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Interactions between alkylglycerols and human neutrophil granulocytes

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We evaluated whether various alkylglycerols would initiate a functional response of human neutrophils or modify responses induced by a formyl peptide (fMLP) *in vitro*. We found that platelet activating factor (PAF) was the most potent with regard to the ability to produce an oxidative response (assessed by cytochrome *c* reduction and/or chemiluminescence), followed by ET-16-OCH3. Lyso-PAF, ET-18-OCH3, batyl- and chimyl alcohols exhibited only a weak activity. PAF was also the most efficient lipid conferring a rise of intracellular calcium concentrations ($[Ca^{2+}]_i$). ET-16-OCH3, ET-18-OCH3 and lysoPAF were less potent, although maximal $[Ca^{2+}]_i$ levels were similar to that of 0.1 μmol/l fMLP. The kinetics of the calcium responses were highly specific for each ether lipid. When neutrophils had been treated with PAF or ET-18-OCH3 and were subsequently stimulated by fMLP, enhancement of the oxidative response was noted. Thus, this study shows that there was an association between the ability of an alkylglycerol to initiate oxidative and calcium responses, indicating strict structure-activity relationships for these lipids.

Key words: alkylglycerols; chemiluminescence; intracellular calcium concentrations; polymorphonuclear granulocytes; superoxide anion formation

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Alkylglycerols are lipids with a glycerol backbone, to which fatty acid derivatives are coupled by means of an ether bond instead of the ester bond that characterizes most mono-, di- and triglycerides and related phospholipids. The ether lipids are present in high concentrations in human bone marrow, spleen and liver [1, 2]. They contain both saturated and mono-saturated 16- and 18-carbon atom fatty acids. Some are methyl/methoxy-substituted at the fatty acid in 1-position [3] or in the 2-position [4].

Alkylglycerols may have biological activities include an antibiotic-like action against various bacteria, fungi and parasites [5, 6], immunostimulation [3, 7] and anti-tumour effects [4, 8–10]. The two latter properties are characteristic for methyl/methoxy-substituted compounds. One example of that class of agents is 1-O-octadecyl,2-methyl-glycerophosphatidyl choline (ET-18-OCH3), where the methyl group appears to be essential for its tumouricidal effect [8, 9]. The best known ether phospholipid possessing discrete biological activity is platelet activating factor (PAF; 1-alkyl,2-acetyl,3-phos-

of cells upon stimulation [11]. Exogenously added, PAF induces functional responses in neutrophils, such as chemotaxis, aggregation and secretion of oxygen radicals and lysosomal constituents [12–14].

In this study we have assessed the ability of a variety of ether lipids to induce a functional response in *in vitro* in human neutrophils and whether they modified that response when it was induced by an unrelated but standard stimulus viz. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). The functional response analysed was initiation of superoxide anion generation. Since a major pathway for initiation of the oxidative response in neutrophils includes mobilization of intracellular calcium, assessments of the cytosolic concentrations ($[Ca^{2+}]_i$) were included in the present analysis. It was found that certain alkylglycerols conferred increases of $[Ca^{2+}]_i$, which were not necessarily followed by superoxide ion (O_2^-) production.

MATERIALS AND METHODS

Chemicals

Hanks' balanced salt solution (HBSS) was obtained from SBL (Stockholm, Sweden). Luminol, cytochrome *c* (horse heart, grade III), human serum albumin (HSA, essential fatty acid free), and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Co (St Louis, MO). Superoxide dismutase (SOD) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was obtained from Peninsula Laboratories (San Carlos, CA). Leukotriene B4 (LTB4) was a kind gift from J. Rokach (Merck Frosst, Dorval, Canada). Ionomycin, A23187 and Fura 2-AM were from Calbiochem (LaJolla, CA).

