antagonist in various tissues [11,12] including human PMN [13]. GKB is one of the bioactive components of extract 761 of the leaves of *Ginkgo biloba* (EGB 761), a standardized extract of the leaves of the fossil tree *G. biloba*, which has been used in traditional Chinese and in occidental medicine for more than 25 years. Beneficial

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Abbreviations: PMN, polymorphonuclear leukocytes; GKB, ginkgolide B; CL, chemiluminescence; ROS, reactive oxygen species; PTX, *Pertussis* toxin; PLD, phospholipase D; DAG, diacylglycerol; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; Paf, platelet-activating factor; EGB 761, extract 761 of the leaves of *Ginkgo biloba*.

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effects of EGB 761 were reported on vascular tonus, stress and cognitive functions (reviewed in [14–16]) during aging or in patients suffering from Alzheimer's disease [17,18]. EGB 761 contains about 3% of ginkgolides A, B and C, among which ginkgolide B is the most potent Paf antagonist. GKB has anti-oxidant properties which are involved in its anti-ischemic and cardioprotective effects in experimental animal models [19,20]. GKB was also found to reduce the mortality rate of a group of patients with Gram negative sepsis [21]. GKB antagonizes various functions of human PMN stimulated by Paf *in vitro*, including chemotaxis [11,22], respiratory burst and exocytosis [22], and aggregation [11]. However, it is not known whether all GKB-promoted biological effects are dependent on its antagonism on the Paf receptor or the existence of other potential mechanisms as suggested by a recent study on the production of cytokines by the rat microglia [23].

In this study, we investigated molecular mechanisms underlying the biological effects of GKB on PMN. The effects of GKB on production of reactive oxygen species (ROS) and phospholipase D activity of human PMN were studied. Our data indicate that both activities were stimulated by GKB and support a role of GKB as partial agonist of the Paf receptor. In addition, GKB was found to enhance the PMN ROS production induced by the bacterial chemotactic peptide fMLP and zymosan particles, indicating it has potential to prime cell functions.

2. Materials and methods

2.1. Reagents

1-*O*-[^{3}H]octadedyl-*sn*-glycero-3-phosphocholine (specific activity 160 Ci/mmol) was from Amersham and precoated silica gel G plates (0.25 mm thick) were from Merck. Dextran T500 was from Pharmacia; *Pertussis* toxin (PTX) was from List biological; SB 203580, SB 202190 was from Calbiochem; CV6209 was from Biomol; WEB 2086 (Apafant) was a gift from Boehringer and other reagents were from Sigma Co. Stock solutions of fMLP, phorbol myristate acetate (PMA), Paf, were prepared in DMSO. All organic solvents were HPLC-grade. Zymosan particles were opsonized by incubating 10 mg zymosan in 1 mL human serum for 30 min at 37°. The zymosan suspension was washed twice in PBS and used at a final concentration of 0.375 mg/mL. Ginkgolide B (BN 52021) provided by Beaufour-Ipsen Pharma, was used in his water soluble formulation, i.e. lyophilisate of the partially salified ginkgolide B by NaOH. The purity of each preparation of GKB (>99%) was checked by HPLC.

2.2. PMN preparation

Human venous blood, heparinized at 10 unit/mL, was obtained from healthy volunteers. PMN were isolated by a first step sedimentation of whole blood on 2% Dextran T500 followed by centrifugation of the granulocyte-rich supernatant on a cushion of a mixture of Ficoll and Hypaque (from Eurobio), as previously described [24]. The purified PMN (97%) were subjected to hypotonic lysis for 20 s, washed and resuspended in Hanks balanced salt solution (HBSS) at pH 7.4 containing 10 mM Hepes.

2.3. PMN respiratory burst

The respiratory burst of PMN was studied by the luminol-enhanced chemiluminescence (CL) assay [25,26] by using a thermostated Packard Picolite luminometer. Suspensions of 10^6 PMN in 300 μL HBSS were incubated at 37° for 10 min then treated with GKB, fMLP, Paf or opsonized zymozan in conditions described in Fig. 1. The final concentration of DMSO did not exceed 0.05% and had no discernable effect on the PMN respiratory burst. Results represent the peak of PMN CL and are expressed either in cpm or as percentages of control values (routinely between 8×10^3 and 50×10^3 cpm). In some experiments, the contribution of ROS production to CL response of PMN was checked by measuring the inhibitory effect of a mixture of superoxide dismutase (4 unit) and catalase (2 unit).

