Dissociation of the effects of the antitumour ether lipid ET-18-OCH$_3$ on cytosolic calcium and on apoptosis

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1 We have compared the effects of 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH$_3$) on the cytosolic free calcium concentration ([Ca^{2+}]$_i$) and on apoptosis in several normal and leukaemia cells, including human polymorphonuclear neutrophils (PMNs), U937 cells, and undifferentiated as well as dimethylsulphoxide-differentiated HL60 cells (uHL60 and dHL60, respectively).

2 ET-18-OCH$_3$ produced apoptosis, as evidenced by DNA degradation into oligonucleosome-size fragments, in U937 and uHL60 cells, but not in dHL60 cells or PMNs.

3 ET-18-OCH$_3$ induced an increase in [Ca^{2+}]$_i$ mediated through the platelet-activating factor (PAF) receptor in U937, dHL60 cells and PMNs, as shown by cross-desensitization experiments and by prevention of the [Ca^{2+}]$_i$ changes by the PAF antagonist WEB-2170. The EC$_{50}$ values for the increase in [Ca^{2+}]$_i$ induced by PAF and ET-18-OCH$_3$ were 5×10^{-11} and 2.5×10^{-7} M, respectively. In uHL60 cells the effect of ET-18-OCH$_3$ on [Ca^{2+}]$_i$ was very small and was not affected by WEB-2170.

4 PAF did not produce apoptosis in any of the cell types tested. WEB-2170 did not prevent the apoptosis induced by ET-18-OCH$_3$.

5 The uptake of [^{3}H]-ET-18-OCH$_3$ was much larger in U937 and uHL60 cells than in dHL60 cells and PMNs.

6 Our results indicate that the apoptotic effect of ET-18-OCH$_3$ is not related to the changes in [Ca^{2+}]$_i$, effected by interaction with plasma membrane PAF receptors, but to other actions which are associated with the uptake of this drug into the cells.

Keywords: Apoptosis; cytosolic calcium; ether lipids; PAF; ET-18-OCH$_3$; antitumour drug

Introduction

Several synthetic ether lipid analogues of platelet-activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, PAF) have been found to be selectively cytotoxic to tumour cells (Andreesen *et al.*, 1978; Okamoto *et al.*, 1987). One of them, 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH$_3$), has been used to purge leukaemic cells from remission marrows in acute leukaemia (Vogler *et al.*, 1992) and behaves as a promising anticancer drug (Dietzfelbinger *et al.*, 1993; Houlihan *et al.*, 1995). This drug has been shown previously to induce apoptosis in several human leukaemia cells (Mollinedo *et al.*, 1997; 1993; Diomede *et al.*, 1993), but the underlying mechanism of its action remains unknown. ET-18-OCH$_3$ has also been found to induce changes in the cytosolic Ca^{2+} concentration ([Ca^{2+}]$_i$) (Seewald *et al.*, 1990; Lazemby *et al.*, 1990; Lohmeyer & Workman, 1993; Bergmann *et al.*, 1994) and several lines of evidence indicate that Ca^{2+} may play a role in regulation of apoptosis in many cell models (McConkey & Orrenius, 1994). Since ET-18-OCH$_3$ is a synthetic 2-*O*-methyl analogue of PAF, we have compared the effects of ET-18-OCH$_3$ and PAF on [Ca^{2+}]$_i$ and on apoptosis in normal and leukaemia cells. We found that ET-18-OCH$_3$ is able to induce a [Ca^{2+}]$_i$ increase acting through the PAF receptor. However, the increase in [Ca^{2+}]$_i$ and the ability to induce apoptosis were not correlated. In addition, PAF was unable to induce apoptosis and PAF-antagonists did not prevent the apoptosis induced by ET-18-OCH$_3$.