Lipids

The acyl- and alkylglycerols (ESTers and EThers, respectively; followed by acronyms used in this study) were obtained as follows: Sigma Chemical Co (St Louis, MO) provided PAF (ET; with C=16), 1-O-hexadecyl,2-OH-glycero,3-phosphatidylcholine (ET; lysoPAF), dipalmitoyl phosphatidyl choline (EST; PPC), distearyl phosphatidyl choline (EST; SPC), chimyl alcohol (1-O-hexadecyl-*sn*-glycerol ET; CA, batyl alcohol (1-O-octadecyl-glycerol; ET; BA), 1-O(2-methoxy)-octadecyl-glycerol (ET; M-BA), 1-monopalmitoyl-*rac*-glycerol (EST; MPG), 1-O-octadecyl,2-methyl-*sn*-glycero-3-phosphatidyl choline (ET; ET-18-OCH3), 1-O-hexadecyl,2-methoxy-glycero-3-phosphatidyl choline (ET; ET-16-OCH3), 1-octadecyl-2-acetyl-glycerol (EST; OAG). Drs G. Ställberg (Gothenburg, Sweden) and P. Munder (Heidelberg, Germany) kindly provided 1-O-(2-methoxy)hexadecyl,2-OH-glycero,3-phosphatidyl choline (ET; M-lysoPAF) and ET-18-OCH3, respectively. Ethanol, used as solvent for the lipids, was always used at the same concentration in control preparations. At the highest concentration, 0.1%, it had a minimal inhibitory effect on fMLP-elicited chemiluminescence, as previously described [15].

Neutrophils

Neutrophils were isolated from peripheral venous blood, obtained from healthy volunteers, by a one-step separation on discontinuous Percoll gradients [16]. The purity and viability were both >95%.

Chemiluminescence (CL)

CL augmented by luminol was assessed essentially as described previously [17, 18]. None of the stimuli used here conferred light emission in a cell-free chemiluminescence system. Results are given as relative chemiluminescence activity when compared with simultaneously run fMLP controls, in order to minimize day-to-day variations. We used fMLP at 0.1 μmol/l since this concentration gives maximal or close to maximal CL- and Fura 2-responses.

Superoxide anion formation

This was assessed with the superoxide dismutase inhibitable cytochrome *c* reduction method in a real time continuous assay at 37 °C [19]. In some experiments cells were pretreated with 5 μg/ml of cytochalasin B.

Intracellular Ca^{2+} concentrations

These concentrations were calculated from the change of Fura 2-AM fluorescence [10]

Neutrophils (5×10^6 cells/ml) in HBSS supplemented with 20 mmol/l HEPES, pH 7.4, were incubated at 37 °C with 0.5 μmol/l Fura 2-AM for 30 min. Loaded cells were washed twice, reconstituted in HBSS (with Ca^{2+} at 1.27 mmol/l) and stored on ice until use. Cells were then warmed at 37 °C with continuous stirring of the cell suspension. Excitation wavelength was set at 340 nm and emission at 510 nm. After a stable baseline had been established, stimulus was added and emitted light recorded until return to baseline. The system was controlled by addition of EGTA, Tris buffer, Triton X-100 and $CaCl_2$, as previously described [20]; calculations of the calcium concentrations were performed according to Metcalf *et al.* [20]. Standard stimuli were fMLP and ionomycin (0.1 μmol/l and 1 μmol/l, respectively).

Statistical analysis was performed with Student's two-tailed t-test.

RESULTS

Neutrophils responded with a burst of chemiluminescence when exposed to fMLP, LTB4, PAF and several of the alkylglycerols and OAG (Table I; Fig. 1). FMLP was the most potent inducer, followed in descending order (based on comparisons at equimolar concentrations) by OAG, PAF and ET-16-OCH3. LysoPAF, ET-18-OCH3, M-lysoPAF, CA, BA, MPG and SPC induced minimal responses, whereas no chemiluminescent activity was associated with PPC or M-BA. Responses to LTB4 were approximately one-tenth of that of FMLP (both at 0.1 μmol/l). OAG produced a very strong response at 10 μmol/l, but only little at lower concentrations. It should be observed that responses to most alkyl-lipids were minute compared with that conferred by fMLP and LTB4 at a 100-fold lower concentration, PAF and ET-16-OCH3 being the exceptions.

