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1-*O*-HEXADECYL-2-METOXY-GLYCERO-3-PHOSPHATIDYLCHOLINE—A METHOXY ETHER LIPID INHIBITING PLATELET ACTIVATING FACTOR-INDUCED PLATELET AGGREGATION AND NEUTROPHIL OXIDATIVE METABOLISM

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Abstract—Whether or not two alkylglycerols could initiate a functional response in human platelets or modify responses induced by platelet activating factor (PAF) was evaluated. It was found that 1–100 μM 1-*O*-hexadecyl-2-metoxy-glycero-3-phosphatidylcholine (Et-16-OCH_3) induced platelet aggregation but 1-*O*-hexadecyl-*sn*-glycerol (chimyl alcohol; CA) did not. Et-16-OCH_3-induced platelet aggregation was abolished by pretreatment with the PAF receptor antagonist WEB 2086. While CA had no effect on platelet aggregation induced by PAF, pretreatment with Et-16-OCH_3 (0.1 μM or higher) significantly inhibited platelet aggregation induced by PAF, but had no effect on aggregation caused by ADP, thrombin or phorbol myristate acetate (PMA). A receptor binding study using radiolabelled [^{3}H]WEB 2086 showed that Et-16-OCH_3 exerts its actions through interaction with the PAF receptor. Moreover, Et-16-OCH_3 inhibited neutrophil chemiluminescence responses induced by PAF, but not reactions to PMA or a formyl peptide. Finally, 1 μM Et-16-OCH_3 induced a rise in the intracellular calcium concentration in platelets equal to that induced by PAF and also had an calcium ionophore-like effect at 100 μM. Thus, this study shows that Et-16-OCH_3 is both a potent inducer of platelet aggregation and an inhibitor of PAF-induced platelet aggregation and neutrophil chemiluminescence, through interaction with the PAF receptor.

Key words: aggregation; alkylglycerol; chemiluminescence; neutrophil; platelet; platelet activating factor

Alkylglycerols are lipids with a glycerol backbone to which fatty acid derivatives are coupled with an ether bond. These ether lipids are present in high concentrations in human bone marrow, spleen and liver [1, 2]. Alkylglycerols possess many biological activities including an antibiotic-like activity against various bacteria, fungi and parasites [3, 4], immunostimulation and anti-tumour effects [5–10]. The best-known ether phospholipid 1-alkyl, 2-acetyl, 3-phosphatidylcholine (PAF; Fig. 1) produces physiological effects such as stimulation of chemotaxis, aggregation and oxidative metabolism in PMN§ [11] as well as inducing aggregation, shape change and secretion in platelets [11]. In a previous study [12] it was shown that other alkylglycerols, such as Et-16-OCH_3 (Fig. 1), its analogue Et-18-OCH_3 (Fig. 1), lyso-PAF and CA (Fig. 1) can induce an oxidative response in PMN, mediated in part by an increase in intracellular calcium levels. Et-18-OCH_3, referred to as Edelfosine [13], has been advocated as a means of purging leukaemic bone marrow cells [14–16]. These results point to discrete functional differences and biochemical similarities between PAF and the Et-16-OCH_3 family of lipids.

The purpose of this study was to determine if these alkylglycerols possess the ability to induce a variety of functional responses in platelets and chemiluminescence in neutrophils and whether they can modify the response induced by PAF. Et-16-OCH_3 was chosen as the primary molecule for evaluation since it has been demonstrated to be more potent than Et-18-OCH_3 for the induction of PMN functional responses [12].