Methods

Human neutrophils were obtained from blood of normal volunteers and prepared as described previously (Montero *et al.*, 1991). HL60 and U937 human leukaemia cells were propagated and cultured in RPMI-1640 culture medium containing 10% heat-inactivated foetal calf serum as described elsewhere (Mollinedo *et al.*, 1994a). HL60 cells were differentiated towards granulocytes by culture in medium containing 1.3% dimethyl sulphoxide (DMSO) for 5 days (Collins *et al.*, 1987).

[Ca^{2+}]$_i$ was measured in cells loaded with fura-2 (Grynkiewicz *et al.*, 1985; Montero *et al.*, 1991; Alonso-Torre *et al.*, 1993). For this purpose the cells were incubated at about 2% cytocrit in standard medium containing 4 μM fura-2/AM for 30–45 min at room temperature. The composition of standard medium was (in mM): NaCl 145, KCl 5, MgCl$_2$ 1, CaCl$_2$ 1, glucose 10 and Na-HEPES 10, pH 7.4. The cells were then washed twice, resuspended at 1% cytocrit and stored at room temperature until used. Fluorescence measurements were performed in 0.5 ml samples of the cell suspension, kept at 37°C under magnetic stirring, in a fluorescence spectrometer constructed by Cairn Research (Newnham, Sittingbourne, Kent, U.K.), which allows quasi-simultaneous (30–300 Hz) measurements of fluorescence excited at up to six different wavelengths. Emission above 510 nm was measured and readings integrated at 1 s intervals. [Ca^{2+}]$_i$ was estimated from the ratio of the fluorescences excited at 340 and 380 nm (Grynkiewicz *et al.*, 1985).

DNA fragmentation was assessed in samples obtained from about 4×10^6 cells by electrophoresis on 1% agarose gels, by use of a 123-bp DNA ladder as standard. Details were as described previously (Mollinedo *et al.*, 1993).

For uptake measurements, cells (10^6) were incubated in 1 ml of standard medium containing 0.03 μCi of [^{3}H]-ET-18-OCH$_3$ and 6 μM of the unlabelled compound. After 1 and 2 h of

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incubation at 37°C the cells were washed with phosphate buffered saline, dissolved in 5 ml of water-miscible liquid scintillator (Ultrafluor, National Diagnostics, Manville, NJ) and counted for radioactivity (Mollinedo *et al*., 1994b).

Fura-2 was obtained from Molecular Probes (Eugene, OR, U.S.A.). PAF was from Calbiochem (San Diego, CA, U.S.A.). ET-18-OCH$_3$ was obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland). A stock solution was prepared at 1 mM in culture medium containing 20% (v/v) foetal calf serum by heating at 50°C for 30 min. [^{3}H]-ET-18-OCH$_3$ was obtained from Amersham Buchler (Braunschweig, Germany). WEB-2170 (5-(2-chloro-phenyl)-5, 4-dihydro-10-methyl-3-[(4-morpholinyl) carbonyl]-2H, 7H-cyclopenta[4,5]thienol [3,2-f][1,2,4]tri-azo[4,3-a][1,4]diazepine), a potent PAF antagonist (Heuer *et al*., 1990) was a generous gift from Boehringer Ingelheim KG (Germany). All other chemicals were either from Sigma (London, U.K.) or from Merck (Darmstadt, Germany).

Results

ET-18-OCH$_3$ induced apoptosis in human myeloid leukaemia U937 and in undifferentiated HL60 (uHL60) cells, as evidenced by DNA degradation into oligonucleosome-size fragments (Figure 1). This corroborates previous findings (Mollinedo *et al*., 1993). Figure 2 illustrates the effects of ET-18-OCH$_3$ on [Ca^{2+}]$_i$ of U937 cells loaded with fura-2. The drug induced an increase of [Ca^{2+}]$_i$ composed of a transient increase followed by a sustained plateau. The effect was very similar to the one induced by PAF (compare the first two traces in Figure 2), and, in fact, the [Ca^{2+}]$_i$ peaks induced by both ET-18-OCH$_3$ and PAF were blocked completely by the PAF antagonist WEB-2170 (Figure 2, last two traces). This suggests that the action of ET-18-OCH$_3$ on [Ca^{2+}]$_i$ was mediated through stimulation of PAF receptors. However, with regard to apoptosis, PAF was not able to induce DNA degradation in U937 cells and WEB-2170 did not prevent the ET-18-OCH$_3$-induced apoptosis (Figure 1a). It is interesting to note that PAF is converted to 1-alkyl-2-acyl-glycero-3-phosphocholine at a rate of about 50% per hour (García *et al*., 1991). However, even at this high rate of conversion the concentration of unmodified PAF would still be above 10^{-9} M after 12 h, which is able to produce over 80% of the maximum effect on [Ca^{2+}]$_i$ (see below, Figure 4).