Respiratory burst was also studied by continuously monitoring the reduction of cytochrome *c* [24] using a Perkin-Elmer Lambda 40 spectrophotometer equipped with thermostated (37°) cuvette holder and magnetic stirrer. Suspensions of 4×10^6 PMN in 2 mL of HBSS were incubated at 37° under stirring for 10 min before treatment with various concentrations of GKB.

2.4. Phospholipase D assay

Phospholipase D (PLD) activity of PMN was studied by two different assays. The first one is based on the use of a radiolabeled precursor of phosphatidylcholine and the other on the measurement of mass choline. PMN labeling was performed in calcium-free HBSS (20×10^6 cells/mL) containing 10 mM Hepes 0.1% BSA and 5 μCi/mL 1-*O*-[^{3}H]-octadecyl-*sn*-glycero-3-phosphocholine for 1 hr [27,28]. PMN were washed twice and suspended in HBSS. Aliquots of 3×10^6 labeled PMN in 400 μL of HBSS were prewarmed for 10 min at 37° and then treated in the absence (control) or presence of GKB in conditions described in Fig. 1. For the measurement of phosphatidylethanol (Pet), ethanol (1%) was added to the medium 3 min before stimulation of PMN by Paf. In some experiments, PLD activity was studied in PMN treated with 5 μg/mL cytochalasin B, a fungal metabolite known to enhance chemoattractant-mediated PLD pathway. The reaction was stopped with 1.5 mL of chloroform/methanol/acetic acid (100/200/4) and lipids were separated by thin layer chromatography. A solvent system consisting of chloroform/methanol/acetic acid (65/15/2) was used to resolve phosphatidic acid (Rf = 0.42) from Pet (Rf = 0.62), as described elsewhere

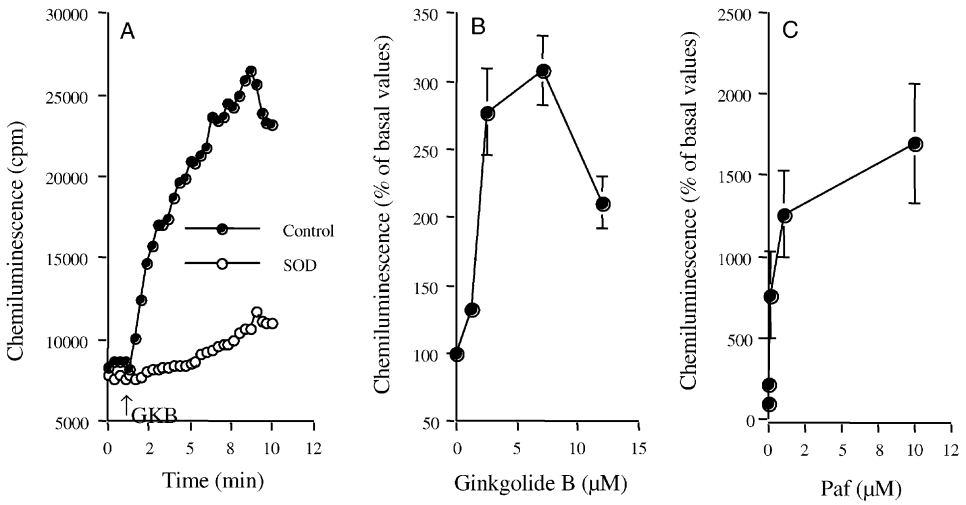

Fig. 1. Comparison of the stimulating effects of ginkgolide B and Paf on PMN CL response. Suspensions PMN were incubated in the presence of luminol for 10 min, then treated with the indicated concentration of ginkgolide B or Paf. (A) Time course study of PMN CL induced by 2.4 μM GKB (indicated by an arrow) in the absence (control) or presence of superoxide dismutase (SOD) and catalase; (B and C) the net peak of CL induced by various concentrations of GKB or Paf, respectively, and are expressed as percentage of basal values obtained before stimulation. Results are the means of six experiments.

[27]. 1-*O*-alkyl-2-acyl-glycerol (EAG, Rf = 0.62) was separated by using chloroform/methanol/acetic acid (98/2/1) [28]. In some experiments, PMN were treated in the absence or presence of 1 μg/mL PTX for 2 hr at 37° and then labeled with tritiated lyso-PC for an additional hour still in the presence of PTX. Under these conditions, most of the PTX-sensitive G proteins were inactivated [29].