MATERIALS AND METHODS

Chemicals. L-Phosphatidylcholine, PAF, Et-16-OCH_3, luminol, thrombin, CA and PMA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ADP and its solvent Dilutent A was supplied by Bio-Zac (Stockholm, Sweden) and HBSS

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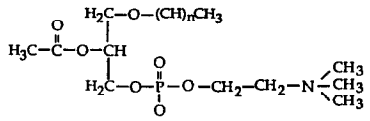
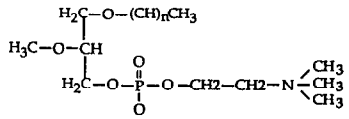
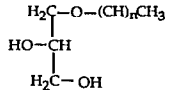
§ Abbreviations: PMN, polymorphonuclear granulocytes; Et-16-OCH_3, 1-*O*-hexadecyl-2-metoxy-glycero-3-phosphatidylcholine; PAF, platelet activating factor; CA, chimyl alcohol; WEB 2086, 3-[4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3, 2-*f*][1, 2, 4]-triazolo-[4, 3*a*][1, 4]-(diazepin-2-yl)-1-(4-morpholinyl)-1-propanone; PMA, phorbol myristate acetate; ADP, adenosine diphosphate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; fura-2/AM; PPP, platelet-poor plasma; PRP, platelet-rich plasma; HBSS, Hank's balanced salt solution; CL, chemiluminescence; PKC, protein kinase C; PV, polycythemia vera; $[Ca^{2+}]_i$, intracellular calcium concentration.



1-O-alkyl-2-acetyl-sn-glycero-3-phosphatidylcholine
(Platelet activating factor; PAF)



1-O-hexadecyl-2-metoxy-sn-glycero
3-phosphatidylcholine (Et-16-OCH_3)



1-O-hexadecyl-sn-glycerol
(Chimylalkohol; CA)

Fig. 1. Molecular structures for PAF, Et-16-OCH_3 and CA.

was from Gibco (Paisley, Scotland, U.K.). fMLP was from Peninsula Lab. (San Carlos, CA, U.S.A.). Calbiochem (La Jolla, CA, U.S.A.) provided fura-2/AM. [^{3}H]WEB 2086 (special activity 388.5 GBq/mmol) was purchased from Dupont (Stockholm, Sweden).

Platelet preparation and aggregation. Blood from healthy members of the hospital staff who stated they had not taken aspirin for at least 10 days was obtained between 8 and 9 a.m. Venous blood was collected in citrated vacuum tubes and centrifuged 1 hr later at 101 *g* for 15 min, leaving no detectable red cells in the upper phase. The platelet-containing supernatant was removed and the remainder centrifuged at 2000 *g* for 20 min to obtain PPP. Platelet-containing and PPP were mixed to provide PRP with a platelet concentration of 300×10^9/L. This mixture was kept at room temperature. A standard sequence was adopted for each series of aggregation readings and tests were always completed within the next 2 hr. Aggregation experiments were performed in siliconized cuvettes by the optical method of Born [17] in a Platelet Aggregation Profiler, model PAP3 (Bio/DATA Corp., Horsham, PA, U.S.A.). Aggregatory agents were diluted in HBSS and the final concentrations were as follows: Et-16-OCH_3 (0.01–100 μM), PAF (0.1–1 μM), ADP (2.2 μM), PMA (1 μM) and thrombin 4 U/mL. The concentrations of the two latter agonists were based on the results of previous experiments [18] and provoked approximately 90% of the maximal response for that agonist. Aggregation is expressed as final maximal aggregation in percent with 100% implying aggregation of all platelets.

Platelet calcium measurements. $[Ca^{2+}]_i$ was calculated from the change in fura-2 fluorescence [19]. Platelets, obtained as described here, were isolated and washed as described [20] with several modifications. The fresh blood was carefully mixed with 0.18 volumes of 3.15% trisodium citrate. Within 30 min it was centrifuged at 612 *g* for 10 min at 20°. The platelet-containing upper phase was removed and diluted in buffer A (134 mM NaCl, 5 mM glucose, 1 mM EDTA, 15 mM Tris, pH 6.3). This mixture was centrifuged at 228 *g* for 20 min at 4°. The supernatant was then discarded and the platelets again resuspended in the same buffer. Fura-2 was added to a concentration of 1 μM and the platelets incubated for 45 min at 37°. Loaded cells were centrifuged at 228 *g* for 20 min at 4° and reconstituted to a concentration of 100×10^9/L in buffer B (129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.4); tests were performed immediately thereafter. Cells were warmed for 5 min at 37° with a continuous stirring of the cell suspension. The fluorescence of the fura-2-loaded platelets was subsequently measured in a Hitachi F-3000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) after stimulation with Et-16-OCH_3 at 100 and 1 μM and PAF at 1 μM. The excitation wavelength was set at 340 nm and emission at 510 nm. After a stable baseline had been established, the stimulus was added and emitted light recorded. The system was controlled by addition of EGTA, Tris buffer, Triton X-100 and $CaCl_2$ as described [21]. Calculations of the calcium concentrations were performed according to Ref. 21.