In human neutrophils, ET-18-OCH$_3$ did not promote apoptosis (results not shown), but this drug produced an increase of [Ca^{2+}]$_i$ (Figure 3a). This effect was mimicked by PAF
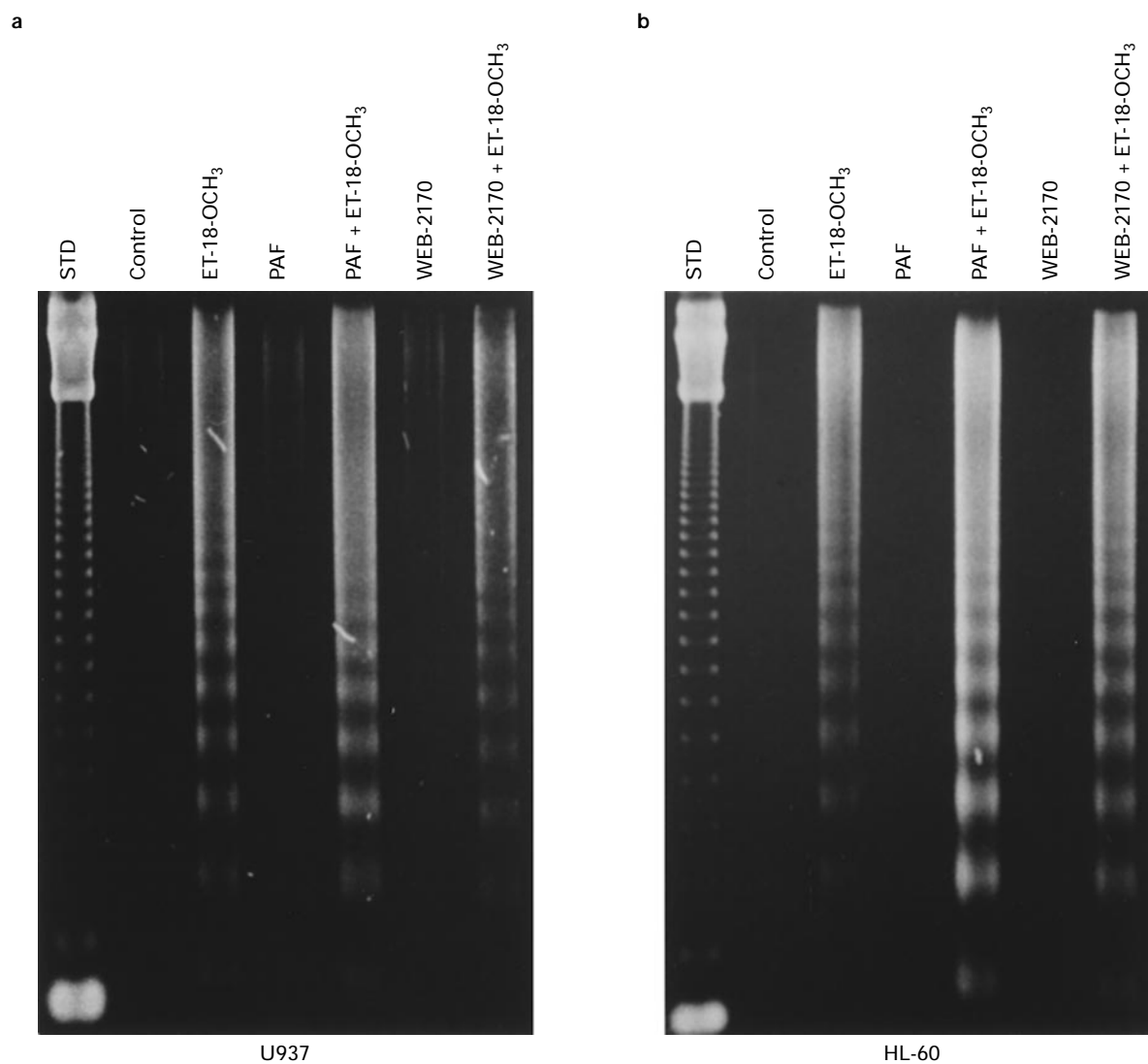