The chemiluminescent responses exhibited clear kinetic differences, as shown in Figure 1. LTB4 conferred a very rapidly emerging and disappearing response (as reported previously) [17–19]. fMLP was associated with a response slower in onset and decline. PAF elicited a double-peaked response [cf. 21]. OAG responses emerged slowly but persisted for nearly 20 min. The other lipids produced responses which were intermediate with regard to response kinetics.

TABLE I. Effect of various ether lipids as activators of neutrophil chemiluminescence

	Relative chemiluminescent activity, in percent, at the concentration of (μmol/l)		
Agent	0.1	1	10
fMLP	100		
LTB4	12±5 (15)		
PAF	4±2 (4)	19±6 (4)	41±8 (4)
LysoPAF	ND	0 (2)	6±2 (3)
M-lysoPAF	ND	0 (2)	2.4±1.2 (6)
PPC	ND	0 (2)	0 (3)
SPC	0 (2)	1.3±0.7 (3)	
ET-16-OCH3	0 (2)	0.4±0.2 (3)	18±11 (3)
ET-18-OCH3	ND	0 (2)	4±2 (5)
CA	ND	0 (2)	2.8±2.1 (5)
MPG	ND	0 (2)	2.4±1.6 (5)
BA	ND	0 (2)	0 (5)
OAG	ND	0 (3)	2900±350 (3)

ND, not determined. Mean and SE values are given with the number of separate experiments in the brackets. Results are expressed as relative chemiluminescent activity when compared to simultaneously run controls stimulated with 0.1 μmol/l fMLP; that stimulation conferred a light emission corresponding to 134±15 mV (n=15). The dose-response curves for fMLP. LTB4 and PAF have been published elsewhere [17, 18]. When a stimulus was associated with a dual peak response (e.g. PAF), calculations were based on the height of the major peak.

Since luminol-enhanced chemiluminescence is a highly sensitive but rather complex process, where several neutrophil secretory products play roles, most notably the superoxide anion and myeloperoxidase–H_2O_2-dependent reactions [17, 18, 22–23], we also analysed superoxide ion formation specifically. With the cytochrome *c* reduction assay an O_2^- response was documented for fMLP and PAF but not for the other compounds (Table II). Cytochalasin B enhanced responses, but did not make previously inactive compounds express activity. The similarities of the kinetics between chemiluminescent and cytochrome *c* reductions have been described previously [24].

We next assessed another secretory response, the extrusion of elastase. Again, fMLP and PAF were active. The PAF response (for 10 μmol/l) was 45% of the fMLP response (for 0.1 μmol/l) reaching maximal degranulation significantly later than did fMLP (data not shown).

