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THE ROLE OF INTRACELLULAR FREE CALCIUM MOBILIZATION IN THE MECHANISM OF ACTION OF ANTITUMOUR ETHER LIPIDS SRI 62-834 AND ET18-OMe

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Abstract—Membrane-active antitumour ether lipids such as ET18-OMe (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) and SRI 62-834 ((±)-2-{Hydroxy[tetrahydro-2-(octadecyloxy) methylfuran-2-yl] methoxyl phosphinyloxy}-*N*,*N*,*N*-trimethylethaniminium hydroxide) are selectively cytotoxic to tumour cells *in vitro*. Their precise mechanisms of action are unclear, but they are known to have effects on cell membranes and cell signalling. A previous report suggested that ether lipids cause a biphasic sustained rise in intracellular free calcium [Lazenby *et al.*, *Cancer Res* **50**: 3327–3330, 1990]. We show here that the second phase is an experimental artefact due to cell membrane permeabilization by ether lipids in serum-free buffers. In serum-free medium, the membrane toxicity of antitumour ether lipids was increased 50–60 fold, when compared to medium containing 10% serum. Membrane disruption was neither dependent on extracellular calcium, nor modulated by preloading cells with the calcium chelators bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid or 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino] quinoline. This indicates that the mechanism of membrane damage by ether lipids does not involve changes in calcium homeostasis. Using indo-1 and fura-2 as calcium probes, we established that lower concentrations of antitumour ether lipids do elicit a genuine monophasic and transient rise in intracellular free calcium, predominantly mobilized from internal stores. This acute calcium agonist activity of ether lipids is distinct from the inhibitory effects on cell signalling reported previously after more prolonged exposure. It appears that the calcium elevation induced by antitumour ether lipids is unlikely to be instrumental in their selective and potent antitumour activity.

Antitumour ether lipids (AELs†) are structurally related to lysolethicin (1-acyl-*sn*-glycero-3-phosphocholine) and the naturally occurring ether lipid platelet-activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) (PAF) (Fig. 1). The latter phosphocholine lipid contains an alkyl moiety linked to the glycerol backbone at the *sn*-1 position by an ether linkage. Numerous synthetic ether lipids, often featuring an additional ether link at the *sn*-2 position, have been synthesized and a number of these were found to be selectively cytotoxic to tumour cells [1]. Structure–activity data on these synthetic ether lipids led to the development of alkylphosphocholines as potential anticancer agents. These lipids differ from ether lipids because they lack the glycerol backbone, retaining only the phosphocholine head group linked directly to an alkyl chain. The alkylphosphocholines have also shown promising antitumour activity [1, 2].

These two classes of structurally and mechanistically novel antineoplastic agents are believed to act in a similar fashion. Examples include ET18-OMe (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine), SRI 62-834 ((±)-2-{hydroxy[tetrahydro-2-(octadecyloxy)-methylfuran-2-yl]methoxyl phosphinyloxy}-*N*,*N*,*N*-trimethylethaniminium hydroxide) and hexadecylphosphocholine (HPC) (Fig. 1). The former has received extensive clinical evaluation and has shown particular promise as a bone marrow purging agent *in vitro* [3]. SRI 62-834 is about to enter Phase 1 clinical studies with the Cancer Research Campaign in the U.K. Topical administration of HPC in breast cancer patients has produced encouraging results [1]. Antitumour lipids exhibit an interesting array of antitumour activities and are of particular interest because they do not appear to kill cells via more conventional targets, such as the DNA structure and its replication process. However, the precise molecular mechanisms of action of AELs have yet to be elucidated [1, 4].

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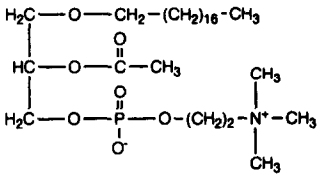
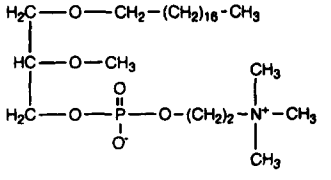
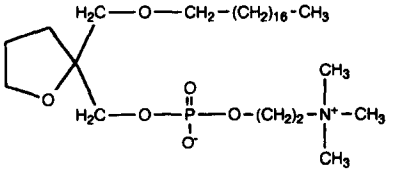
† Abbreviations: SRI 62-834, (±)-2-{hydroxy [tetrahydro-2-(octadecyloxy)methylfuran-2-yl] methoxyl phosphinyloxy}-*N*,*N*,*N*-trimethylethaniminium hydroxide; ET18-OMe, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; HPC, hexadecylphosphocholine; PAF, 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, platelet-activating factor; AEL, antitumour ether lipid; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; quin2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy] methyl]-6-methoxy-8-[bis (carboxymethyl) amino] quinoline; BAPTA, bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; MTT, [3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.



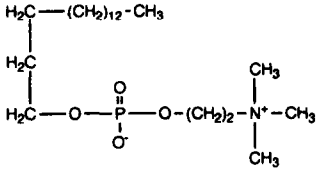
Platelet-activating factor (PAF) C-18



ET18-OMe



SRI 62-834



Hexadecylphosphocholine (HPC)

Fig. 1. Molecular structures for PAF, ET18-OMe, SRI 62-834 and HPC.

Recent studies on the antitumour effects of AELs are focusing on the cell membrane and cellular signalling mechanisms as potential targets [5–9]. Alterations in membrane structure [10], a membrane lytic activity [11, 12] and an increase in membrane permeability to fluorescent dyes [13] have been reported in response to AELs. Inhibition of the signalling enzymes protein kinase C and phospholipase C has also been described [1, 6, 8, 9, 14, 15]. Furthermore, there is evidence that AELs can depress growth factor-induced inositol-1,4,5-trisphosphate formation and thus inhibit the associated calcium elevations [9].