PLD activity was also studied by measuring mass choline formation using a CL assay [26]. Briefly, resting and stimulated PMN (10^7 cells/mL) were sonicated in 200 mM phosphate buffer, pH 8.6 and centrifuged (11 000 *g*) for 5 min. Supernatants were kept on ice and choline content was assessed in 500 μL phosphate buffer containing 20 μM luminol, horse peroxidase (1 unit) and choline oxidase (1.25 unit). The reaction was initiated in the dark by addition of choline oxidase and the light emission was monitored every 5 s. Calibration curves were established in the presence of exogenous choline chloride. The height of the luminescence peak was used to calculate choline content [26].

2.5. Statistical analysis

Each experiment was performed in duplicate and repeated at least four times. Unless otherwise indicated, data represent means ± SEM. Statistically significant differences between means were identified by using Student's paired *t*-test with a threshold of $P < 0.05$.

3. Results

3.1. Stimulation of PMN respiratory burst by GKB and its down-regulation by protein kinase inhibitors

The action of GKB was investigated on PMN ROS production determined by the highly sensitive luminol-enhanced CL assay which offers the advantage of combining the measurement of both respiratory burst and release of granular content (exocytosis). Peroxidases released during this latter process catalyze the oxidation of luminol in the presence of hydrogen peroxide, a reaction in which light emission is enhanced [25]. Treatment of PMN with GKB triggered a rapid and transient CL response (Fig. 1A). The maximal CL response was obtained in the presence of 2–5 μM GKB whereas a weaker CL response was induced by higher concentrations of GKB (Fig. 1B). In some experiments, the peak of CL occurred at early times (between 3 and 8 min). This variability, which may due to differences in the basal state of PMN activation, was also observed to a lesser extent with potent stimuli of PMN such Paf or fMLP. The maximal GKB-promoted CL response was weak (approximately 3-fold increase over basal levels) and was five times weaker than that induced by Paf (15–20-fold increase over basal levels) (Fig. 1C). The CL response induced by GKB was inhibited by approximately 80% ($P < 0.01$) in the presence of superoxide dismutase (4 unit) and catalase (2 unit), consistent with the production of ROS by PMN (Fig. 1A). A similar inhibition was observed on Paf-induced PMN CL response (data not shown).

To further explore the mechanism of CL stimulation by GKB, we investigated the contribution of major intracellular signaling effectors such as PKC, tyrosine kinases and the p38 MAPK, which were previously shown to be involved in chemoattractant-mediated PMN respiratory burst [30,31]. For this purpose, PMN were treated for 10 min with either a selective inhibitor of PKC, GF 109203X, a non-selective inhibitor of tyrosine kinases, genistein, or the selective p38 MAP kinase inhibitors SB 203580 or SB 202190, before the stimulation with GKB. These drugs induced a concentration-dependent inhibition of the GKB-stimulated CL ($P < 0.01$) with an IC_{50} value of

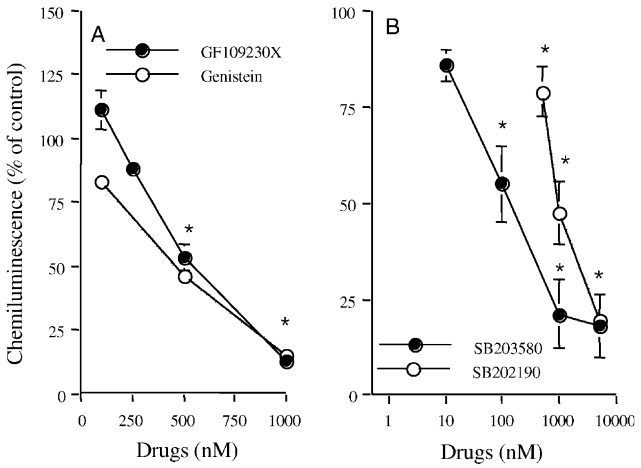

Fig. 2. Effect of GF 109203X, genistein (A), SB 203580 and SB 202190 (B) on the PMN CL induced by ginkgolide B. PMN were treated in the absence (control) or presence of various concentrations of each inhibitor for 10 min at 37°, then stimulated with 2.4 μM GKB. Results are expressed as percentage of control values and represent the peak of CL response (mean of four to six experiments), ∗ represents statistically significant difference between control and drug-treated PMN ($P < 0.05$).

approximately 0.5 μM for GF 109203X and genistein (Fig. 2A); 0.2 μM for SB 203580 and 1.1 μM SB 202190 (Fig. 2B). The inhibition induced by these drug affected predominantly the peak of CL and to a lesser extent its duration (data not shown). We confirmed that the Paf-induced CL response was also blunted by the inhibitors with an IC_{50} value close to that observed in the presence of GKB (data not shown), suggesting that Paf and GKB may stimulate respiratory burst through similar mechanisms.

