Microphysiometric Measurement of PAF Receptor Responses to Ginkgolides

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Dedicated to Professor Duilio Arigoni on the occasion of his 75th birthday

Microphysiometry was used to evaluate the effects of terpene trilactone and flavonoid constituents of Ginkgo biloba on human platelet-activating-factor receptor (PAFR). Inhibition of the platelet-activating factor response by terpene trilactones was confirmed using this functional assay. Ginkgolide B (GB) and 10-O-benzyl-GB showed the strongest inhibition (81 and 93%, resp.) of the PAFR response, while the flavonoids rutin, quercetin, and kaempferol showed negligible response inhibition. G. biloba extract mixtures were also tested, and results indicate possible synergistic effects among various components.

Introduction²). – Standardized Ginkgo biloba extracts, such as EGb 761® (Schwabe Group) [1], which is sold under a variety of trade names including Tanakan®, Rōkan® and Tebonin forte®, and BioGinkgo® 2777 (Pharmanex) are among the best-selling herbal supplements on the market [2]. G. biloba extract is thought to increase cerebral blood flow, and has generated immense interest for its reputed value in treating memory-related afflictions [3]. In fact, EGb 761 has been suggested as a treatment for Alzheimer’s disease [4], since reports have linked beneficial effects on dementia and memory to this extract [5]. Recent studies using a computerized test battery have shown positive effects of G. biloba extract on short-term working memory on healthy adults [6].

The Ginkgo tree, Ginkgo biloba L., is the only surviving member of the family of trees Ginkgoaceae that appeared in the Jurassic period 170 million years ago, when dinosaurs roamed the earth. According to the Cretaceous fossil record, the Ginkgo tree has hardly changed over the last 100 million years, and is, thus, called the living fossil [7]. A number of G. biloba natural products have been identified (Fig. 1), including ginkgolides A–C, J, and M (1–5) [8], and bilobalide (6) [9], together termed terpene trilactones (TTLs). The ginkgolides are diterpene trilactones with a cage-like skeleton consisting of six five-membered rings, i.e., a spiro[4.4]nonane carbocyclic ring, three lactones, and a tetrahydrofuran moiety. Bilobalide, a C₁₅ compound, is also a trilactone

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²) Abbreviations. CHO, Chinese hamster ovary; CNS, central nervous system; ECAR, extracellular acidification rate; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; LTP, long-term potentiation; mcPAF, methylcarbamyl PAF; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; TTL, terpene trilactone.
with a cage-like structure. The TTLs from *G. biloba* are the only known terrestrial natural products containing a \( t \)-Bu group.

Through biosynthetic studies of ginkgolides, Arigoni and co-workers showed the surprising existence of an alternative route for the genesis of these unique molecules [10]. Although it was well-known that isopentenyl diphosphate and dimethylallyl diphosphate serve as the universal precursors in terpenoid biosynthesis leading to the key intermediate mevalonate, they found the existence of a second, mevalonate-independent biosynthetic pathway starting from pyruvate and glyceraldehyde phosphate. Around the same time, Rohmer and co-workers also discovered this alternate route during biosynthetic studies of bacterial hopanoids [11].

Commercial *G. biloba* herbal extracts are complex mixtures of TTLs and other components, and contain 6–7% TTLs (roughly equal composition of ginkgolides and bilobalide) and 24–27% flavonoids. Little is known concerning the efficacy of individual extract components. Thus, the molecular basis for the action of *G. biloba* constituents on the central nervous system (CNS) and memory is poorly understood. Although TTLs comprise a fraction of the extract, these are the unique constituents of this herbal extract and are logically the primary focus of investigations into the mechanism of *G. biloba* medicinal benefits. We are interested in clarifying the effects of these terpenoid elements of the *G. biloba* extract.