Hickman and co-workers [16, 17] have reported that HL-60 human promyelocytic leukemia cells responded to acute exposure to SRI 62-834 with a concentration-dependent biphasic rise in cytosolic free calcium concentration ($[Ca^{2+}]_i$), which was maintained at a high level for over 10 min. This sustained elevation in $[Ca^{2+}]_i$ is distinct from the usually more immediate and transient elevations associated with the effects of mitogens and growth factors in cancer cells expressing appropriate membrane receptors [18]. A genuine prolonged calcium rise in response to the AELs would suggest a link with calcium-induced cell killing, as is observed in a number of model systems in response to xenobiotic agents [19, 20].

We have investigated the calcium-mobilizing potential of AELs with particular focus on possible correlations with the cytotoxic and membrane-damaging activities of ET18-OMe and SRI 62-834 in HL-60 cells. Cytotoxicity was determined using the standard tetrazolium MTT {[3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide} dye-reduction assay with 4 days of continuous drug exposure, while short-term membrane permeability changes were examined by [^{51}Cr]chromate ion radiolabel release. The calcium responses were monitored spectrofluorimetrically using the new generation of calcium-sensitive fluorophores indo-1 and fura-2 [21]. These new calcium indicators are greatly superior to the previously used quin2 {2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline} probe [17] or the aequorin luminescence protein [9], because analysis of the ratio of readings obtained at different wavelengths allows a more precise determination of $[Ca^{2+}]_i$ changes without artefacts introduced by non-uniform dye loading, cell size and cell density [21]. Fura-2 and indo-1 are also more sensitive to slight changes in $[Ca^{2+}]_i$ and offer superior magnesium ion discrimination [21].

Our studies show that the apparent biphasic and sustained rise in $[Ca^{2+}]_i$ observed previously in response to cytotoxic concentrations of AELs is, in fact, an artefact due to membrane disruption in serum-free buffer. However, a monophasic and more short-lived elevation in $[Ca^{2+}]_i$ was seen following exposure to lower concentrations of AELs which did not induce substantial membrane damage. We conclude that calcium changes induced by AELs are unlikely to be involved in their selective and potent antitumour activity.

MATERIALS AND METHODS

Cell culture. The human promyelocytic leukemia cell line HL-60, obtained from Prof. J. A. Hickman (University of Manchester, U.K), was cultured routinely in antibiotic-free RPMI 1640 medium and supplemented with 10% foetal calf serum and 1 mM glutamine. Stock cultures were maintained in 75 cm^2 plastic tissue culture flasks at 37° in a humidified atmosphere of 92% air and 8% CO_2. Cells were mycoplasma free and used in log-phase throughout.

Calcium responses. Cellular calcium responses were measured using the indo-1 and fura-2 fluorophores [21] (Molecular Probes, Eugene, OR, U.S.A.). Cells in full medium were loaded with 2 μM of the membrane-permeable acetoxymethyl ester indo-1-AM or fura-2-AM for 40 min and then washed and resuspended in full medium at a density between 2 and 5 $\times$ 10^6 cells/mL. After loading, the cells were kept in a gassing incubator. For each assay, an aliquot of cell suspension was withdrawn,

washed and resuspended in 3 mL of serum-free assay buffer [145 mM NaCl, 5 mM KCl, 2.8 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 60 μM $MgSO_4$, 5.6 mM glucose and 15 mM HEPES, pH 7.2] at a density of 10^6 cells per mL, as described previously [17]. Cells were allowed to acclimatize to the buffer for about 5 min at 37° before being transferred to a 3 mL quartz cuvette for analysis. In some experiments, cells were resuspended in calcium-free buffer containing 2.5 mM EDTA. Fluorescence measurements were performed on a Perkin–Elmer LS50 spectrofluorimeter fitted with a thermostatted stirring cell holder (Perkin–Elmer, Beaconsfield, U.K.). Data were collected using the LS50s Intracellular Biochemistry software in its alternating (medium-speed) ratio mode. Indo-1 was excited at 355 nm and fluorescence responses were monitored alternately at 410 and 480 nm. Fura-2 was excited alternately at 340 and 380 nm while emission was collected at 510 nm [21].

Appropriate controls for membrane integrity were performed at the end of each experiment. This included fluorescence analysis of the medium after pelleting the cells to determine the amount of dye which had leaked into the medium. Measurements were carried out at both calcium-sensitive and insensitive (isosbestic) wavelengths. A further control involved determination of the amount of fluorescence quenching, following addition of excess EDTA or EGTA [22].

Membrane damage. This parameter was measured independently by monitoring the release of [^{51}Cr]-chromate ion (Amersham, Aylesbury, U.K.) from previously loaded cells using a standard procedure [23], which we adapted for use with cytotoxic drugs. Briefly, 2–5 × 10^6 cells were harvested, pelleted and resuspended in 100–200 μL of sodium [^{51}Cr]chromate in Dulbecco's phosphate-buffered saline (total activity = 100 μCi). The cells were incubated in the presence of [^{51}Cr]chromate for 1 hr, then washed and incubated in full medium for a further hour to avoid analysis during the initial period of high spontaneous release. The cells were then washed twice, counted and seeded into 96-well plastic microtitre plates at a density of 10^4 cells per well. The test agents were added immediately thereafter. After 3–4 hr of incubation, the plates were spun and 100 μL aliquots of supernatant were drawn from each well for analysis on a γ counter (LKB 1282 Compugamma-CS, South Croydon, U.K.). The activity in cpm was calculated for each well from duplicate 1-min counts with background and half-life correction. The results from four replicate wells per treatment dose were then averaged and the degree of membrane perturbation was calculated as a fraction of label incorporation according to the formula: (EXR − SR)/(TU − SR), where EXR is the observed experimental release, SR is the spontaneous release and TU represents total uptake of the ^{51}Cr-label, all in cpm [23]. The concentration causing 50% release of the isotope (R_{50}) was then interpolated from the resulting graph. In some experiments, cells were loaded with intracellular calcium buffer BAPTA [bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid]-AM or quin2-AM (Molecular Probes). The cells were exposed to 7.5 μM of the membrane-permeable chelators during the initial period of spontaneous [^{51}Cr]chromate release and again before cells were seeded into the microtitre plates. In other experiments, cells were resuspended in serum-free medium containing 2.5 mM EGTA before seeding into assay plates.