Figure 1 DNA fragmentation induced by ET-18-OCH$_3$ in several cell types. DNA was extracted and analysed as described in Methods. U937 (a) and undifferentiated HL60 cells (b) were treated with the indicated agents for 12 h. Cells were incubated with no additions (control), with 6 μM ET-18-OCH$_3$, with 6 μM PAF, with a mixture of both PAF (6 μM) and ET-18-OCH$_3$ (6 μM), with 10 μM WEB-2170 or with 6 μM ET-18-OCH$_3$ and 10 μM WEB-2170. The first lane of each panel shows a 123-bp DNA ladder, used as standard (STD). Fragmented DNA from 6 × 10^5 cells was loaded in each lane. The experiment shown is representative of three performed.
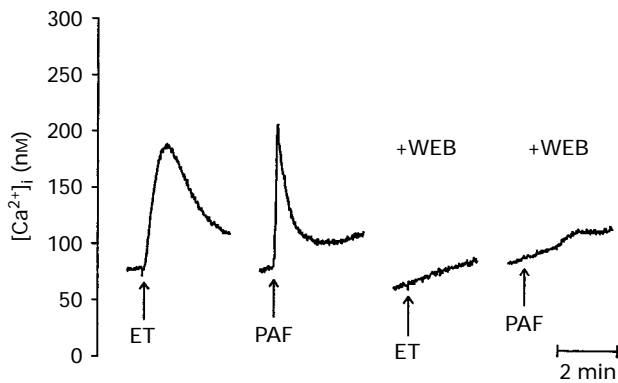
Figure 2 Effects of ET-18-OCH$_3$ (ET, 6 μM) and PAF (1 μM) on [Ca^{2+}]$_i$ in U937 cells. In the last two traces, 10 μM WEB-2170 was added to the cells 1 min before stimulation with ET-18-OCH$_3$ or PAF. The experiment shown is representative of 3 similar ones.

(Figure 3b) and cross-desensitization between both drugs was observed (compare the relative size of the [Ca^{2+}]$_i$ peaks obtained when each drug was added either before or after the other one in Figure 3a and b). This was also observed when two consecutive additions of either ET-18-OCH$_3$ or PAF were used (results not shown). In contrast, cross-desensitization was not observed between ET-18-OCH$_3$ and leukotriene B$_4$, an agonist acting through an entirely different membrane receptor (Figure 3c and d). In addition, the [Ca^{2+}]$_i$ increase induced by ET-18-OCH$_3$ was fully blocked by the PAF antagonist WEB-2170 (Figure 3e). As shown previously for PAF (Montero *et al.*, 1991) the [Ca^{2+}]$_i$ increase induced by ET-18-OCH$_3$ had a component due to Ca^{2+} release from the intracellular Ca^{2+} stores and another one due to Ca^{2+} entry. This is shown by the observation that removal of Ca^{2+} from the incubation medium decreased but did not abolish the increase of [Ca^{2+}]$_i$ induced by ET-18-OCH$_3$ (compare traces a and f in Figure 3). Figure 4 compares the dose-response curves for the effects of ET-18-OCH$_3$ and PAF on [Ca^{2+}]$_i$ of neutrophils. The values estimated for EC$_{50}$ were 2.5×10^{-7} and 5×10^{-11} M, respectively. Thus, synthetic ET-18-OCH$_3$ seems to activate PAF receptors with a much smaller affinity than the natural lipid, in spite of their similarity in molecular structure.