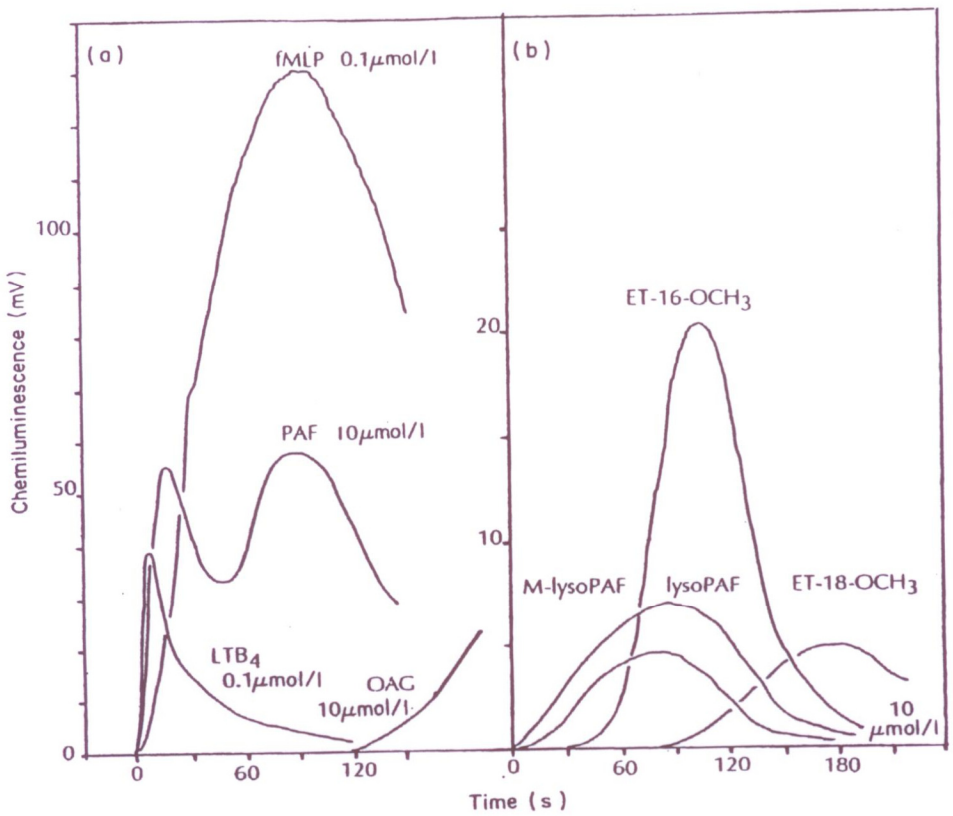

FIG. 1. The chemiluminescent responses to fMLP, LTB4 and various lipids. The figure depicts an actual tracing from a representative experiment repeated at least three times with similar results. Only the beginning of the OAG response is shown here; it increased gradually and continued for at least 20 min.

A rise of intracellular calcium concentrations $[Ca^{2+}]_i$, is a prerequisite for an oxidative response to fMLP and LTB4 [19, 25]. Thus, we next analysed whether the lipids would confer an increase of Fura 2 fluorescence, since this is a probe for $[Ca^{2+}]_i$; as shown in Table III and Figure 2 fMLP and LTB4 did so. The PAF and lysoPAF responses were also marked, the latter being half of the former with regard to peak heights. M-lysoPAF was half as potent as lysoPAF. ET-16-OCH3 and, to a lesser extent, ET-18-OCH3 were also active, the former

TABLE II. Effect of ether lipids on cytochrome *c* reduction

Stimulus	Concentration (μmol/l)	Cytochachalasin B addition	O_2^- generation: direct stimulation	O_2^- generation: as priming agent for 0.1 μmol/l fMLP
fMLP	0.1	–	0.65±0.09	0
		+	4.47±0.29	ND
PAF	10	–	0.15±0.04	3.91±1.47 (305)
		+	0.16±0.04	ND
M-lysoPAF	10	–	0	5.64±0.30 (441)
		+	0	ND
M-BA	10	–	0	0.99±0.01 (78)
		+	0	ND
Ethanol	0.1%	–	0	1.28±0.81 (100)

Results are given as mean±SE values for three separate experiments. The superoxide ion generation is given as maximal μmol/l O_2^-/2×10⁶ PMN/ml. ND, not determined. Figures in brackets denote the increase, as percent of the control (i.e. ethanol-treated cells).