Inhibition of [^{3}H]*WEB 2086 binding to the human platelet PAF receptor.* The binding assay is a modification of the procedure of Kloprogge [22] and Ukena [23]. Platelets were washed as described above and resuspended in the same buffer B to a concentration of 250×10^9/L. Triplicate aliquots of platelets (500 μL) were mixed with [^{3}H]WEB 2086, 300 nM. In competition experiments the final concentrations were Et-16-OCH_3 10–100 μM. Non-specific binding was measured by adding an excess of cold WEB 2086 (10 μM final concentration).

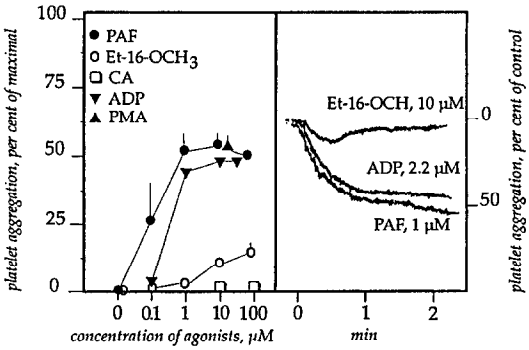


Fig. 2. Platelet aggregation induced by various agonists. The left panel shows platelet aggregation (%) induced by PAF (N = 7), Et-16-OCH_3 (N = 10), CA (N = 4), PMA (N = 11) and ADP (N = 3) in concentrations ranging from 0.1 to 100 μM. The right panel depicts typical aggregation curves for PAF and Et-16-OCH_3 at 1 μM as well as ADP at 2.2 μM.

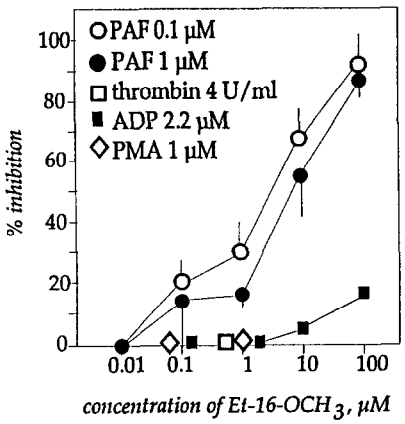


Fig. 3. Effect of Et-16-OCH_3 on platelet aggregation induced by PAF, PMA, ADP and thrombin. After 5 min preincubation with or without Et-16-OCH_3, platelet aggregation was induced by PAF 0.1 (○) or 1 μM (●, N = 8), PMA 1 μM (◇, N = 3), ADP 2.2 μM (■, N = 4) or thrombin 4 U/mL (□, N = 7). For each agonist, results are expressed as mean inhibition (%) conferred by Et-16-OCH_3 of the response obtained in cells that had not been pretreated with Et-16-OCH_3. Aggregation induced by 1 μM PAF was significantly inhibited by 1 μM Et-16-OCH_3 ($P < 0.01$), whereas 0.1 μM did not significantly hamper the response ($P > 0.1$). Moreover, the response to 0.1 μM PAF was significantly inhibited by both 0.1 and 1 μM Et-16-OCH_3 ($P < 0.01$ for both).