HL60 leukemia cells do not respond to PAF, but express PAF receptors when they are induced to differentiate towards granulocytes by incubation with DMSO (Vallari *et al.*, 1990; Müller *et al.*, 1991). Thus, it would be most informative to compare the actions of ET-18-OCH$_3$ in non-differentiated (uHL60) and DMSO-differentiated HL-60 (dHL60) cells. Results from these experiments are summarized in Figure 5. The first trace in (a) shows that PAF had little or no effect in uHL60 cells. ET-18-OCH$_3$ had a very small [Ca^{2+}]$_i$-increasing effect (second trace in Figure 5a), which was not prevented by WEB-2170 (third trace in Figure 5a). This small [Ca^{2+}]$_i$ transient seems to be due to Ca^{2+} entry, as it was fully prevented by Ca^{2+} removal or by addition of Ni^{2+}, an antagonist of Ca^{2+} entry (Figure 5a). For comparative purposes, the last trace in Figure 5a illustrates the effect of adenosine 5'-triphosphate (ATP), for which uHL60 cells do possess functional receptors (Montero *et al.*, 1995). In some batches of uHL60 cells PAF also produced a small [Ca^{2+}]$_i$ increase which was insensitive to WEB-2170. In contrast to the poor effects of ET-18-OCH$_3$ on [Ca^{2+}]$_i$ in uHL60 cells, this compound was able to induce apoptotic DNA degradation in these cells, which, as in U937 cells, was neither reproduced nor prevented by PAF or by the PAF antagonist WEB-2170 (Figure 1b).

In DMSO-differentiated HL60 cells (dHL60) both PAF and ET-18-OCH$_3$ were able to induce a transient increase of [Ca^{2+}]$_i$ which was blocked by WEB-2170 (Figure 5b). In contrast, it has been shown that HL-60 cells become resistant to the cytotoxic action of the ether lipid following cell differentiation towards granulocytes with DMSO (Vallari *et al.*, 1988).
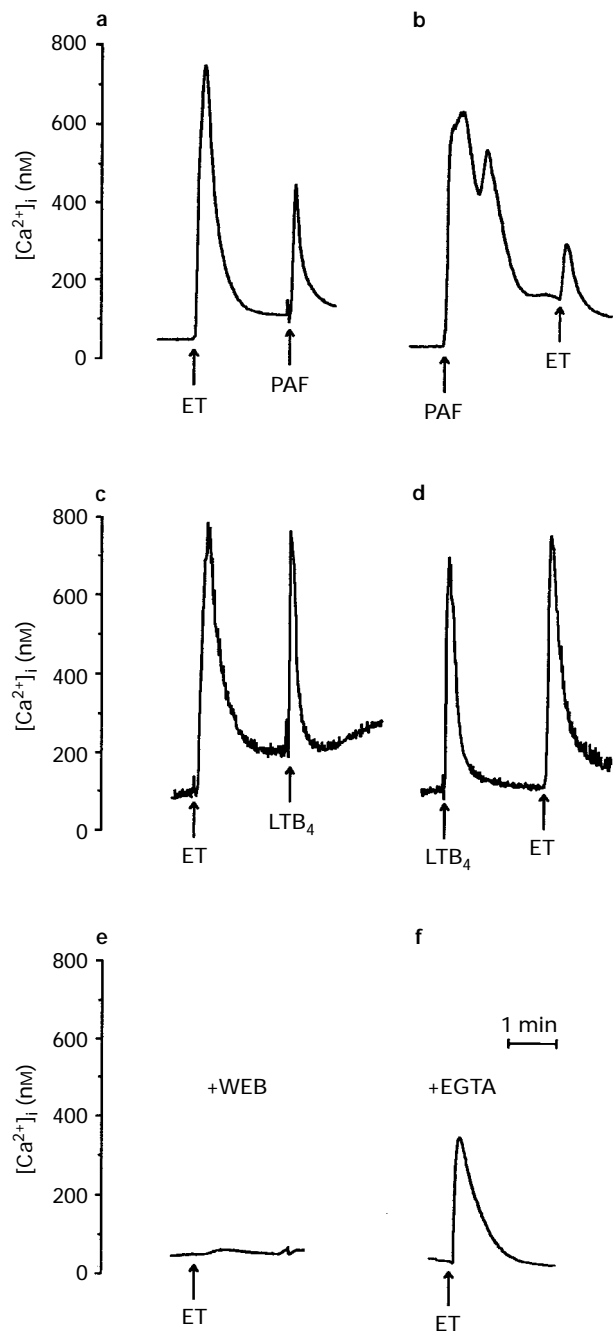