TABLE III. Fura 2 fluorescence responses to ether lipids

Stimulus	Concentration (μmol/l)	Rise of $[Ca^{2+}]_i$ (nmol/l)	n
fMLP	0.1	+253±55	6
LTB4	0.1	+248±60	3
Ionomycin	1	+381±42	3
PAF	10	+512±150	5
LysoPAF	10	+234±60	5
M-lysoPAF	10	+138±34	5
ET-16-OCH3	10	+308±54	5
ET-18-OCH3	10	+224±36	5
M-BA	10	0	4
OAG	10	0	3

The figures are given as the maximum rise above the $[Ca^{2+}]_i$ of resting cells, which was 74±10 nmol/l.

being more than half as active as PAF. In contrast, OAG, PPC and M-BA did not produce a rise in $[Ca^{2+}]_i$.

The kinetics of the $[Ca^{2+}]_i$ responses were highly characteristic. As shown in Figure 2 increases were rapid and transient for LTB4 and biphasic for fMLP. Fluorescence responses to ET-18-OCH3 (at 10 μmol/l) were similar to those of fMLP, but the second peak was more prominent for the lipid. In contrast, PAF, lysoPAF and ET-16-OCH3 were associated with kinetics resembling those of the calcium ionophore ionomycin, i.e. a sustained elevation of $[Ca^{2+}]_i$. With lower ether lipid concentrations responses were of shorter duration (Fig. 2).

The question of whether the various lipids would prime a neutrophil for a subsequent oxidative response to fMLP was assessed next. We studied PAF, ET-18-OCH3, M-lysoPAF, BA, M-BA and CA, since these substances were associated with either distinct, minute or no direct chemiluminescent and calcium responses. The substance under test was incubated with polymorphonuclear leukocytes (PMNs) at 37 °C and after 15 min luminol and fMLP were added. The ensuing light emission was compared to fMLP-activated controls incubated for 15 min with HBSS and the appropriate concentrations of the solvent

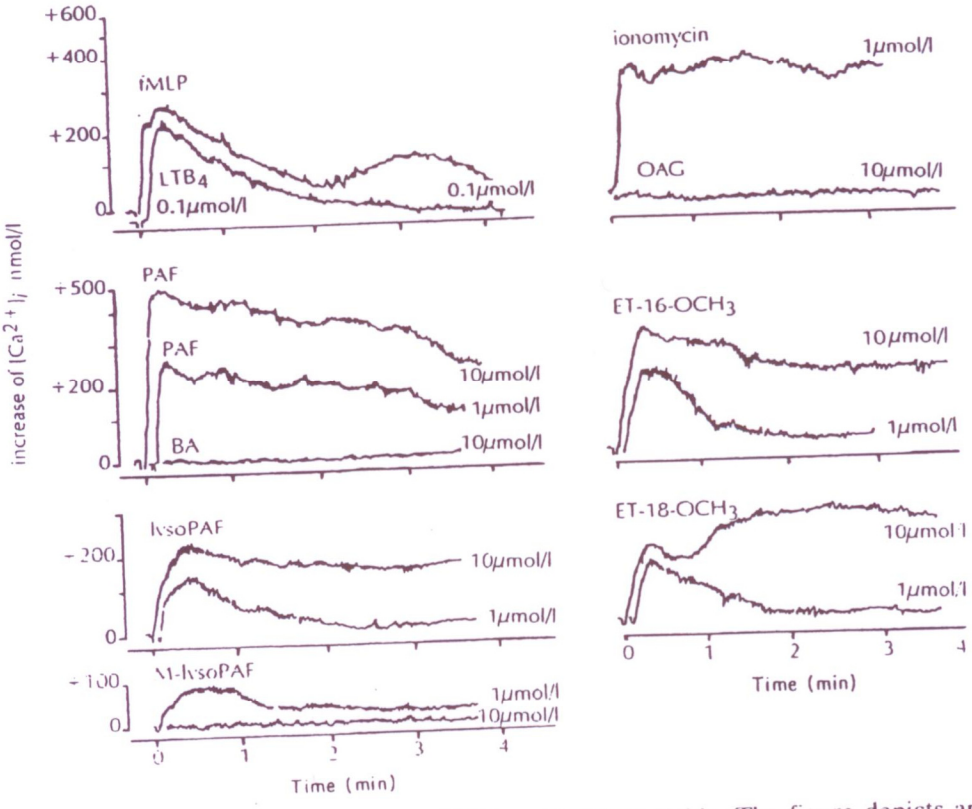

FIG. 2. Fura 2-fluorescent responses to fMLP, LTB4 and various lipids. The figure depicts an actual tracing from one experiment which was repeated at least three times with similar results. The y-axis gives the increase of $[Ca^{2+}]_i$ from the basal level, which was 74±10 nmol/l.