In 1985, ginkgolide B (2; GB and BN 52021) was found to be a potent *in vitro* antagonist of the platelet-activating factor receptor (PAFR) [12], a G protein-coupled receptor (GPCR) expressed both peripherally and in the CNS [13]. The mechanism by which PAFR and PAF are involved with the CNS is unclear, but it is likely that the PAFR is an important target in gaining an increased understanding of the neuro-modulatory effects of TTLs. PAF is involved in several events in the CNS, including modulation of long-term potentiation (LTP). In LTP, PAF is believed to act as a retrograde messenger [14], but this role has not been settled [15]. It is unclear whether

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R^1 )</th>
<th>( R^2 )</th>
<th>( R^3 )</th>
<th>( R^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginkgolide A (GA, 1)</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Ginkgolide B (GB, 2)</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Ginkgolide C (GC, 3)</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Ginkgolide J (GJ, 4)</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Ginkgolide M (GM, 5)</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>10-O-Bu-GB (7)</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>( \text{OCH}_3 \text{C}_6 \text{H}_3 )</td>
</tr>
</tbody>
</table>

Fig. 1. TTLs from *G. biloba*. GA (1), GB (2), GC (3), and BB (6) are found in the leaves and root bark of the tree, while GJ (4) is found only in the leaves and GM (5) is found only in the root bark. 10-O-Bu-GB (7) is a synthetic analog.
ginkgolide antagonism of the PAFR modulates the role of PAF in memory, perhaps by increasing cerebral blood flow, or whether TTLs influence memory via other as yet uncharacterized targets. Increased understanding of the structure and function of the PAFR on a molecular level and the interconnection between PAFR, ginkgolides, and memory may have a significant impact on the development of medicinal therapies targeting cognitive disorders.

The microphysiometer, manufactured by Molecular Devices Corporation under the name Cytosensor® (3) is a bioassay device developed to measure proton excretion rates of living cell populations [16]. Nearly all cells acidify their environment by release of metabolic products. Hence, cell physiology and metabolism are linked to acidification of the extracellular environment. The microphysiometer uses a light-addressable potentiometric sensor (LAPS) [17] to take one pH measurement per second in each cell sample chamber with a root-mean-square noise between 0.0005 and 0.001 pH units. Extracellular acidification rate (ECAR) data is generated and plotted against time to display changes in cell metabolism coincident with the addition of receptor ligands. Notably, in contrast to a binding assay, which simply gives the relative binding affinities of various compounds, the microphysiometer provides functional information regarding the activity of applied drugs. Moreover, since radioligand-displacement studies are inherently limited to screening compounds that bind at the same site as the radioligand, this functional, nonradioactive microphysiometry assay has the additional advantage of determining the overall nature of the drug applied, such as whether it is an agonist or antagonist. With this instrument, we have characterized a subtle structure–activity relationship amongst two locked conformational isomers of ouabain-phosphate for Na⁺, K⁺-ATPase inhibition [18]. Here, we describe the use of Cytosensor microphysiometry as a valuable tool in evaluating the functional activity of PAFR antagonists.

Results. — We observed consistent and reproducible ECAR responses following the application of mcPAF (9), while much variability was observed in response to application of PAF (8; data not shown). The mcPAF analog has a response profile similar to PAF but is far more stable to hydrolysis by serum [19]. Therefore, mcPAF was used throughout our experiments in place of the native agonist PAF. To verify that the observed responses were indeed PAFR-dependent, untransfected Chinese hamster ovary (CHO) cells were treated with mcPAF. These cells did not respond to the mcPAF agonist at all (black data trace ±, Fig. 3), confirming a PAFR-mediated effect.

Additionally, our experiments showed that the fetal bovine serum (FBS) concentration in the Cytosensor cell media was critical to observing antagonist inhibition of the PAFR agonist response. Although serum starvation has been reported to potentiate ECAR responses [20], FBS was essential in our assay medium to maintain the health of transfected CHO cells used in this study. In the absence of serum, the cells died throughout the experiment and did not respond to PAF application. Experiments were

3) The Cytosensor is no longer being manufactured by Molecular Devices Corporation; instead, it is now replaced by the FlexStation or FLIPR, which perform similar functional assays with greater efficiency. These fluorescence-based instruments are designed to monitor calcium flux or membrane potential rather than extracellular pH (Molecular Devices, Sunnyvale, CA).
Fig. 2. Structure of the native PAFR agonist, PAF (8), the methylcarbamyl analog, mcPAF (9), and the PAFR antagonist, WEB2086 (10).

