1-*O*-Octadecyl-2-*O*-methylglycerophosphocholine inhibits protein kinase C-dependent phosphorylation of endogenous proteins in MCF-7 cells

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Studies with leukaemic cells, based primarily on *in vitro* assays, have suggested that antitumour ether lipids have only a moderate effect on protein kinase C (PKC) activity, and, furthermore, inhibition of PKC is unlikely to be involved in the mechanism of inhibition of cell proliferation by these compounds. To determine if this is also the case for epithelial cancer cells, we examined the effect of 1-*O*-octadecyl-2-*O*-methylglycerophosphocholine (ET18-OCH₃) on PKC-induced phosphorylation of endogenous proteins in MCF-7 cells under incubation conditions where the drug inhibited cell proliferation. As expected, stimulation of quiescent ³²P-labelled MCF-7 cells with 1 μ M PMA resulted in the phosphorylation of a number of proteins. The PMA-induced phosphorylation of the proteins was abolished by preincubation of the cells with Ro 31-8220 (5 μ M) for 20 min, or 10 μ g/ml ET18-OCH₃ for 3 h before stimulation with PMA. Thus under

incubation conditions where ET18-OCH₃ inhibited the proliferation of MCF-7 cells, the ether lipid potently inhibited the activity of PKC in intact cells. This inhibition was unlikely to be due to the effect of the compound on PKC translocation since there was little effect of ET18-OCH₃ on the translocation of the α , γ and ϵ species of PKC. These results suggest that a role for the inhibition of PKC activity by ET18-OCH₃ in the mechanism of inhibition of cell proliferation by ET18-OCH₃ cannot yet be discounted in epithelial cancer cells. In addition, we also observed that ET18-OCH₃ enhanced the phosphorylation of selected proteins under basal unstimulated conditions. Although some of these proteins were also observed to be phosphorylated in response to PMA stimulation, the phosphorylation induced by ET18-OCH₃ was not inhibited by Ro 31-8220, indicating that this was not mediated by PKC.

INTRODUCTION

Alkyl-lysophospholipids (ALPs) are a class of synthetic ether lipids that have significant antiproliferative properties. 1-*O*-Octadecyl-2-*O*-methylglycerophosphocholine (ET18-OCH₃), the prototype of these compounds, perturbs a large number of cellular processes [1,2]. Although the mechanism of action has yet to be unequivocally established, evidence exists to suggest that the antiproliferative effect of the compounds may be due to perturbation of signal transduction mechanisms [1–6]. We have recently demonstrated that, under preincubation conditions with ET18-OCH₃ that inhibit the proliferation of MCF-7 cells, activation of the mitogen-activated protein kinase cascade is perturbed due to the premature termination of Raf-1 activity [6]. Whether this inhibition of Raf-1 activation is solely responsible for the inhibition of proliferation still remains to be resolved. Another signalling molecule affected by antitumour ether lipids is protein kinase C (PKC) [3,7–9], a ubiquitous protein kinase that transduces growth signals. ET18-OCH₃ inhibits PKC activity in cell-free systems [8], and its ability to inhibit PMA-induced differentiation of HL-60 cells in an additive manner with H-7 suggested that the compound inhibits PKC in intact cells [9]. However, H-7 is not as specific for PKC as previously thought [10], and conclusions implicating PKC in cellular events based on the effects of H-7 may need to be re-evaluated [11]. In direct contrast with reports of the inhibition of PKC by ET18-OCH₃, there is one that suggests that ET18-OCH₃ may activate PKC [12]. The question of whether inhibition of PKC by antitumour ether lipids is necessary for their antiproliferative action has been addressed [13,14]. Moderate (20–30 %) inhibition

of PKC activity in a number of leukaemic cell lines was noted but this was observed in antitumour ether lipid-sensitive and -resistant cell lines [13,14]. In addition, one of these studies reported similar inhibitory effects with an active and inactive anomer of an ether thioglycolipid [13]. The conclusion from both studies was that the ability of antitumour ether lipids to inhibit PKC was unrelated to their ability to inhibit cell growth. While the results of the studies support these conclusions, the limitations in the approach used leave unanswered the question of whether ET18-OCH₃ and related compounds inhibit PKC activity in intact cells following stimulation. In the above studies, cells were pretreated with antitumour ether lipids and subsequently stimulated with phorbol esters followed by quantification of the total PKC activity in membrane and cytosolic fractions using *in vitro* assays. Underpinning this approach is the assumption that translocation of PKC is synonymous with activation, but Trilivas et al. have reported that redistribution of PKC following cell stimulation may not reflect activation of the enzymes [15]. Also, since the *in vitro* assays require the addition of exogenous compounds to activate PKC, the methods do not discriminate between the effect of the ether lipids on the active PKC fraction in the sample and the total activatable PKC. The consequence of this is that although the effect of the antitumour ether lipid on the total PKC fraction may be moderate, it could conceivably severely inhibit the active PKC fraction alone. We have circumvented the shortcomings of the above approaches by examining the effect of ET18-OCH₃ on the phosphorylation of endogenous cellular proteins by PKC in intact cells. We also assessed the effect of ET18-OCH₃ on the translocation of selected PKC isoforms.