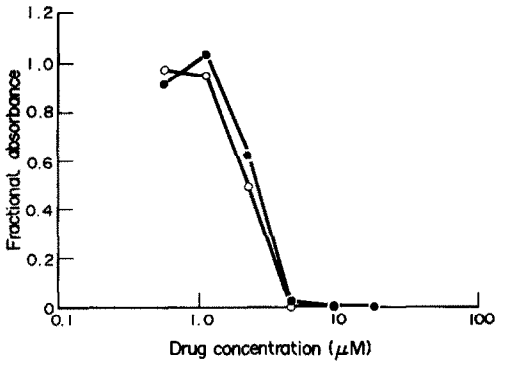


Fig. 2. Cytotoxicity dose–response profiles for ET18-OMe and SRI 62-834 in HL-60 cells. The data are representative of at least four independent experiments where cells were exposed to ET18-OMe (○) or SRI 62-834 (●) as detailed in Materials and Methods. The ID_{50} concentrations (see text) are those at which MTT dye absorbance was reduced to 50% of control values. Error bars have been omitted as standard deviations of the four replicate wells were routinely below 15% of the mean. Means and standard deviations for replicate experiments are given in Results.

Cytotoxicity testing. The cytotoxic potencies of ET18-OMe and SRI 62-834 were determined by the tetrazolium MTT assay [24] using a protocol adapted from Coley *et al.* [25] for the HL-60 cell line. Cells (10^4/mL) were seeded into 96-well plastic microtitre plates and drug was then added to the wells in a volume of 20 μL to give the required final concentrations. The cells were given a nominal 4-day drug exposure under normal cell culture conditions, during which control cells increased in number by a factor of 12–16. On day 4, 20 μL of MTT solution (5 mg/mL in Dulbecco's phosphate-buffered saline) was added to each well and the cells were incubated for a further 3–4 hr. The formazan crystals were dissolved in 200 μL of dimethyl sulphoxide, aided by gentle agitation on a plate shaker for 10 min. Absorbances were read on a Titertek Multiskan MCC MKII ELISA plate reader (Flow Laboratories, Helsinki, Finland) using a test wavelength of 540 nm and a reference of 690 nm. The values from four or eight replicate wells per treatment dose were averaged. Results were expressed as a fraction of untreated vehicle controls to establish the dose at which MTT–formazan conversion was reduced to 50% of control (ID_{50}). Experiments were carried out with HL-60 cells in order to establish that absorbance was linear with viable cell number.

RESULTS

Figure 2 illustrates the dose–response curves for the cytotoxicity of ET18-OMe and SRI 62-834

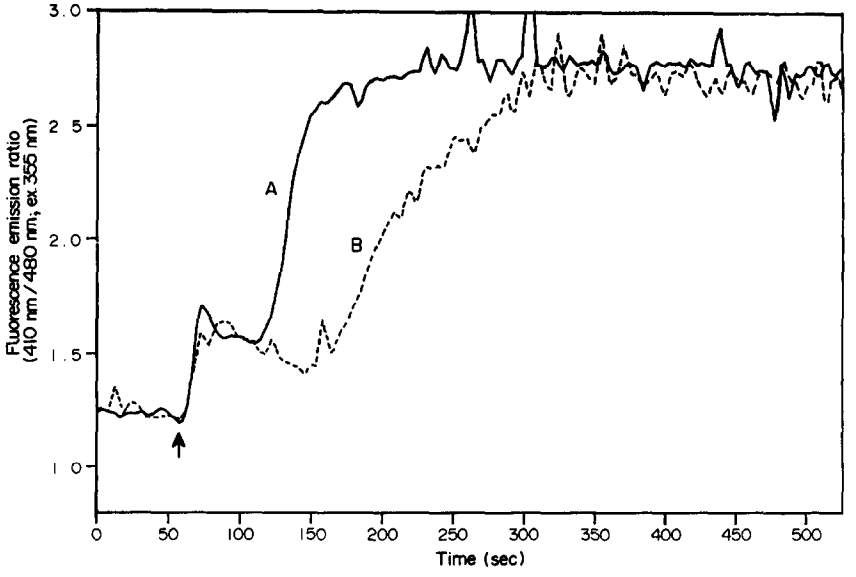


Fig. 3. Biphasic indo-1 dye fluorescence profiles in response to membrane-damaging concentrations of ET18-OMe and SRI 62-834. HL-60 cells were challenged with 3.3 μM ET18-OMe (A) or SRI 62-834 (B) where indicated (arrow). The calcium-sensitive fluorescence emission ratio of 410/480 nm is plotted on the Y-axis (see Materials and Methods). Similar results were obtained with the fura-2 fluorophore (not shown).

against HL-60 cells following 4-day exposure in full growth medium, as measured by the MTT assay. The ID_{50} values for the two agents in 10% serum were 2.3 ± 0.3 and 2.7 ± 0.3 μM, respectively (mean ± SD of values obtained from four independent experiments, i.e. N = 4). These results compare well with cytotoxicity data obtained for other AEL-sensitive cell lines [13, 15, 26, 27].