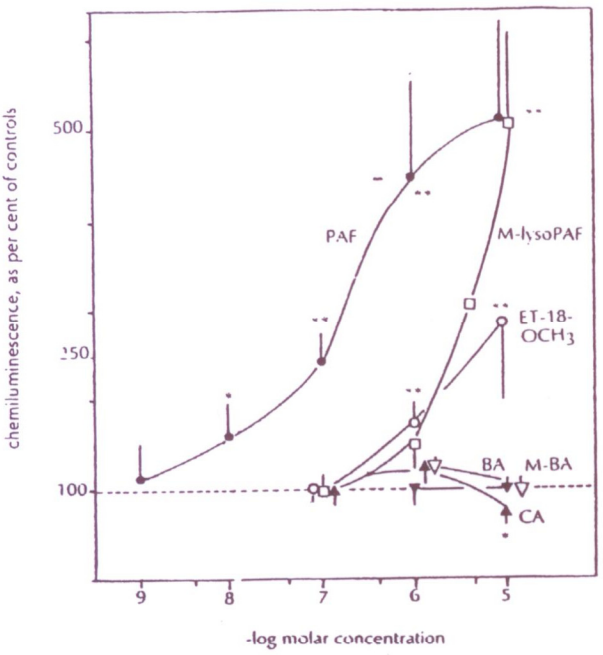

FIG. 3. The priming capacity of alkylglycerols for chemiluminescence induced by 0.1 μmol/l fMLP. Neutrophils were treated for 15 min with the indicated concentration of the lipid at 37 °C, subsequently cells were stimulated by fMLP. The maximal chemiluminescent response was related to that induced by fMLP in solvent-treated cells (i.e. 0.1% ethanol in HBSS). Symbols: ●=PAF, □=M-lysoPAF, ○=ET-18-OCH3, ▲=CA, ▼=BA, ▽=M-BA.

ethanol. We found that PAF and, to a lesser extent, M-lysoPAF and ET-18-OCH3 enhanced the fMLP response dose-dependently, whereas BA, CA and M-BA did not. In contrast, CA was associated with a significantly inhibitory effect (Fig. 3). These results were confirmed by analysis of superoxide ion generation with the cytochrome *c* reduction assay. As shown in Table II, priming with 10 μmol/l PAF and M-lysoPAF, but not with M-BA, enhanced fMLP-induced O_2^- three- to five-fold.

DISCUSSION

This study has shown that a number of alkyl-lipids stimulated a functional response in human neutrophils. The tested response was either activation of the oxidative metabolism or a rise of $[Ca^{2+}]_i$ or both. The lipids were also able to prime neutrophils to an enhanced oxidative response when stimulated by an unrelated agent, fMLP.

PAF was, on an eqimolar basis, the most potent of tested ether lipids in conferring a secretory as well as a $[Ca^{2+}]_i$ response. The fact that PAF can induce these reactions has been reported previously [12–14, 21, 26, 27]. ET-16-OCH3, one of several structurally modified ether lipids, which hitherto have not been evaluated as stimulus for neutrophil function, was also able to stimulate both functions. Some of the other phosphatidylcholine-containing ether lipids were associated with a rise of $[Ca^{2+}]_i$, but only with minute or no oxidative responses (lysoPAF > ET-18-OCH3 > M-lyso-PAF). CA, BA and M-BA, being simple alkyl-glycerols, were only weak stimulants for chemi-luminescence. The ability to prime neutrophils paralleled the direct chemiluminescence-activating capacity.