3.2. Stimulation of PLD activity of PMN by ginkgolide B and its inhibition by Pertussis toxin

The respiratory burst induced by classical chemoattractants such as Paf is dependent on generation of second messengers by various phospholipases [32,33]. PLD is a major source of phosphatidic acid (PA) and diglycerides and was shown to regulate cellular functions in many cell types including the respiratory burst in PMN [34,35]. We therefore investigated whether GKB-stimulated PLD activity in PMN labeled with 1-*O*-[^{3}H]octadecy-*sn*-glycero-3-phosphocholine. Treatment of PMN in the presence of GKB triggered a significant increase in the basal amount of tritiated PA ($P < 0.05$) which was maximal at 1 min and was transient (Fig. 3A). The PA level increased up to 50% above basal values and did not appear to be dephosphorylated by PA hydrolase since the basal level of 1-*O*-alkyl-2-acyl-3-glycerophosphocholine measured during this period was not changed (data not shown). PA formation was proportional to GKB concentration in the range of 0.12–1.2 μM (Fig. 3B). Higher concentrations (2.4 and 4.8 μM) did not further increase the PA production rather GKB at a higher concentration (7.1 μM) was less effective. Activation of PLD by GKB was confirmed by measuring choline formation. A concentration–response curve similar to that of PA formation was obtained (Fig. 3C).

Stimulation of PLD activity in human PMN can be achieved through distinct pathways with or without the involvement of PTX-sensitive Gi proteins [36,37]. Activation of phospholipases can also be induced by agents which activate directly PTX-sensitive G proteins such as staurosporine, a protein kinase inhibitor [38], or mastoparan [29]. To determine whether G proteins are involved in the mechanism of PLD activation by GKB, PMN were treated with PTX using experimental conditions where most of the PTX-sensitive G proteins are inactivated [29]. Under these conditions, the production of PA induced by GKB was completely inhibited (Fig. 4A). Paf-induced formation of PA, although of higher amplitude than that elicited by GKB, was also completely inhibited by PTX (Fig. 4B). This inhibition was not due to a cytotoxic effect of PTX since PTX did not alter the PA production induced by

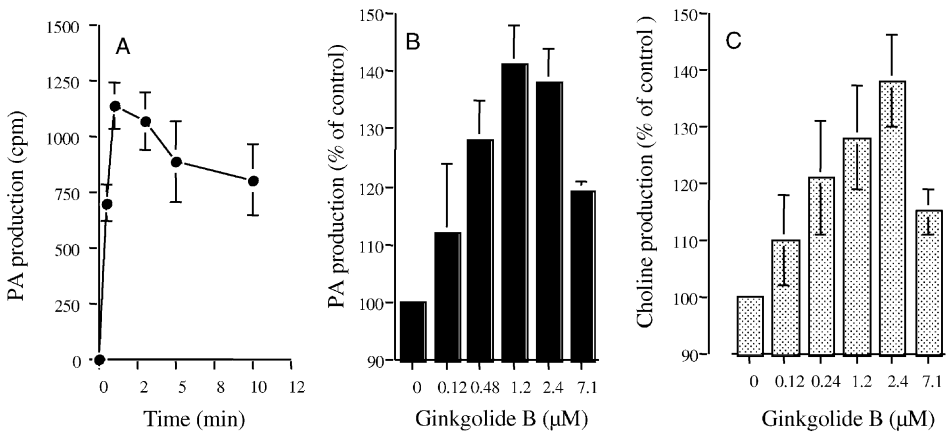

Fig. 3. Ginkgolide B stimulates PLD activation in PMN. Labeled PMN were incubated in the absence (control) or presence of various concentrations of GKB. (A) Time course of net production of PA induced by 2.4 μM GKB and expressed in dpm per 3×10^6 PMN; (B) GKB concentration effect on the production of tritiated PA expressed as percentage of control values (1325 ± 180 dpm per 3×10^6 PMN); (C) formation of mass choline expressed as percentage of resting cells (610 ± 80 pmol/10^7 cells). Values represent the means of four experiments.