It is standard practice to carry out experiments involving calcium-sensitive fluorophores in serum-free assay buffers [22]. This eliminates the effects of absorbing or fluorescing serum components [22]. Serum-free assay buffers were also used by previous investigators [9,16,17] to study the effects of AELs on $[Ca^{2+}]_i$. In the first experiments, we used AEL concentrations around the ID_{50} values for cytotoxicity in serum-containing medium (i.e. 3–5 μM). Using these concentrations, but now in serum-free buffer, both ET18-OMe and SRI 62-834 produced apparent biphasic calcium elevations (Fig. 3) closely resembling those reported previously by Lazenby *et al.* [17]. In fact, using the improved calcium probes indo-1 (Fig. 3) and also fura-2 (not shown), we were able to resolve the two components of the biphasic fluorescence responses more clearly than was possible with quin2 [17]. However, the appropriate controls detailed below revealed that the apparently biphasic and sustained elevation of fluorescence was not truly representative of changes in $[Ca^{2+}]_i$. Extensive membrane damage, resulting in the release of fluorescent indicator into the high-calcium buffer and equilibration of extracellular with intracellular calcium, was detected by two independent methods.

Firstly, chelating extracellular free calcium by adding excess EDTA or EGTA to the cells in assay buffer after exposure to AELs caused a precipitous drop in fluorescence (Fig. 4). This indicated that a significant amount of the loaded fluorophore had either leaked into the extracellular buffer or was located in membrane permeable cells. Measuring the fluorescence of the assay buffer supernatant after centrifugation revealed an increase in dye leakage, presumably due to cell lysis or membrane permeabilization. Thus, with emission collected at 450 nm, the calcium-insensitive isosbestic point for indo-1, we have demonstrated that concentrations of 3.3 μM SRI 62-834 or ET18-OMe in serum-free assay buffer can release between 29 and 46% of the total incorporated fluorophore (36.6 ± 8.8%; mean ± SD, N = 3). Dye fluorescence at 410 nm, the calcium-sensitive wavelength, was elevated by 63–126% of the resting cellular fluorescence levels before AEL exposure. Such a high level of calcium-sensitive fluorescence is expected in a situation where leaked dye will fluoresce maximally in the calcium-containing buffer, while fluorescence from intact cells is limited due to the much lower intracellular calcium concentration. Note that in untreated cells, routinely more than 95% of the total fluorescence intensity could be pelleted, indicating minimal dye leakage.

Both of the above procedures independently showed that AEL concentrations of 3.3 μM in conventional serum-free assay buffer caused extensive membrane perturbation in HL-60 cells. It was noted that further addition of AELs to concentrations of 10–19 μM did not produce any further marked increases in fluorescence levels. As reported previously [17], the addition of Triton X-100 (to 0.1% v/v) after AEL exposure could yield a further

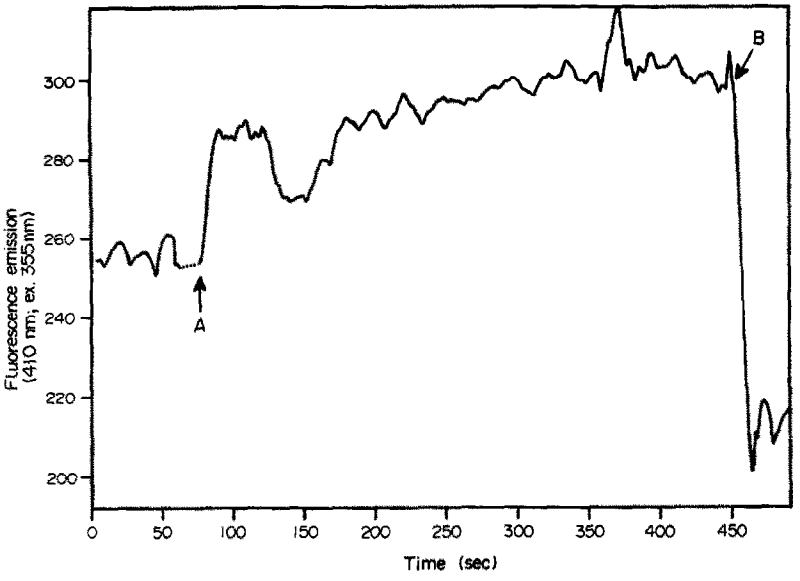


Fig. 4. Quenching of AEL-induced extracellular dye fluorescence at 410 nm by the addition of 1 mM EDTA. The calcium changes were evoked by addition of 3.3 μM ET18-OMe at point A and 1 mM EDTA was added at point B. This figure also shows that indo-1 can be used as a sensitive single wavelength probe, because the two phases of fluorescence increase are clearly resolved in time. The trace was smoothed using the 19 point smooth function of the fluorimeter software.

increase in fluorescence levels to a new plateau value. However, rather than proving the absence of any membrane damaging activity, as proposed previously [17], this in fact indicates that not all cells had been lysed by AEL exposure (see results of [^{51}Cr]chromate experiments).