These observations may form a basis for discussions of structure–activity relations. It is apparent that the phosphatidyl group in 3-position was essential for both calcium and oxidative responses. This is exemplified by lysoPAF, on the one hand, and CA and BA, on the other. Likewise, the introduction of a methyl group in 2-position increased the tested biological activity (cf. ET-16-OCH3 and ET-18-OCH3 with lysoPAF, which differ only in this respect) [4, 8, 9, 27], although the methyl group was less active than an acetyl group (cf. ET-16-OCH3 and ET-18-OCH3 with PAF). Insertion of a methoxy side-group on the fatty acid in 1-position reduced the assessed biological activity (cf. lysoPAF with M-lysoPAF and BA with M-BA). An acetyl group in 2-position was associated with greater efficacy, as demonstrated by comparisons of PAF with lysoPAF and OAG with MPG, and by other investigators [28, 29]. The main issue, whether an ether bond changes the efficacy in relation to an ester bond cannot be unequivocally answered because of lack of substances for appropriate comparisons. However, comparisons of PAF and lysoPAF with PPC and SPC, and of BA and CA with MPG, suggest that ether compounds were associated with enhanced biological activity with regard to the functions analysed here.

This study also shows that some ether lipids induced oxidative as well as $[Ca^{2+}]_i$ responses (PAF, ET-16-OCH3), whereas others mainly conferred a $[Ca^{2+}]_i$ rise (lysoPAF, ET-18-

OCH3, M-lysoPAF). This suggests that a calcium rise is not sufficient in its own right to activate the NADPH-oxidase, catalysing conversion of oxygen to superoxide ion. On the other hand, substantial oxidative responses were noted only for substances with $[Ca^{2+}]_i$-enhancing properties, which may indicate that $[Ca^{2+}]_i$ plays a role in the induction of O_2^- by these substances. The reason for the discrepancy of responses is not known, but similar phenomena have been reported previously [30].

It is not yet known whether the ether lipids may activate the other major pathway for oxidative responses in the neutrophil, mediated by protein kinase C (PKC). OAG was used as an example of a stimulus strictly dependent on this pathway. Preliminary data, obtained by adding PAF, M-lysoPAF, BA, CA, M-BA, or MPG to purified PKC *in vitro* and assessing the phosphorylation of the PKC substrate histone-III in the presence or absence of calcium or phosphatidyl serine, suggested that no major activation of PKC occurred (A. Hansson, J. Palmblad, J. Samuelsson and J. Brohult, unpublished observations). Similar results have been presented [31, 32].

It is interesting to note that the kinetics of the rise of $[Ca^{2+}]_i$ was also reflected in the kinetics of the chemiluminescent response, e.g. the rapid responses to LTB4, the slower but more sustained responses to fMLP, PAF and ET-16-OCH3, and the considerably slower response to ET-18-OCH3 (Figs 2 and 3). It is not known whether the difference between the two latter relates to the length of the alkyl group.

Ether lipids have attracted interest as anti-tumour agents. ET-18-OCH3 has been advocated as a bone marrow purging substance [33]. The intake of a mixture of CA, BA, M-BA and methoxy-substituted alkyl lipids, similar to M-lysoPAF, has been shown to reduce the 5-year mortality from cervical cancer [10] and also to reduce the radiation-induced neutropenia in such patients [34]. Moreover, ether lipids may increase blood concentrations of neutrophils (J. Brohult, J. Samuelsson, J. Palmblad, unpublished observations) and enhance antibody formation [3, 7]. Taken together, these observations and the present study may establish a platform for further studies of the biological effects of alkylglycerols which are, for as yet unknown reasons, abundant in the bone marrow, spleen, liver and neutrophils [1, 2].

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