Figure 3 Cross-desensitization between the effects of ET-18-OCH$_3$ (ET, 6 μM) and PAF (100 nM) on [Ca^{2+}]$_i$ in human neutrophils. In traces (a) and (b) the cells were stimulated as shown. Traces (c) and (d) show similar experiments, but with ET-18-OCH$_3$ (6 μM) and leukotriene B$_4$ (LTB$_4$, 20 nM) as the stimulators. In trace (e) 10 μM WEB-2170 was added 1 min before ET-18-OCH$_3$. In trace (f) excess EGTA (1 mM) was added 1 min before ET-18-OCH$_3$. The experiment shown is representative of 3 similar ones.

Finally a comparative study of the uptake of ^{3}H-labelled ET-18-OCH$_3$ by the different cell types used in this study was performed. U937 and uHL60 cells showed much larger uptake of ET-18-OCH$_3$ than either dHL60 or neutrophils (Figure 6).

Discussion

Ether lipids have been shown to have multiple biological activities (Munder & Westphal, 1990). Among them, a prominent one is their ability to induce apoptosis in tumour cells (Mollinedo *et al.*, 1993; Diomede *et al.*, 1993; Mollinedo *et al.*,
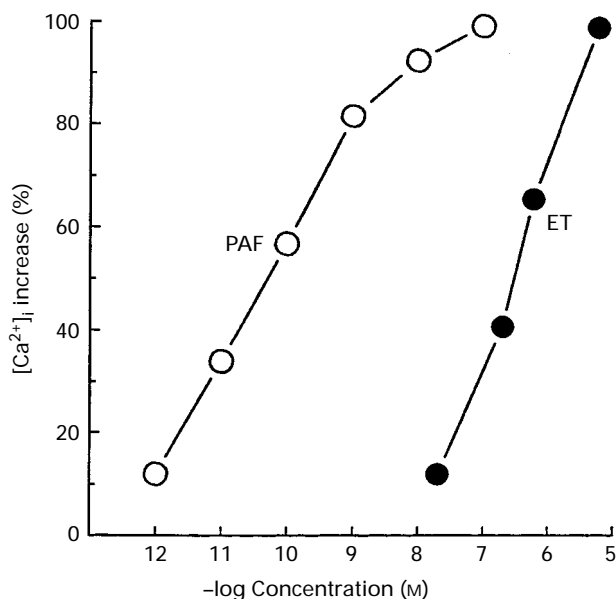
Figure 4 Comparison of the dose-response curves for the effects of ET-18-OCH₃ (ET) and PAF on $[Ca^{2+}]_i$ in human neutrophils. Results were quantified by the height of the $[Ca^{2+}]_i$ peak and are expressed as % of the maximum response. Each value is the mean of two data obtained in different experiments.
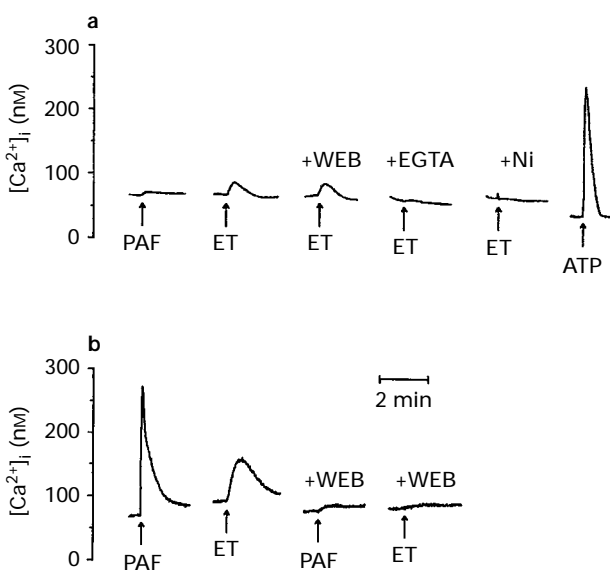