Fig. 3. Desensitization of the PAFR upon repeated application of mcPAF agonist
initially done with 0.2% FBS, and higher concentrations showed little benefit toward the CHO cells. Reduction of the serum concentration to 0.1% was found to increase the observed percent inhibition while only modestly attenuating the ECAR response. Thus, we proceeded with 0.1% serum.

Agonist activation of GPCRs is known to induce receptor desensitization as a component of signal transduction. Receptor desensitization results from various processes, including endocytosis or sequestration of receptors into intracellular compartments and interaction with cellular arrestins [21]. The PAFR is known to desensitize extensively [22], and this was confirmed in our experiments. Repeated mcPAF applications of increasing concentration resulted in diminished PAFR responses (grey data trace —, Fig. 3), compared with CHO-1F8 cells that were used only for a single mcPAF application (solid circles •, Fig. 3). Even at low mcPAF concentrations (1 nM), desensitization of the PAFR was apparent. Because of desensitization, each cell capsule was used for only one determination, and fresh cells were used for each inhibition experiment.

Since the TTLs are nearly insoluble in H2O, organic co-solvent was required to expose the CHO-1F8 cells on the Cytosensor to our sample TTLs. We tested a variety of organic solvents (EtOH, polyethylene glycol, DMSO) and found that these were tolerated to <10%. In high concentrations, organic solvents can destabilize the plasma membrane [23], or have an effect on functional assays [24]. We determined that a maximal co-solvent concentration of 0.1% minimized any adverse effects. At this level, an acceptable and relatively small ECAR decrease was observed upon introduction of co-solvent. DMSO was selected, since the TTL stock solutions could be prepared at the highest concentration in this solvent.

A mcPAF dose-response curve was prepared with 5 × 10^4 cells per capsule. With mcPAF concentrations ranging from 0.1 to 50 nM, and a different capsule for each concentration, the maximum ECAR (ca. 40%) was observed at 30 nM, at which point the response plateaued (solid circles, Fig. 3). Further experiments were performed with 5 nM mcPAF, which elicited the half-maximal response.

Ginkgolides, known PAFR antagonist WEB2086, and other compounds (Fig. 1, 2, and 4) were assayed at 100 μM with 5 × 10^4 cells per capsule, as in the dose-response experiment. In the Cytosensor experiments, CHO-1F8 cells were pre-treated with the test compound for 30 min prior to mcPAF application. This allowed equilibration of the cells with both the antagonist and the co-solvent. Following 10-s mcPAF application, ECAR increases were monitored. The maximum mcPAF response was usually seen within 4 min (two Cytosensor cycles), and the ECAR returned to baseline levels within 20 min. Representative raw data is depicted in Fig. 5. As shown, the extracellular acidification rate is significantly inhibited in the presence of GB (2), a PAFR antagonist. The inhibited PAFR responses in the presence of antagonists are shown in Table 1. GB (2) was the most-potent native ginkgolide (81% inhibition), followed by GA (1), GJ (4), and GC (3), which were almost equipotent and showed ca. 25% inhibition. The benzylated GB analog, 10-O-Bn-GB (7), was more potent than the native compounds, and demonstrated inhibition similar to synthetic PAFR antagonist WEB2086 (10) (93% for both). Conversely, ginkgolide M (5), bilobalide (6), and the flavonoids rutin (11), quercetin (12), and kaempferol (13) did not exhibit significant antagonism of the PAFR.
Three of the most-potent antagonists, GB (2), 10-O-Bn-GB (7), and WEB2086 (10), were tested at lower concentrations, since nearly complete inhibition was observed at the first concentration used. As shown in Fig. 6, 10-O-Bn-GB (7) and WEB2086 (10) are very potent and showed nearly equal inhibition strengths at all concentrations examined. GB (2) was less potent and, in contrast, demonstrated a non-linear dose-response relationship in the 25–100 µM region.