Incubations were carried out for 60 min at 20°. Bound and free ligand were separated by centrifugation in an Eppendorf Microfuge (10,000 *g*, 60 sec) and then washed twice with cold 900 μL buffer B and resuspended in 1% Triton X-100 in buffer B. The pellets were resuspended in 5 mL liquid scintillation fluid and the tubes mixed and counted for 240 sec in a liquid scintillation spectrometer.

Neutrophil preparation and in vitro *function.* Neutrophils were isolated from venous blood, obtained from healthy volunteers, by a one step separation on discontinuous Percoll gradients [24]. Purity and viability were both > 95%. CL augmented by luminol was assessed essentially as described previously [25]. None of the stimuli used here conferred light emission in a cell-free CL system. Results are given as the maximal light emission. We used fMLP, PAF and PMA at 0.1 μM since this concentration gives maximal or close to maximal CL.

Statistics. All results are expressed as the mean ± SEM. Statistical analyses were performed using Wilcoxon's test of paired samples.

RESULTS

The ability of alkylglycerols to induce a functional response in platelets was assessed. As demonstrated in Fig. 2, PAF was the most potent inducer of platelet aggregation followed by Et-16-OCH_3, whereas CA did not promote measurable platelet aggregation. Et-16-OCH_3 induced an aggregation kinetically resembling that induced by PAF at 0.1 μM. Higher concentrations of Et-16-OCH_3 were needed in order to achieve comparable results to those with regular agonists for platelet aggregation, e.g. thrombin, PMA and ADP.

It was investigated whether Et-16-OCH_3 or CA could modify platelet aggregation induced by PAF. As depicted in Fig. 3, preincubation for 5 min with Et-16-OCH_3 at concentrations ranging from 0.01 to 100 μM conferred an inhibition of PAF (1 μM)-induced aggregation with an apparent ID_{50} of 9 μM Et-16-OCH_3 (N = 8). As also shown in Fig. 3, a 10-fold reduction in PAF concentration shifted the dose–response curve to the left. This gave an apparent ID_{50} of 5 μM Et-16-OCH_3 (N = 8). In contrast, pretreatment with Et-16-OCH_3 (0.1–100 μM) had no effect on aggregation induced by ADP (2.2 μM), which was 44% without Et-16-OCH_3 as compared to 44% with Et-16-OCH_3 (0.1 μM) (mean, N = 4); 46% with Et-16-OCH_3 (1 μM) (mean, N = 4) and 41% with Et-16-OCH_3 (10 μM) (mean, N = 6). Pretreatment with Et-16-OCH_3 (100 μM), a concentration that in itself can cause platelet aggregation, still only slightly impaired ADP-induced aggregation, which was 44% without Et-16-OCH_3 and 35% with Et-16-OCH_3 (100 μM) (mean, N = 6). Accordingly, ET-16-OCH_3 (1 μM) had no effect on aggregation elicited by PMA (1 μM), 53% without Et-16-OCH_3 and 53% after pretreatment with Et-16-OCH_3 (1 μM) (mean, N = 3). Aggregation induced by thrombin was equally unaffected. An average of 53 ± 2% with thrombin 4 U/mL, it became 56 ± 1% after preincubation with Et-16-OCH_3 (1 μM) (mean, N = 7). Pretreatment with CA (10 μM) had no effect on PAF (1 μM)-induced aggregation, which was 49% both with and without pretreatment (mean, N = 4).

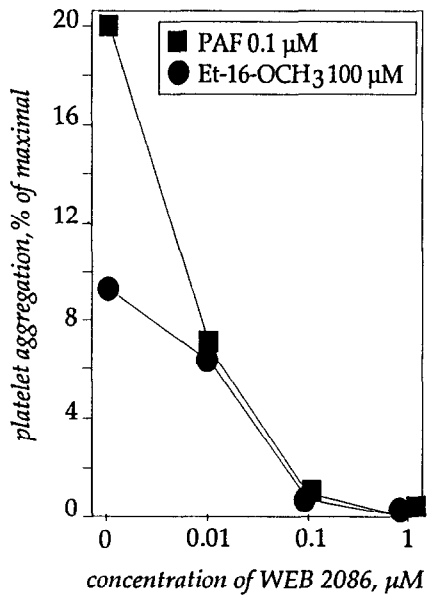


Fig. 4. Inhibition by WEB 2086 of platelet aggregation. Total aggregation induced by PAF 0.1 μM (■, means of two experiments) and Et-16-OCH_3 (●, means of four experiments) after 5 min preincubation with WEB 2086.