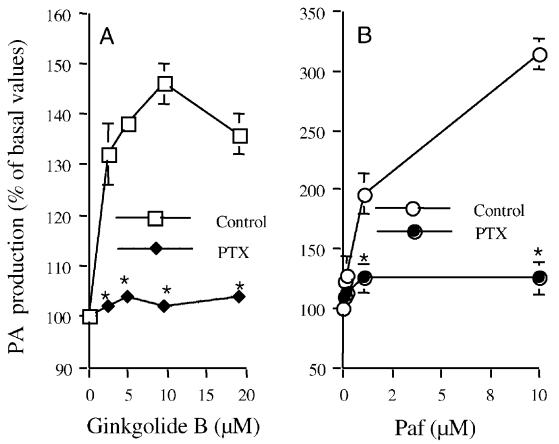

Fig. 4. Effect of *Pertussis* toxin on the ginkgolide B and Paf-induced PA formation. PMN were treated in the absence (control) or presence of PTX. The PA production was induced by various concentrations of GKB (A) or Paf (B) and is expressed as percentages of control basal values (means of the experiments), ∗ represents statistically significant difference between control and PTX-treated PMN ($P < 0.05$).

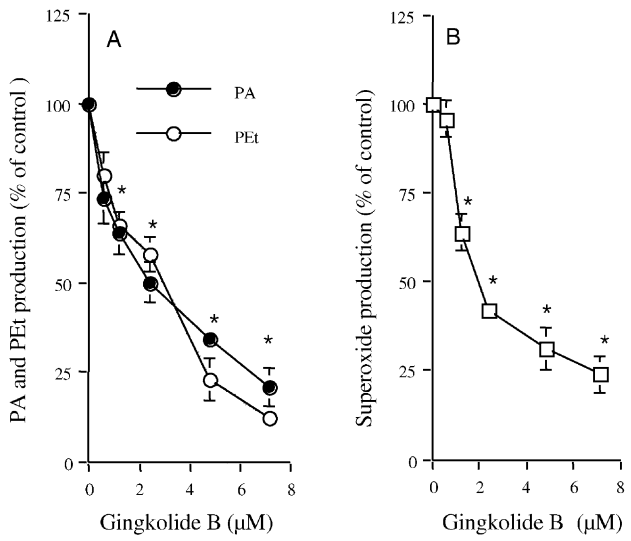

Fig. 5. Ginkgolide B inhibited Paf-mediated PLD activity and respiratory burst of PMN. (A) PLD activity was measured in PMN which were treated in the absence (control) or presence of various concentrations of GKB for 10 min and stimulated with 10 μM Paf for 1 min for measurement of tritiated PA. For measurement of tritiated Pet, PMN treated or not with GKB were incubated in the presence of 1% ethanol for 3 min before stimulation with Paf. Results are the means of three experiments and are expressed as percentage of control values (9407 ± 399 and 4421 ± 230 dpm for PA and Pet, respectively). (B) Superoxide production was measured after treatment of PMN in the absence (control) or presence of GKB for 10 min and stimulation with 10 μM Paf. Results are expressed as percentage of control values (8 ± 2 nmol), ∗ represents statistically significant difference between control and GKB-treated PMN ($P < 0.05$).

100 nM phorbol myristate acetate (data not shown). These data suggest a role of Gi protein in the mechanism of PLD activation by GKB.

3.3. GKB prevented Paf-mediated PLD activity and respiratory burst of PMN

Previous studies indicated that GKB is a Paf receptor antagonist in various tissues [11,12] including PMN [13]. Since Paf potently stimulates PLD and the respiratory burst in PMN [39], we studied the antagonistic effects of GKB on Paf-mediated activation of PLD. PMN were treated with various GKB concentrations for 10 min and then stimulated with Paf for 1 min. GKB strongly inhibited PA production in a concentration effect experiments with an IC_{50} of approximately 2 μM (Fig. 5A, $P < 0.01$). The antagonistic effect of GKB on PLD activity was confirmed by measuring the formation of Pet, a specific marker of PLD activation. Similarly, GKB inhibited the Paf-mediated respiratory burst of PMN studied by the cytochrome *c* reduction assay ($P < 0.05$, Fig. 5B). However, under the conditions used here for superoxide measurement, GKB *per se* did not induce detectable production of superoxide. This may be due to the low sensitivity of the cytochrome *c* assay [13,24] versus CL.