Figure 5 illustrates the different fluorescence changes obtained by the addition of either highly membrane-damaging or apparently innocuous concentrations of AELs. The data show that 1.0 μM SRI 62-834 in serum-free buffer causes a genuine, transient rise in $[Ca^{2+}]_i$ which returns to basal levels

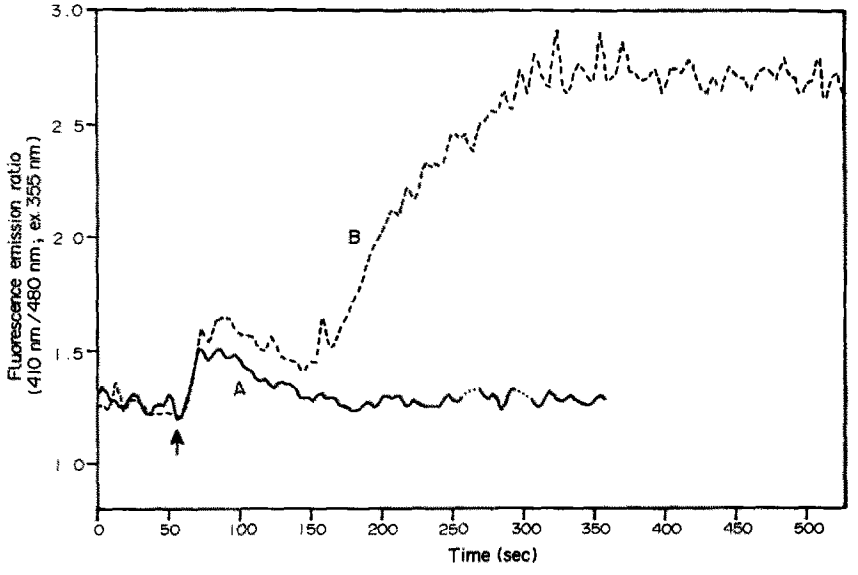


Fig. 5. "Genuine" and "artefactual" intracellular calcium elevations respectively in response to 1.0 μM (A) and 3.3 μM (B) SRI 62-834. The agent was added where indicated (arrow). Trace B is reproduced from Fig. 3 for comparison. Similar results were obtained with ET18-OMe (not shown). The second prolonged increase in calcium fluorescence can not be attributed to the opening of conventional calcium channels since the strong fluorescence could be entirely quenched by later addition of extracellular EGTA/EDTA (Fig. 4), while significant leakage of the fluorophore from the cells was also evident. These data are consistent with membrane permeabilization as the cause for the prolonged increase in fluorescence (see also Fig. 8).

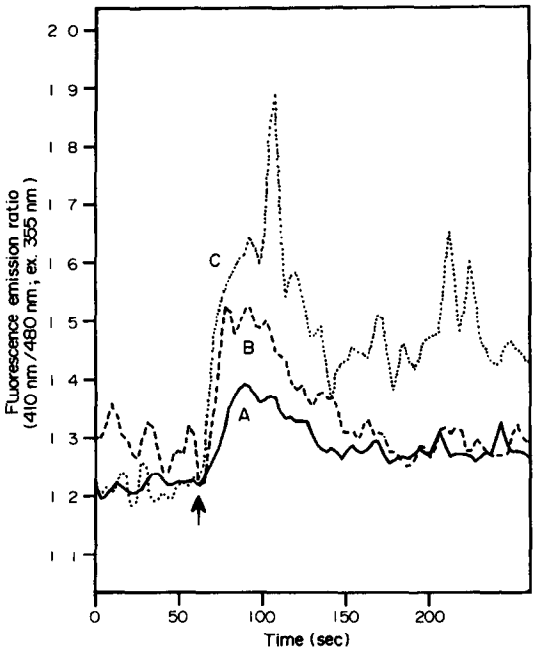


Fig. 6. Changes in intracellular calcium levels induced by ET18-OMe and SRI 62-834. The kinetics of the calcium rise in response to the two agents are similar and the magnitude of the measured response is increased at higher doses. The dose–response curve is steep and the precise concentrations eliciting fluorescence changes varied slightly between repeated experiments. The figure shows calcium elevations elicited by 0.5 μM ET18-OMe (A), 1.0 μM SRI 62-834 (B) and 3.3 μM ET18-OMe (C) added where indicated (arrow). Trace (A) illustrates an instance where a measurable calcium response was elicited by less than 1 μM AEL, the normal threshold. Trace (B) shows a characteristic calcium response. Trace (C) illustrates the maximal calcium elevation achievable without incurring extensive membrane damage. In this case, addition of 3.3 μM AEL, which is normally the threshold for extensive lysis, did not prove to be quite as damaging; however, a small degree of dye leakage was evident since fluorescence levels did not return fully to their previous basal level. Elevated baseline fluorescence was reduced to normal levels by the addition of EDTA or EGTA, as demonstrated in Fig. 4.

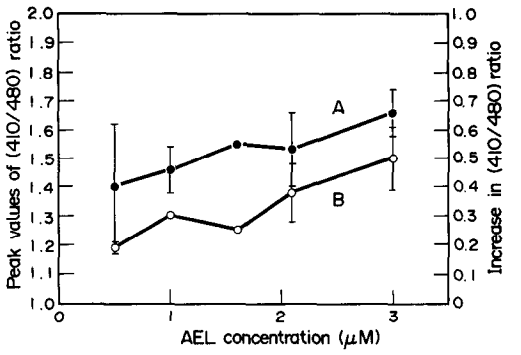


Fig. 7. Calcium elevations in response to increasing concentrations of ether lipid. Since the calcium increases stimulated by SRI 62-834 and ET18-OMe were very similar, data using both agents were pooled. Trace A (●) illustrates the maximal fluorescence ratios obtained with increasing AEL dose, while trace B (○) shows the increase in the fluorescence ratio obtained by subtracting baseline from peak fluorescence values. Measurements of the initial calcium rise were taken only from traces where no significant membrane damage was apparent for the duration of the genuine response. Error bars show the SD of three to four experiments per data point, except for the dose of 1.6 μM, where only two experiments were available for analysis.

after 2 min. High concentrations (3.3 μM) produce a similar initial rise in $[Ca^{2+}]_i$, but fluorescence levels do not return back to basal. Instead, they rise to a sustained high level, resulting in the biphasic pattern reported previously [17]. In the biphasic rise, the early elevation appears similar to the monophasic rise seen at lower AEL concentrations. However, the late kinetics of this rapidly elicited calcium response are masked by the increasing fluorescence of released extracellular dye. It is also apparent from Fig. 5 that the initial increase in intracellular calcium precedes the increase in membrane permeability. Similar results were obtained for ET18-OMe (see Figs 3 and 6).