In an effort to clarify the mechanism(s) whereby Et-16-OCH_3 inhibits PAF-enhanced platelet aggregation, we incubated platelets for 5 min with the PAF receptor antagonist WEB 2086 (0.01–1 μM). As shown in Fig. 4, pretreatment with WEB 2086 at 0.1 and 1 μM abolished PAF (0.1 μM)-induced platelet aggregation in all experiments, and a 10-fold lower concentration, 0.01 μM, also reduced it markedly. Interestingly, WEB 2086 proved to have a similar effect on the Et-16-OCH_3 response, i.e. Et-16-OCH_3-induced platelet aggregation was completely abolished after pretreatment with the high WEB 2086 concentrations (0.1 and 1 μM) but less, yet consistently, affected by the low WEB 2086 concentration (0.01 μM). To confirm our suspicions that Et-16-OCH_3 exerted its actions through interaction with the PAF receptor, a receptor binding study using [^{3}H]WEB 2086 was performed. This showed that preincubation with Et-16-OCH_3 (100 μM) inhibited [^{3}H]WEB 2086 binding by 56% and preincubation with Et-16-OCH_3 (10 μM) inhibited [^{3}H]WEB 2086 binding by 29% (mean; N = 3).

We further investigated the effects of Et-16-OCH_3 on platelets by measuring the intracellular rise in calcium after stimulation. Et-16-OCH_3 (1 μM) increased $[Ca^{2+}]_i$ by 118 ± 13 nM (N = 4), which is equal to the rise induced by PAF at the same concentration, 127 ± 19 nM (N = 11). The kinetics of the $[Ca^{2+}]_i$ responses to the two stimuli were similar (Fig. 5). After stimulation of the platelets with Et-16-OCH_3 (1 μM) a subsequent addition of PAF (1 μM) caused no further increase in $[Ca^{2+}]_i$. Characteristic for the $[Ca^{2+}]_i$ response induced by a higher concentration of Et-16-OCH_3 (100 μM) were kinetics resembling those of the calcium ionophore ionomycin, which is also shown in Fig. 5.

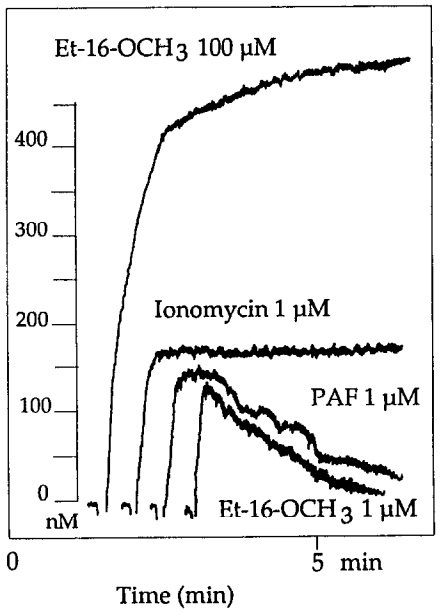


Fig. 5. Effect of Et-16-OCH_3 (1 and 100 μM), PAF (1 μM) and ionomycin (1 μM) on platelet $[Ca^{2+}]_i$ assessed by fura-2 fluorescence. The figure depicts actual tracings from one experiment which was repeated at least four times with similar results. They *y*-axis gives the increase in $[Ca^{2+}]_i$ from the basal level (34 ± 9 nM).