Figure 5 Effects of PAF (100 nM) and ET-18-OCH₃ (ET, 6 μM) in either undifferentiated HL60 cells (uHL60, a) or DMSO-differentiated HL60 cells (dHL60, b). WEB-2170 (10 μM), EGTA (2 mM) or NiCl₂ (5 mM) were added 1 min before ET-18-OCH₃ or PAF. The concentration of ATP was 100 μM. Data shown are representative of 2–4 different experiments.

1997). Ca^{2+} may play a role in regulation of apoptosis in many cell types (McConkey & Orrenius, 1994). Previous studies on the effects of ET-18-OCH₃ and the related antitumour ether lipid SRI 62–834 have shown the ability of these drugs to increase $[Ca^{2+}]_i$ (Seewald *et al.*, 1990; Lazemby *et al.*, 1990). Some of the early studies may be biased by the known ability of these drugs to disrupt membranes (Lohmeyer & Workman, 1993; Bergmann *et al.*, 1994). In the present study, we showed that ET-18-OCH₃ is able to induce a $[Ca^{2+}]_i$ increase, composed of a transient peak and a sustained plateau, in human leukaemia U937 and dHL60 cells as well as in human PMNs. This effect seems to be mediated by stimulation of the PAF receptor since WEB-2170, a well known PAF-receptor an-
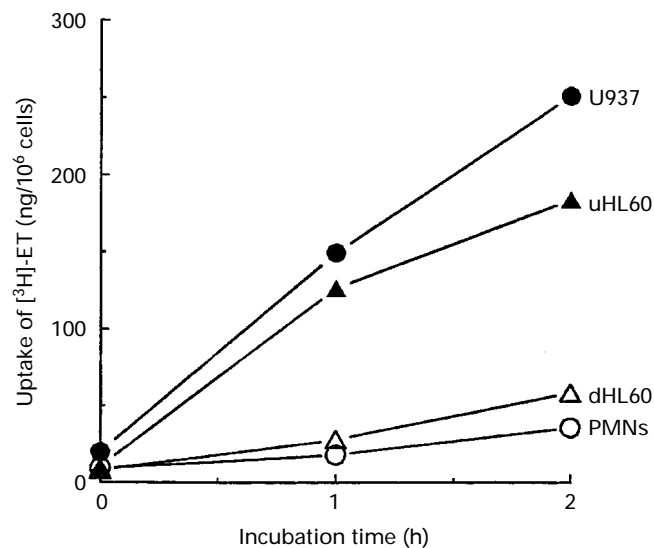

Figure 6 Uptake of ET-18-OCH₃ (ET) by U937 cells, undifferentiated (uHL60) and DMSO-differentiated (dHL60) HL60 cells and human neutrophils (PMNs). The data shown are the means of 2 values obtained in different experiments.