3.4. Effect of the Paf antagonist CV 6209 and WEB 2086 on PMN respiratory burst

To determine whether the stimulating effects of GKB may result from a direct interaction of GKB with Paf receptors or from a distinct mechanism, Paf receptors were blocked with a potent Paf antagonist, CV 6209 [40]. Unexpectedly, the treatment of PMN with CV 6209 stimulated a bell-shaped increase of the CL response in PMN, maximal at 1 nM (Fig. 6A, $P < 0.05$), which was inhibited by superoxide dismutase and catalase (data not shown). Interestingly, low concentrations of CV 6209 (10^{-11} to 10^{-9} M) inhibited the peak of CL response induced by GKB (Fig. 6B, $P < 0.05$). CV 6209 also inhibited the PMN CL induced by Paf consistent with its antagonistic effect (Fig. 6C, $P < 0.05$).

The ability of CV 6209 to prevent the stimulation of respiratory burst by GKB indicates that GKB may stimulate the Paf receptors as a partial agonist. Alternatively, the inhibitory effects of CV 6209 may result from an heterologous desensitization process, independently of CV 6209 antagonistic effect on Paf receptors. If this would be the case, CV 6209 would also inhibit the PMN response induced by other PMN stimuli. To investigate this possibility, the effects of CV 6209 and GKB were studied on the CL response of PMN induced by fMLP, which like Paf, stimulates membrane receptors coupled to G protein. Both compounds potentiated the CL induced by 0.1 μM fMLP (Figs. 6C and 7). GKB also improved the CL induced by the stimulation of PMN with zymosan particles (Fig. 7). These priming effects of GKB are more consistent with its role as a partial Paf receptor agonist rather than with the induction of a desensitization process.

The effect of another Paf antagonist, WEB 2086 (Apafant) was also investigated on GKB and Paf-induced CL.

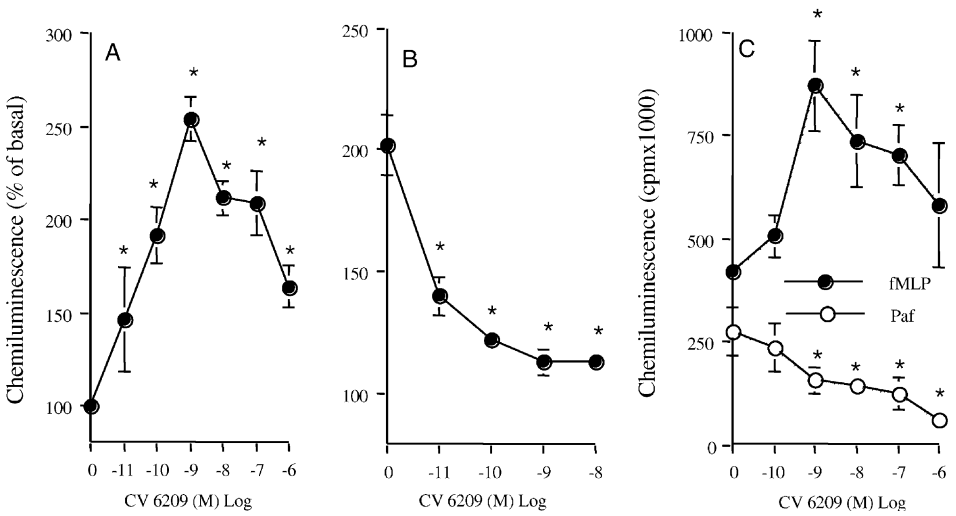

Fig. 6. Effect of CV 6209 on the CL of resting and stimulated PMN. (A) PMN were treated with various concentrations of CV 6209 alone and the CL response of PMN was expressed as percentage of basal values. After the CL response returned to a stable value, CV 6209-treated and untreated (control) cells were stimulated with 2.4 μM GKB (B), results are expressed as percentage of basal values of A. (C) PMN were treated in the absence (control) or presence of various concentrations of CV 6209 for 10 min and stimulated with 1 μM Paf or fMLP, results represent the net CL response of PMN expressed in cpm. Data represent the peak of CL and are the means of four to six experiments, ∗ represents statistically significant difference between control and CV 6209-treated PMN ($P < 0.05$).