Additionally, various G. biloba extracts such as the BioGinkgo extract and a TTL-enriched extract [25] were assayed for their ability to inhibit the mcPAF response (Table 2). The compositions of these extracts are detailed in Table 3. The enriched
Table 1. ECAR Response to 5 nM mcPAF in the Presence of 100 µM Antagonists, Compared with Kᵢ Values from Radioligand-Displacement Assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Responseᵃ) ± SEM</th>
<th>p Valueᵇ)</th>
<th>Kᵢᶜ) [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>mcPAF (control)</td>
<td>100.0 ± 4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA (4)</td>
<td>73.7 ± 3.1</td>
<td>**</td>
<td>1.46</td>
</tr>
<tr>
<td>GB (2)</td>
<td>19.1 ± 1.9</td>
<td>***</td>
<td>0.56</td>
</tr>
<tr>
<td>GC (3)</td>
<td>79.4 ± 5.6</td>
<td>**</td>
<td>12.6</td>
</tr>
<tr>
<td>GJ (4)</td>
<td>76.7 ± 1.6</td>
<td>**</td>
<td>9.9</td>
</tr>
<tr>
<td>GM (5)</td>
<td>101.1 ± 3.7</td>
<td></td>
<td>&gt; 50</td>
</tr>
<tr>
<td>BB (6)</td>
<td>114.6 ± 5.7</td>
<td></td>
<td>&gt; 50</td>
</tr>
<tr>
<td>10-O-Bn-GB (7)</td>
<td>6.9 ± 2.5</td>
<td>***</td>
<td>0.12</td>
</tr>
<tr>
<td>WEB2086 (10)</td>
<td>6.2 ± 1.8</td>
<td>***</td>
<td>ND</td>
</tr>
<tr>
<td>Rutin (11)</td>
<td>96.2 ± 4.3</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Quercetin (12)</td>
<td>98.7 ± 7.9</td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

ᵃ) Values are means of at least three experiments.ᵇ) Student–Newman–Keuls test: **: p < 0.01, ***: p < 0.001 compared with mcPAF control.ᶜ) Data from previous experiments (J. Med. Chem. 2002, 45, 4038 and J. Med. Chem. 2002, 46, 601); ND = not determined.

Fig. 6. Percent inhibition of 5 nM mcPAF ECAR response by potent PAFR antagonists, GB (2), 10-O-Bn-GB (7), and WEB2086 (10).

extract, which contains 66% TTLs, showed 38% inhibition at 100 µg/ml and 8% inhibition at 10 µg/ml. The BioGinkgo extract contains 7% TTLs and showed pH-dependent inhibition (13–28%) at 100 µg/ml. When the BioGinkgo assay media was prepared as usual (see Exper. Part) the 100-µg/ml assay solution was only pH 6.9 (as opposed to blank media, pH 7.4) due to acidic flavonoids; this solution resulted in a PAFR response inhibition of 28%. Adjusting the pH to 7.4 after addition of the BioGinkgo extract produced 13% inhibition.

Discussion. – Inhibition of the PAFR determined by microphysiometry correlates well with relative $K_i$ values determined in complementary platelet-aggregation assays
and radioligand-displacement experiments [27][28] (Table 1). Ginkgolide B (2) was the most potent of the native TTLs but, as predicted by $K_i$ values, it was still less active than either the benzylated GB analogs or the synthetic PAFR antagonist, WEB2086 (10). Screening these compounds by microphysiometry required higher concentrations than in earlier radioligand displacement studies. It should be noted, however, that, in the previous binding experiments, the radioactive PAFR antagonist [3H]-WEB2086 was used, in part because the compounds tested were less potent against the PAFR agonist [3H]-PAF [27].

The 10-O-Bn group certainly confers on the antagonist greater inhibitory properties than seen in the native ginkgolides, as demonstrated by comparison of the activities of 10-O-Bn-GB (7) and GB (2). WEB2086 (10) and 10-O-Bn-GB (7) inhibited the PAFR response potently to 25 μM (the lowest concentration tested), while GB (2) was significantly less effective at this concentration. Other native ginkgolides A, C, J, and M (1 and 3–5) showed PAFR response inhibition values much lower than that of GB (2). Interestingly, the ginkgolides all share the same cage-like structure and differ only in their OH-group substitution pattern. Although bilobalide (6) is also a TTL, it is a C15 sesquiterpene and has a C skeleton considerably different from the C20 ginkgolide diterpenes (Fig. 1). Bilobalide showed no PAFR response inhibition.