Table 1 Summary of the effects of ET-18-OCH₃ on apoptosis and $[Ca^{2+}]_i$ in U937, undifferentiated and differentiated HL60 cells and human neutrophils

Cell type	Apoptosis	$\Delta[Ca^{2+}]_i$	Uptake	PAFR
U937	Yes	Yes	Yes	Yes
uHL60	Yes	No	Yes	No
dHL60	No	Yes	No	Yes
Neutrophils	No	Yes	No	Yes

The last two columns give information on the ability to take up the ether lipid and the presence or the absence of PAF receptors (PAFR) in each cell type.

tagonist (Heuer *et al.*, 1990), produced complete inhibition. On the other hand, we observed cross-desensitization between PAF and ET-18-OCH₃. This effect was not unexpected as the chemical structures of PAF and ET-18-OCH₃ are very similar, ET-18-OCH₃ being an unnatural synthetic 2-*O*-methyl analogue of the natural lipid PAF. The affinity of the PAF receptor for ET-18-OCH₃ was about 5000 times smaller than for the natural PAF lipid, as estimated from the respective EC_{50} values for the $[Ca^{2+}]_i$ increases. In uHL60 cells, which do not possess PAF receptors (Vallari *et al.*, 1990; Müller *et al.*, 1991), the effect of ET-18-OCH₃ was very small, due only to Ca^{2+} entry and insensitive to WEB-2170.

We found no correlation between the effects of ET-18-OCH₃ on $[Ca^{2+}]_i$ and on apoptosis. Thus, ET-18-OCH₃ was unable to produce apoptosis in dHL60 cells and in neutrophils, where the effects on $[Ca^{2+}]_i$ were bigger. In contrast, ET-18-OCH₃ induced apoptosis in uHL60 cells, where it had little effect on $[Ca^{2+}]_i$ (compare to a 'real' agonist such as ATP, Figure 5a). On the other hand, PAF, which reproduced the effects of ET-18-OCH₃ on $[Ca^{2+}]_i$, was not able to produce apoptosis in any of the cells tested. Finally, WEB-2170 prevented the effects of ET-18-OCH₃ on $[Ca^{2+}]_i$ but had no effect on ET-18-OCH₃-induced apoptosis. The increased $[Ca^{2+}]_i$ seen in uHL60 cells was so small that it could be due to minor nonspecific perturbations of the plasma membrane induced by ET-18-OCH₃. Thus, the data presented here demonstrate that ET-18-OCH₃ can induce an increase in $[Ca^{2+}]_i$ through its interaction with the PAF receptor in cells that express them, but this rise in $[Ca^{2+}]_i$ is not involved in the apoptotic action. Also, the present results indicate that the apoptotic effect of ET-18-OCH₃ does not involve its binding to the PAF receptor. Table

1 shows a summary of the main effects of ET-18-OCH$_3$ on apoptosis and on $[Ca^{2+}]_i$, the correlation with the presence or absence of PAF receptors and with the ability of the cells to take up the ether lipid.

We found a good correlation between the uptake of $[^3H]$-ET-18-OCH$_3$, which was $5-10$ times higher in uHL60 and U937 cells than in dHL60 cells and PMNs, and the apoptotic ability. It has also been shown that leukaemia Jurkat cells take up ET-18-OCH$_3$ and are sensitive to apoptosis induced by this drug, whereas human peripheral blood lymphocytes do not take up ET-18-OCH$_3$ and are insensitive to apoptosis (Mollinedo *et al.*, 1994b). Thus, the apoptotic effect of ET-18-OCH$_3$ is not related to the changes in $[Ca^{2+}]_i$ effected by interaction with the PAF receptor, but to other actions which require access of the antitumour ether lipid to the cell interior. Recent results documenting activation of AP-1 transcription factor by ET-18-OCH$_3$ (Mollinedo *et al.*, 1994b; 1997) are consistent with this proposed mechanism.

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