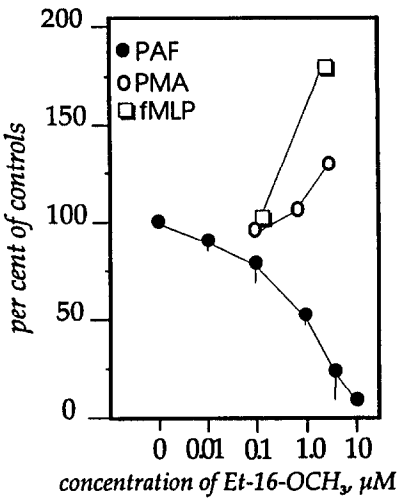


Fig. 6. Effect of Et-16-OCH_3 on neutrophil chemiluminescence. Mean values for two to six separate experiments. Results are expressed as relative chemiluminescent activity when compared to simultaneously run controls treated with buffer alone. Agonists (PAF = ●, PMA = ○ and fMLP = □) were used at 0.1 μM. That stimulation conferred a light emission corresponding to 81.3 ± 22 mV for PAF, 1785 ± 226 mV for PMA and 852 ± 97 mV for fMLP (N = 15). When a stimulus was associated with a dual peak response (e.g. PAF), calculations were based on the height of the major peak. Et-16-OCH_3 elicited a CL response when used as an agonist at 10 and 100 μM (but not at a lower concentration) corresponding to 20 mV and 248 mV, respectively. Statistically significant inhibitions were noted for 0.1 μM Et-16-OCH_3 ($P < 0.05$), and ≥1 μM ($P < 0.01$).

In order to see if another cell reacted similarly as platelets to the phospholipids we assessed neutrophil chemiluminescence. PMN responded with a burst of CL when exposed to fMLP, PAF, PMA and Et-16-OCH_3. PMA was the most potent inducer, followed in descending order (based on comparisons at equimolar concentrations) by fMLP, PAF and Et-16-OCH_3 (Fig. 6).

The question whether Et-16-OCH_3 would interact with the CL response elicited by PAF (or by fMLP and PMA) was approached by incubating neutrophils with the lipid (or solvent only) for 10 min, followed by addition of agonists for CL. It was found that PAF, but not fMLP and PMA, responses were inhibited in the presence of Et-16-OCH_3 in a dose-dependent way (Fig. 6).

DISCUSSION

Due to their antibiotic-like activity [3, 4], their ability for immunomodulation and their anti-tumour effects [5–10], alkylglycerols have been the object of increasing interest in recent years. Different alkylglycerols have been shown to stimulate platelets in different ways [26–29]. The precise molecular

mechanisms of action of alkylglycerols have not yet been elucidated. It has been shown previously that Et-16-OCH_3, Et-18-OCH_3 and lyso-PAF can induce neutrophil chemiluminescence, an effect paralleled by their ability to increase PMN intracellular calcium concentration [12]. In the present study we were able to show that Et-16-OCH_3 could induce a CL response in PMN as well as inhibit CL elicited by PAF. This suggests that Et-16-OCH_3 might interact with the PAF receptor, presumably as a competitive partial antagonist. We also extended our studies to platelets. Here, it was found that Et-16-OCH_3, but not CA, could induce platelet aggregation with kinetics resembling PAF-induced platelet aggregation. This is in accordance with the previous findings by Hanahan *et al.* [26] and others [27–29] that alkylglycerols with a metoxy-substituent in the 1- or 2-position are able to stimulate platelets.