We proceeded to establish the maximum Ca^{2+}-mobilizing dose of AELs which could be tolerated by cells in serum-free buffer without giving rise to extensive membrane damage. At concentrations of around 2–3 μM, both ET18-OMe and SRI 62-834 stimulated significant $[Ca^{2+}]_i$ elevations, while concentrations of AELs below 1 μM only occasionally had any discernible Ca^{2+}-mobilizing effects (Fig. 6). Thus in serum-free buffers, there is a steep dose–response effect within a very narrow window between 1 and 3 μM. The dose–response relationship within this window is illustrated in Fig. 7. At concentrations below 0.5 μM AELs fail to elicit measurable calcium responses, while extensive membrane perturbation results at concentrations slightly above the upper critical threshold. These findings were reproducible across at least four independent repeat experiments, although the precise concentration thresholds varied slightly between repeats. Figure 6 also highlights that the genuine monophasic calcium rises elicited by ET18-OMe and SRI 62-834 exhibit similar kinetics. Although it is difficult to quantitate the exact change in $[Ca^{2+}]_i$ because of the likely heterogeneity of the effect between different cells, the average increase across the cell population in response to 2–3 μM AEL is of the order of 30–40 nM.

It was noted that increases in $[Ca^{2+}]_i$ were not blocked, nor were their kinetics altered, in Ca^{2+}-free or EDTA-containing assay buffers (data not shown). The effect was confirmed in two independent experiments for both SRI 62-834 and ET18-OMe. This finding indicates that the initial increase in $[Ca^{2+}]_i$ is due to calcium mobilization from intracellular stores and is independent of extracellular calcium concentrations and the degree of membrane perturbation (Fig. 5).

As an alternative and more quantitative measure of the membrane-damaging potential of AELs both in serum-containing and serum-free media, we used

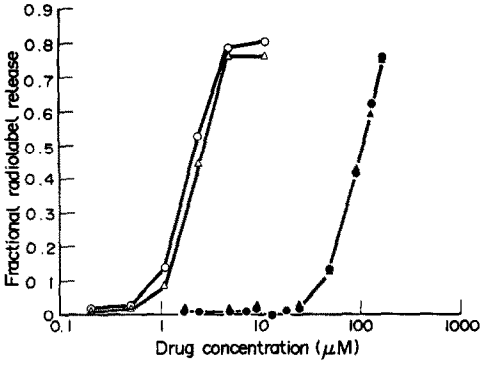


Fig. 8. The comparative membrane-damaging potencies of ET18-OMe and SRI 62-834 in serum-free and 10% serum-containing media. HL-60 cells in full growth medium were exposed to ET18-OMe (●) and SRI 62-834 (▲) as detailed in Materials and Methods. Similarly, cells in serum-free buffer were exposed to AELs [ET18-OMe (○), SRI 62-834 (△)]. Fractional radiolabel release was calculated as described in Materials and Methods, where the R_{50} concentrations are those at which [^{51}Cr]chromate release reached 50% of total [^{51}Cr]chromate incorporation. The data shown are from one of three independent experiments and represent the average of four replicate wells. Error bars have been omitted for clarity since the per cent error, as calculated by the γ counter software, was routinely well below 10%.

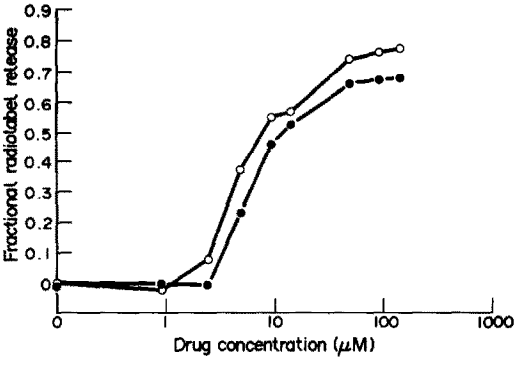


Fig. 9. [^{51}Cr]Chromate release profiles as a measure of acute membrane damage for SRI 62-834 in serum-free control medium (●) and serum-free 2.5 mM EGTA-containing medium (○). Data were pooled from two experiments and each point represents the mean of four replicate determinations. Error bars have been omitted for clarity, as the per cent error was routinely well below 10%.