Treatment of PMN with various concentrations of WEB 2086 (25–500 nM) for 15 min also induced a concentration-dependent inhibition of the CL response triggered by 2.4 μM GKB or 1 μM Paf, with a similar IC_{50} of 68 ± 12 and 75 ± 15 nM, respectively. However, as observed with CV 6209, WEB 2086 also induced weak stimulation of the peak of CL response (data not shown) suggesting it may stimulate Paf receptors.

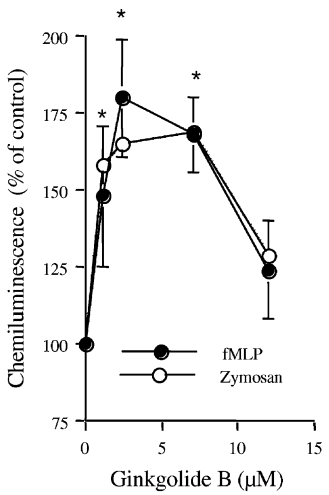

Fig. 7. Priming effect of ginkgolide B on the CL of PMN induced by fMLP and zymosan. PMN treated in the absence (control) of presence of various concentrations of ginkgolide B. After the response returned to a stable value (10 min), cells were stimulated with 1 μM fMLP or 0.375 mg/mL zymosan. Results are expressed as percentage of the CL response of control PMN stimulated with fMLP or zymosan ($833 \pm 160 \times 10^3$ and $2040 \pm 238 \times 10^3$ cpm, respectively). Values represent the means of six experiments, ∗ represents satistically significant difference between control and GKB-treated PMN ($P < 0.05$).

4. Discussion

GKB was initially described as a Paf receptor antagonist and was thus largely used to prevent the biological effects of Paf *in vitro* and in pharmacological models. The antagonist effect of GKB was further confirmed here on Paf-mediated PLD activity and respiratory burst. In this study, we show that GKB is actually a partial agonist of the Paf receptor, which may stimulate signaling pathways and production of oxidant in human PMN. As a consequence of being a partial agonist of Paf receptors, GKB sensitized PMN functions induced by various stimuli, a novel property which may be of potential pharmacological relevance.

The stimulation of Paf receptors by GKB is supported by the observation that both GKB and Paf-induced CL response in PMN could be inhibited by the Paf antagonists CV 6209 or WEB 2086. These compounds may also act as a partial agonist as they stimulate a weak respiratory burst with a characteristic bell-shaped dose–response curve similar to that induced by GKB, although CV 6209 is active on a wider range of concentrations (10^{-11} to 10^{-6} M) than GKB (0.1–12 μM). This weak stimulatory effect of CV 6209 somewhat complicates our interpretation of the subsequent inhibition observed on GKB-induced CL. Indeed, this inhibition could be interpreted as being due to a biochemical desensitization of CL response and/or an CL response induced by fMLP and zymosan strongly argues against desensitization. Note that the modifications of CL response described here were calculated with the peak value of CL which we previously showed to be a good parameter of PMN respiratory burst [26]. Some changes in the duration of the peak and lag time of the CL response were observed (data not shown).

The signaling pathways leading to the stimulation of the respiratory burst by GKB are similar to those activated by Paf and classical chemoattractant (fMLP, C5a). They include a rapid and transient PTX-sensitive activation of PLD and a positive regulation by PKC, tyrosine kinases and p38 MAP kinase. However, some differences in the regulation of CL response by high versus low GKB concentrations suggest that GKB may have a more complex action (Fig. 1). Unlike Paf, high concentrations of GKB (7–12 μM) were less active than low concentrations (0.5–5 μM), whereas higher concentrations were completely inactive (data not shown). One possible interpretation of this phenomenon is that high GKB concentrations may change the receptor conformation allowing a deactivating process, such as the activation of cyclic AMP production. The mechanism of respiratory burst deactivation by high GKB and CV 6209 concentrations is not known and may involve several processes such as the phosphorylation of the Paf receptor by protein kinases [41] or the transition between different forms of the Paf receptor, i.e. monomeric and oligomeric. This process, whose functional significance is still unclear, was reported for a number of receptors coupled to G proteins including human Paf receptors [42]. Alternatively, high GKB concentration may inhibit PKC-dependent signaling [43].