In an attempt to clarify the mechanism(s) whereby Et-16-OCH_3 induces a response in platelets, platelets were incubated with Et-16-OCH_3 prior to stimulation with PAF, and found that Et-16-OCH_3 strongly inhibited PAF-induced platelet aggregation. In contrast, Et-16-OCH_3 was not able to influence platelet aggregation induced by ADP, PMA or thrombin. CA did not hamper platelet aggregation induced by PAF. These findings suggest that Et-16-OCH_3 exerts its activity on platelets through interaction with the PAF receptor, similar to what is assumed for PMN. Further proof for this hypothesis was found by showing that incubation of platelets with the PAF receptor antagonist WEB 2086 [30] completely abolished the aggregatory response both to PAF and Et-16-OCH_3. The dose–response curves for inhibition were similar for both stimuli at equimolar concentrations. To confirm the assumption that Et-16-OCH_3 binds to the PAF receptor a receptor-binding study was performed. In accordance with previous findings on the properties of SRI 62-834, a cyclic analogue of Et-18-OCH_3 [31], the present experiments showed that Et-16-OCH_3 inhibited [^{3}H]WEB 2086 binding to the human PAF receptor.

Following binding of PAF to its receptor on the cell, G-protein(s) are first activated followed by phospholipases C and D. Such activation results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate, which induces intracellular calcium mobilization [32] and 1,2-diacylglycerol formation, which causes activation of PKC. It has previously been shown that purified brain PKC can be activated by naturally occurring ether-linked diglycerides [33], a reaction requiring calcium. The authors have previously described that alkylglycerols elevate $[Ca^{2+}]_i$ in PMN [12] and this has also been found in HL-60 cells [34, 35]. The agonist-induced rise in intracellular calcium in platelets was therefore investigated. Even though Et-16-OCH_3 at the concentration 1 μM could not produce visible platelet aggregation, it was equally potent to PAF in increasing intracellular calcium, with kinetics also being identical. This indicates that a rise in calcium is not enough to induce platelet aggregation. It is a well-known fact [36] that an elevation in $[Ca^{2+}]_i$ is an essential, but in itself insufficient, event for inducing a functional response in neutrophils. In accordance, it has been shown a defective neutrophil oxidative metabolism in PV in spite of a normal elevation in intracellular calcium after stimulation with a cell surface receptor-dependent stimulus such as fMLP or PAF [37, 38]. Finally, we have also found that PAF-induced platelet aggregation is impaired in PV compared to healthy controls [18] and recently discovered that this occurs despite a normal PAF-induced intracellular calcium elevation (Le Blanc *et al.*, unpublished observations).

The addition of PAF 5 min after stimulation with Et-16-OCH_3 caused no further increase in calcium. It has previously been reported [35] that the biphasic changes in fluorescence which alkylglycerols produce in HL-60 cells may not be truly representative of changes in intracellular calcium but rather the result of membrane perturbation and leakage of the fluorescent dye. With Et-16-OCH_3 (100 μM) we were able to produce a similar, but monophasic, sustained rise in fluorescence. Lohmeyer and Workman [35] noted that addition of a chelating agent such as EGTA caused a precipitous drop in fluorescence, indicating that the loaded ionophore had either leaked out of the HL-60 cells or was located in membrane permeable cells. In our experiments, however, addition of EGTA caused no decrease in fluorescence. Furthermore, subsequent addition of ADP (2.2 μM) to the platelets caused normal to near normal aggregation, contradicting a possible membrane damage. A check in the light microscope after aggregation with Et-16-OCH_3 and after preincubation with Et-16-OCH_3 and subsequent stimulation with ADP or collagen showed normal-looking platelet aggregates.

It has thus been shown that stimulation with the alkylglycerol Et-16-OCH_3, but not CA, causes functional responses, i.e. CL in PMN and platelet aggregation, with kinetics resembling that induced by PAF. It has also been shown that Et-16-OCH_3 is a potent inhibitor of PAF-induced CL and aggregation. The PAF receptor antagonist WEB 2086 is equally potent in inhibiting aggregation elicited by either PAF or Et-16-OCH_3. Receptor binding experiments show that Et-16-OCH_3 interacts with the platelet PAF receptor. Also, stimulation of platelets with Et-16-OCH_3 leads to an increase in $[Ca^{2+}]_i$ which cannot be further potentiated by additional stimulation with PAF. A high concentration of Et-16-OCH_3 causes a ionophore-like rise in calcium that is unlikely due to membrane damage to the platelets since they are still viable.

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