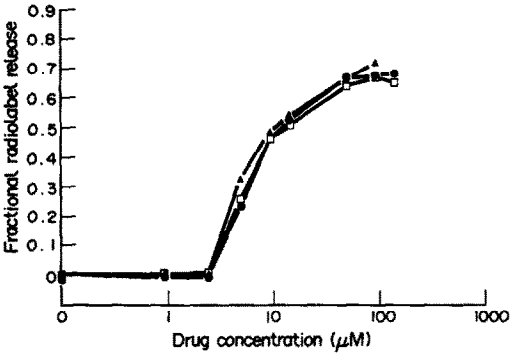


Fig. 10. [^{51}Cr]Chromate release profiles for SRI 62-834 in control cells (●) and cells pre-loaded with the intracellular calcium buffer BAPTA (□) or quin2 (▲) (see Materials and Methods). Data were pooled from two experiments. Each point represents the mean of four replicate determinations. Error bars have been omitted for clarity, as the per cent error was routinely well below 10%.

the [^{51}Cr]chromate-radiolabel release assay. Figure 8 illustrates the greatly increased membrane-damaging effect of AEL agents in serum-free buffers. As with the cytotoxicity data, SRI 62-834 and ET18-OMe were equipotent in the radiolabel release assay. R_{50} values indicate the drug concentrations at which half-maximal radiolabel release was detected. The R_{50} values for ET18-OMe in serum-containing and serum-free medium were 115 ± 14 and 2.2 ± 0.6 μM, respectively (mean ± SD, N = 3). The corresponding values for SRI 62-834 were 108 ± 4 and 2.7 ± 0.7 μM, respectively. Thus, AEL membrane toxicity was augmented about 50–60-fold in the absence of serum. Note that the concentrations of AELs causing more than 50% radiolabel release in serum-free medium are exactly in the range where the biphasic and sustained elevation of dye fluorescence becomes significant. In contrast, concentrations around 1 μM which can elicit a genuine monophasic rise in $[Ca^{2+}]_i$ cause minimal radiolabel release over 4 hr (Fig. 8).

Given that the initial increase in intracellular calcium levels precedes membrane permeabilization, we investigated whether influx of extracellular calcium or fluctuations in intracellular calcium are important for the membrane-disrupting effects of AELs. Previous experiments (see above) had shown that the calcium mobilized during the initial phase originated mainly from intracellular stores. To further eliminate the influx of extracellular calcium as a possible initiator of membrane perturbation, we carried out the [^{51}Cr]chromate assay with cells suspended in serum-free medium containing 2.5 mM EGTA. Comparing the results obtained with those for the regular calcium-containing medium, it is apparent that chelation of extracellular calcium has little effect on the membrane-damaging potency of SRI 62-834 (Fig. 9). The R_{50} values for control and EGTA-treated cells are 11.8 and 7.8 μM, respectively. Clearly, rather than protecting cells from permeabilization by AELs, the absence of extracellular calcium may even sensitize cells marginally to the membrane-perturbing effect of AELs.

To investigate the importance of changes in intracellular calcium levels for the membrane-damaging effects of AELs, the cells were pre-loaded with the intracellular calcium chelator BAPTA or quin2 [28]. Similar loading concentrations were used by Nasmith and Grinstein [29] to buffer changes in intracellular calcium. Figure 10 illustrates that buffering intracellular calcium elevations did not

affect membrane integrity in any discernible way. The R_{50} values for control, BAPTA- and quin2-treated cells are 11.8, 12.9 and 10.2 μM respectively (data from one experiment). Thus changes in intracellular calcium levels are not responsible for effecting the delayed membrane permeabilization seen with higher doses of AELs. These experiments have shown that neither intracellular calcium elevation nor the presence of extracellular calcium mediates membrane permeabilization by AELs.

DISCUSSION

We have shown previously that cell membrane permeability to fluorescent dyes increases in response to AEL exposure [13]. AELs also alter the biophysical properties of lipid membranes [10] and electron microscopy studies have illustrated that AELs cause cell membrane damage, bleb formation and the appearance of holes in the plasma membrane [11, 12]. Interestingly, these dramatic membrane perturbations were seen in the murine YAC-1 lymphoma cells and not in similarly treated normal lymphocytes [12]. A wide variation in cytotoxic potency is also seen across a range of different tumour cell lines [1, 26, 27, 30]. Moreover, ester lipids at the same concentrations did not cause any membrane damage [11]. These results argue against a simple detergent-like effect on the cells at pharmacologically active concentrations.

The fact that serum content can markedly affect the membrane-damaging potency of AELs has been mentioned in the literature [8, 9, 13, 31–33], but so far, few data concerning the magnitude of the toxicity differential have been published. Powis *et al.* [34] showed recently that Swiss 3T3 fibroblasts and BG1 ovarian adenocarcinoma cells are more sensitive (62 and 7 times, respectively) to a 1 hr ET18-OMe exposure in the absence of serum. The IC_{50} values reported for serum-free conditions were near-identical for the two cell lines, and we suggest that direct membrane damage sustained in the first hour of exposure may be responsible for the overall reduction in cell number. Early studies by Storch and Munder [31] established that ET18-OMe, and by implication other related lipids, display the same high affinity binding to serum albumin as naturally occurring lysophosphatidylcholine. Using the [^{51}Cr] chromate radiolabel release assay, we have provided the first quantitative assessment of the importance of serum for AEL-mediated membrane cytotoxicity. We found that AELs are 50–60-fold more lytic in serum-free buffer than in full medium containing 10% serum. The presence of serum proteins can thus protect cells from lysis by reducing the available concentration of free AEL.

Diomede *et al.* [35] reported that the toxicity of ET18-OMe and related AELs towards tumour cells increased when cholesterol-containing full medium was diluted with cholesterol-free, serum-free medium. This effect was attributed to a lowering of the cholesterol content. However, in view of the present results, it seems likely that the variation observed may have been due at least partly to the reduction in serum content. On the other hand, the same group have used a cholesterol abstraction procedure to confirm that membrane cholesterol content is directly protective against short-term, high-dose AEL-induced membrane damage [36].