Of note is the difference between inhibition caused by the BioGinkgo extract and the TTL-enriched extract. The BioGinkgo extract contains 7% TTLs, while the enriched extract contains 66% TTLs. The enriched extract was diluted ten-fold, for direct TTL-level comparison with the BioGinkgo extract. When these two extracts are

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Table 2. ECAR Response to 5 nm mcPAF in the Presence of G. biloba Extract Mixtures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Extract concentration</th>
<th>TTL concentration</th>
<th>% Response ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>mcPAF (control)</td>
<td>–</td>
<td>–</td>
<td>100.0 ± 4.5</td>
</tr>
<tr>
<td>BioGinkgo extract, pH 6.9</td>
<td>100 μg/ml</td>
<td>7 μg/ml</td>
<td>72.4 ± 3.4 **</td>
</tr>
<tr>
<td>BioGinkgo extract, pH 7.4</td>
<td>100 μg/ml</td>
<td>7 μg/ml</td>
<td>87.3 ± 1.8</td>
</tr>
<tr>
<td>TTL-enriched extract</td>
<td>10 μg/ml</td>
<td>7 μg/ml</td>
<td>92.2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>100 μg/ml</td>
<td>66 μg/ml</td>
<td>61.7 ± 5.9 ***</td>
</tr>
</tbody>
</table>

*) Student–Newman–Keuls test: **: p < 0.01, ***: p < 0.001 compared with mcPAF control.

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Table 3. Compositions of G. biloba Extracts (from [25])

<table>
<thead>
<tr>
<th>Compound</th>
<th>BioGinkgo extract [%]</th>
<th>TTL-Enriched extract [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpene trilactones *)</td>
<td>7</td>
<td>65.6</td>
</tr>
<tr>
<td>GA (1)</td>
<td>2.94</td>
<td>34.8</td>
</tr>
<tr>
<td>GB (2)</td>
<td>0.98</td>
<td>11.8</td>
</tr>
<tr>
<td>GC (3)</td>
<td>0.98</td>
<td>6.6</td>
</tr>
<tr>
<td>GJ (4)</td>
<td>0.35</td>
<td>3.2</td>
</tr>
<tr>
<td>BB (6)</td>
<td>1.75</td>
<td>9.2</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>27</td>
<td>ND *)</td>
</tr>
</tbody>
</table>

*) Ginkgolide M (5) is not present because these extracts were taken from the leaves of the tree and GM is found only in the root bark. *) ND = not determined.
compared at similar TTL composition (7 μg/ml), the BioGinkgo extract, which contains a higher percentage of flavonoids than the enriched extract, more potently inhibits the PAFR response (13% inhibition for BioGinkgo extract, 8% inhibition for TTL-enriched extract). Although not extremely large, the difference is noteworthy, and the contributing effects of flavonoid components of the natural G. biloba extract may need to be considered to help explain this disproportion.

Therefore, we assessed whether the PAFR response was inhibited by G. biloba flavonoids. There are few reports of flavonoid effects on PAF-related activity. Vasange et al. found that rutin and other flavonoids did not show PAF-specific effects [29], while Chen et al. showed that rutin inhibited PAF-induced platelet aggregation in a concentration-dependent manner [30]. Our results indicate that rutin (11), quercetin (12), and kaempferol (13) do not significantly antagonize the PAFR (Table 1). Furthermore, these flavonoids did now show any synergistic potentiation of the PAFR inhibitory activity of GB when screened together with this potent ginkgolide (50 μM each; data not shown). However, the G. biloba extract is a complex mixture of terpene trilactones, flavonoids, and other compounds. A challenge in elucidating the mechanism of action of herbal extracts is the potential for synergistic interactions that contribute to overall efficacy of a product. An effect such as this cannot be ruled out with the non-TTL components of G. biloba.

Microphysiometry, requiring no radioligand for assay, was shown to be a valuable tool in evaluating the functional activity of PAFR antagonists. Native ginkgolides displayed the same relative inhibitory activity as in previously reported radioligand binding experiments. Additionally, G. biloba extracts, mixtures of terpene trilactones and flavonoids, were tested, with results indicating possible synergism. The present results suggest that a functional assay using FLIPR3 should provide an efficient, high-throughput alternative to radioligand displacement for assaying ginkgolides and analogs.