The discrepancies in the effects of high and low Paf and GKB concentrations also raise the possibility that GKB may interact with different Paf receptor subpopulations. Three distinct Paf binding sites has been described in the rat cerebral cortex on the basis of different affinities, i.e. 25 pM, for plasma membranes; 25 nM, for microsomes, and a lower affinity binding site for synapstosomal plasma membranes [44]. It is not known whether these differences result from structural properties of the receptors or biochemical modifications. GKB exhibited a higher potency as an antagonist in the synapstosomal membranes than in microsomal membranes, whereas CV 6209 showed inverse properties [44]. In the membrane of human PMN, two distinct populations of Paf receptors have been described [45,46]. One with a high affinity of 0.2 nM ($\sim 10^3$–10^4 receptors per cell) was proposed to couple to G proteins [46] and the other, with a lower affinity of 500 nM ($\sim 10^5$–10^6 receptors per cell) was less well characterized. GKB was shown to inhibit the binding of Paf acether to its specific receptors on human PMN [13] and to displace Paf acether binding with a IC_{50} of 0.25 μM, although its structural relationship with Paf is not obvious [11]. Nevertheless, this interaction appears to be specific since GKB does not interact with more than 20 other receptors tested so far [11].

The ability of GKB to increase the PMN CL response to the bacterial peptide fMLP and zymosan particles represents a novel property of this compound and indicates that the priming of PMN [7] can be induced by partial agonists. This priming effect was relatively weak (a 2-fold increase) and is in the physiological range of PMN activation. In addition, the GKB priming effect was restricted to a narrow range of drug concentrations between 0.5 and 3 μM (Fig. 7). In some experiments with human PMN primed *in vivo* or *in vitro* with the calcium ionophore A23187, the active concentrations of GKB were reduced to 0.2–0.6 μM (80–250 ng/mL). These concentrations remain relatively high in comparison with the plasma concentration of GKB in healthy volunteers (4–10 ng/mL) after oral administration of EGB [47]. It is thus unclear whether GKB may provide direct or indirect enhancement of PMN functions compatible with a pharmacological use *in vivo*. Nevertheless, it has been observed that oral administration of the *G. biloba* extract EGB 761 to healthy subjects induced a priming of PMN *in vivo* as determined by their *in vitro* CL response induced fMLP and zymosan [48]. The relevance of this observation has not been studied. A possible interpretation of this PMN priming *in vivo* is that it may result from the combined action of several bioactive components of EGB 761. One of them, GKA, a structural analog of GKB, has also anti-Paf receptor properties. GKA also primed fMLP-induced PMN CL response in our studies (data not shown) and has similar anti-oxidant properties to GKB in other studies [20,49]. GKA reaches higher plasma concentrations (20–30 ng/mL) than GKB with a better bioavailability [47]. The concentration of GKA or GKB associated with PMN *in vivo* is also a parameter that may contribute to priming of PMN. It is also worth noting that GKB was previously found to reduce the rate of mortality in a group of patients with Gram negative sepsis [21] although a smaller non-significant treatment effect was observed in a subsequent study [50] due to a larger number of patients carefully selected [51]. In these human clinical studies, it is not known whether beneficial effects of GKB were the consequence of its Paf receptor antagonism or of its priming of phagocyte function. The mechanism of PMN priming by GKB has not been investigated in detail here and may involve an activation of Paf receptor-mediated early signaling events upstream of phospholipases, particularly PLD (Fig. 3). PA directly generated by PLD has already been implicated in the priming of PMN respiratory burst by various substances such as cytokines [36,52] or drugs [53]. PLD is a major source of PA and choline in various cell types [34]. Both PA and its derivative diacylglycerol (DAG) are activators of PKC. Choline is a precursor of acetylcholine in the nervous system [54]. It may thus be of interest to study whether the GKB priming property contributes to the various beneficial effects of the *G. biloba* extract EGB 761, particularly in cognitive function, vascular tonus [15,16] and in Alzheimer's diseases [18].

In conclusion, this study provides evidence of partial agonist effects of GKB on Paf receptors which promotes stimulation of the respiratory burst in PMN and PLD activity. As a consequence of this partial agonist effect, GKB improves the PMN respiratory burst induced by heterogeneous stimuli such as bacterial peptides and

opsonized zymosan. These properties are potentially beneficial to boost the defense activity of phagocytes. This study also emphasizes the utility of considering antagonists of receptors coupled to PTX-sensitive G proteins as potential priming agents.

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