Hickman and co-workers [16, 17] showed that AELs ET18-OMe and SRI 62-834 cause a rise in $[Ca^{2+}]_i$ and suggested that this effect may be involved in the mechanism of cell death induced by these agents. In HL-60 cells, they observed a sustained, biphasic increase in fluorescence of the calcium probe quin2 with concentrations of 30 μM SRI 62-834 in serum-free assay buffer. We have confirmed the observation of an apparent rise in $[Ca^{2+}]_i$, this time using the improved calcium probes indo-1 and fura-2. However, in contrast to the previous conclusion [17], we have shown that the true elevation of $[Ca^{2+}]_i$ elicited by SRI 62-834 or ET18-OMe is not biphasic. The putative second phase of calcium mobilization was, in fact, an artefact caused by extensive cell membrane damage which results in both an influx of extracellular calcium into permeabilized cells and leakage of the calcium indicator into the high calcium assay buffer. Due to the inferior performance of the quin2 fluorophore, the genuine initial increase in $[Ca^{2+}]_i$ was not well resolved and appeared only as a small shoulder on the steeply rising curve.

Seewald *et al.* [9] have also observed the disparate behaviour of AELs on the fluorescence of the calcium-sensitive protein aequorin in serum-containing and serum-free media. They reported that a sustained apparent increase in $[Ca^{2+}]_i$ was only seen in serum-free conditions using ET18-OMe at concentrations of 10 μM and above. The apparent calcium rise was assumed to be genuine. Moreover, the absence of a sustained calcium rise in calcium-free assay buffers was advanced as evidence for an AEL-induced calcium influx from the extracellular medium [9]. In the light of our findings, we propose that the observed calcium ion influx was probably due to the membrane damage artefact, rather than a physiological activity of ET18-OMe.

Despite the involvement of a measuring artefact at high AEL concentrations, we were nevertheless able to demonstrate that lower, non-membrane toxic concentrations of ET18-OMe and SRI 62-834 do stimulate a genuine, dose–dependent, monophasic rise in $[Ca^{2+}]_i$ which decays back to basal levels within 100–200 sec. The kinetics of the calcium rises were similar for the two AELs, and proved to be independent of extracellular calcium concentrations. This indicates that the mechanism probably involves the release of calcium from internal stores.

It has been suggested that AELs may elicit calcium responses by displacing natural growth factors from serum proteins [9]. We have shown the ability of AELs to generate calcium responses directly in serum-free conditions. Moreover, the kinetics of the calcium responses were somewhat different from those normally seen with neuropeptides and growth factors [9, 18]. The AELs appear to elicit $[Ca^{2+}]_i$ increases which are less sharp and return to basal levels over a longer period of time. Displacement of growth factors may occur in serum-containing media, but we would consider it unlikely to play a part in the potent cytotoxic activity of AELs.

In contrast to the effects associated with acute

exposure to AELs (described above), studies by Seewald *et al.* [9] and ourselves (Lohmeyer, Brunton, Workman, unpublished data) show that more prolonged (e.g. 18 hr) exposure to AELs *inhibits* calcium signalling in response to natural mitogenic membrane receptor agonists such as bombesin, neurotensin and platelet-derived growth factor. Moreover, AELs are among the most potent known inhibitors of phospholipase C and modulation of protein kinase C activity has also been widely reported [1, 6, 8, 9, 14, 15, 37]. Thus it appears that AELs feature at least two different modes of action with respect to intracellular calcium signalling: they are directly acting, acute calcium agonists as well as signalling inhibitors upon chronic exposure.

The importance of calcium changes for both the acute and chronic antitumour effects of AELs remains controversial. Our results with acute exposures suggest that a calcium rise is not directly responsible for cytotoxicity. The previously considered sustained calcium rise is an artefact associated with membrane damage. The initial, transient elevation in intracellular calcium is genuine. Moreover, flow cytometry experiments with complimentary fluorescent probes for cell calcium and membrane permeability have confirmed that this genuine rise in $[Ca^{2+}]_i$ within intact, viable cells precedes an increase in membrane permeability [38]. The intracellular calcium elevations are seen at non-toxic concentrations of AELs and the magnitude of the initial response does not increase further once membrane-toxic concentrations are exceeded. In addition, the effects of AEL exposure on intracellular calcium levels are very similar to those seen with lyso-PAF, a structural analogue which is nevertheless 10 to 15 times less cytotoxic in long-term culture [27]. It now appears unlikely that the initial calcium rise is instrumental in the selective and potent antitumour activity of AELs.

It is tempting to speculate that, rather than inducing cytotoxicity, the initial rise in calcium seen at non-toxic concentrations may even be mitogenic. van Corven *et al.* [39] have shown that lysophosphatidic acid and similar simple phospholipids can cause a rise in $[Ca^{2+}]_i$ and stimulate quiescent fibroblasts by activating three different cell signalling cascades. It is thus not inconceivable that AELs too may be mitogenic at sub-cytotoxic concentrations, and studies are in progress to examine this. As far as the inhibitory effect of chronic exposure to AELs on growth factor-induced calcium elevations is concerned, further work is required to determine whether this is a major mechanism of AEL action.

In summary, we have shown that serum is a crucial factor affecting the membrane-damaging and calcium-mobilizing activity of AELs. In the absence of serum, AEL membrane toxicity was augmented 50–60 fold. This is of great importance for the application of fluorescent calcium indicators which are routinely used in serum-free assay buffers. We have further established that the apparently persistent elevation in $[Ca^{2+}]_i$ in response to high concentrations AELs is an artefact caused by cell membrane permeabilization. However, lower concentrations of AELs do elicit a genuine monophasic and transient rise in $[Ca^{2+}]_i$ which appears to differ from those routinely observed in response to growth factors and mitogens. Changes in $[Ca^{2+}]_i$ do not appear to be involved in the acute membrane toxicity of AELs, but the importance of AEL-induced modulation of calcium signalling by mitogenic growth factors remains to be elucidated.

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