**Experimental Part**

*Cell Culture.* CHO Cells transfected to express the cloned human PAFR (CHO-1F8) were obtained from Prof. Takao Shimizu, Department of Biochemistry, Faculty of Medicine, University of Tokyo. This CHO-1F8 cell line [31] was grown at 37°C in a humidified atmosphere of 95% air/5% CO2 in nutrient F-12 mixture (Ham’s, Sigma, MO; N6658) containing 10% FBS (Atlanta Biologicals, GA; S11550), 0.3 mg/ml geneticin disulfate (Sigma; G9516), and 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen, CA; 15240-062). Cells at 80–90% confluence were passaged every 3–4 days by mild trypsinization (BioWhittaker, MD; 17-160), followed by centrifuging at 1000 rpm, 20° for 4 min, counting with a hemacytometer, and reseeding in 25-cm² flasks with 1–3 × 10⁶ cells. Individual stocks were maintained until passage 20, at which time a new frozen stock was started.

Cells for use in the Cytosensor microphysiometer (Molecular Devices, CA) were plated onto microporous membrane capsules (Corning Costar, MA; 3401) at a density of 5 × 10⁴ cells/insert, and were grown under the conditions described above. Microphysiometry experiments were performed two days after subculture. Ham’s powdered media without bicarbonate (Sigma; N6760) containing 0.1% FBS (Atlanta Biologicals, GA; S11550), 0.3 mg/ml genetin disulfate (Sigma; G9516), and 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen, CA; 15240-062). Cells at 80–90% confluence were passaged every 3–4 days by mild trypsinization (BioWhittaker, MD; 17-160), followed by centrifuging at 1000 rpm, 20° for 4 min, counting with a hemacytometer, and reseeding in 25-cm² flasks with 1–3 × 10⁶ cells. Individual stocks were maintained until passage 20, at which time a new frozen stock was started.

**Cytosensor Microphysiometry:** Measurement of Extracellular Acidification Rate. Cell capsules were prepared for microphysiometry experiments as described in [32]. Briefly, a capsule spacer (Molecular Devices; R7026B), a capsule insert (Molecular Devices; R7025), and 1 ml running media were added sequentially to
each capsule. This assembly was then transferred to a silicon sensor chamber. The cells were equilibrated on the Cytosensor for at least one hour prior to the assay.

At 37 °C, running medium was pumped over the cells at 50% maximum flow (50 μl/min) with a 120-s pump cycle: 80-s pump on and 40-s pump off, during which time the ECAR was measured for 30 s (88 – 118 s). Agonist mcPAF was applied at the half-maximal dose (5 nM) for 10 s (cycle time 70 – 80 s). Putative antagonists were pre-applied for 30 min prior to agonist introduction, and the selected antagonist was also present during and after agonist application. In each experiment, one of the four Cytosensor channels was randomly designated the vehicle-only control, while the responses of the other channels were averaged to calculate the percent inhibition.

Materials. Terpene trilactone ginkgolides 1–5 and bilobalide (6) from G. biloba were isolated through established methods [8][25][33]. Ginkgolide M (5) was extracted from tree root bark in 1967, and the stability of this original sample was recently confirmed by 1H-NMR. The GB analog, 10-O-Bn-GB (7), was prepared as described in [34]. The BioGinkgo extract, which contains flavonoids and TTLs in a ratio of 27:7, similar to EGB 761, was a generous gift of the Pharmarx Company. This extract was enriched to 66% TTL content according to established methodology to yield the TTL-enriched extract [25]. PAFR Antagonist WEB2086 (10) was generously provided by Boehringer-Ingelheim Pharmaceuticals. PAF (8, Sigma; P4904), methylcarbamyl PAF (9, mPAF, Sigma; H4648), and the flavonoids rutin hydrate (11, Sigma; R5143), quercetin dihydrate (12, Aldrich; 17,196-4), and kaempferol (13, Sigma; K0133) are commercially available.

Statistical Analysis. Data were evaluated for statistical significance with one-way ANOVA and, if significant, group means were compared by post-hoc analysis with Student–Newman–Keuls test for multiple comparison of means. The confidence level for statistical significance was set at a probability value of 0.05.

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