Abbreviations used: ALP, alkyl-lysophospholipid; DiC₈, 1,2-dioctanoyl-*sn*-glycerol; ET18-OCH₃, 1-*O*-octadecyl-2-*O*-methylglycerophosphocholine; IEF, isoelectric focusing; PKC, protein kinase C.

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MATERIALS AND METHODS

Materials

ET18-OCH₃ was obtained from Medmark (Gruenwald, Germany) and Ro 31-8220 was obtained from Roche (Welwyn, Herts, U.K.). Coomassie protein assay reagent was a product of Pierce (Rockford, IL, U.S.A.). 1,2-Dioctanoyl-*sn*-glycerol (DiC₈) was purchased from Serdary Research Laboratories (Englewood Cliffs, NJ, U.S.A.). Fetal-bovine serum and [³²P]orthophosphate were obtained from ICN Pharmaceuticals (Montreal, Quebec, Canada). Cell culture medium and subculturing reagents were purchased from Gibco BRL (Burlington, Ontario, Canada). Tissue culture ware (Falcon) were obtained from VWR Scientific (Winnipeg, Canada). BSA was procured from Sigma Chemical Co (St. Louis, MO, U.S.A.). PMA and 4- α -PMA were obtained from LC Laboratories (Woburn, MA, U.S.A.). Enhanced chemiluminescence detection system was a product of Amersham (Oakville, Ontario, Canada). Bio-Lyte were obtained from Bio-Rad (Mississauga, Ontario, Canada) and Pharmalyte from Pharmacia Biotech Inc. (Baie D'Urfé, Quebec, Canada). PKC antibodies and Jurkat and HeLa cell lysates were obtained from Transduction Laboratories (Lexington, KY, U.S.A.).

Cell culture

MCF-7 (human breast adenocarcinoma) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (w/v) fetal-bovine serum [16]. The cells were propagated from frozen stocks originally obtained from ATCC (Rockville, MD, U.S.A.). Proliferating cells in 100-mm dishes were made quiescent by incubating in medium containing 0.5 mg/ml fatty-acid-free albumin for 4–6 days. The proliferative rate was determined by monitoring the 24 h change in cell number of parallel cultures in 6-well plates seeded at the same density as the cells in the dishes. Cells were considered quiescent when the 24 h increase in cell number was 15 % or less.

One- and two-dimensional gel analysis of phosphorylated proteins

Quiescent cells were labelled with [³²P]orthophosphate (150–300 μ Ci/ml) in phosphate-free Dulbecco's modified Eagle's medium containing 0.5 mg/ml BSA for 3 h. The cells were then washed and preincubated with ET18-OCH₃ (10 μ g/ml, 3 h) or Ro 31-8220 (5 μ M, 20 min) as required. After this the cells were stimulated with PMA (1 μ M, 10 min) or DiC₈ (20 μ M, 10 min), washed in ice-cold PBS and harvested in ice-cold buffer A [20 mM Tris/HCl (pH 7.4)/2 mM EGTA/100 mM β -glycerophosphate/1 mM Na₃VO₄/aprotinin and leupeptin (10 μ g/ml)/0.2 mM aminoethylbenzenesulphonyl fluoride/0.2 mM benzamidine/0.1 mM PMSF/0.5 % (v/v) Nonidet P40]. The cells were sonicated and aliquots were taken for determination of protein content using the Coomassie protein assay, with BSA dissolved in lysis buffer as the standard. For one-dimensional gels 3 vol. of 4 $\times$ SDS sample buffer was added to 1 vol. of the cell lysate, followed by boiling for 5 min. For two-dimensional gel electrophoresis, the urea sample buffer [9.5 M urea/2 % Nonidet P40/5 % (v/v) β -mercaptoethanol/0.4 % (v/v) Bio-Lyte (pH 3–10) and Bio-Lyte (pH 5–7)/0.6 % (v/v) Bio-Lyte (pH 6–8) and Pharmalyte (pH 4–6.5)] was added to the lysates to a final urea concentration of 6 M. The samples were incubated at room temperature for 30 min. The lysates for both one- and two-dimensional gels were centrifuged at 20000 *g* for 1 min. Proteins were resolved on 10 % (v/v) SDS gels for analysis by one-dimensional gel electrophoresis or isoelectric focusing (IEF) gels (pH 3–10) using a miniprotean IEF apparatus followed by separation on SDS/PAGE [12 % (w/v) gel] for analysis by

two-dimensional gel electrophoresis [17]. The experiments were carried out three times with different cultures of MCF-7 cells.

PKC translocation

Quiescent MCF-7 cells were preincubated with or without ET18-OCH₃ (10 μ g/ml, 3 h) followed by stimulation with PMA (1 μ M, 10 min). The cells were harvested and sonicated in buffer B (buffer A without Nonidet P40). Unbroken cells were removed by centrifugation at 7000 *g* for 10 min, and the cytosolic fractions were obtained by centrifugation of the resultant supernatant at 200000 *g* for 30 min at 4 °C. The pellets were dissolved in the buffer B plus 1 % (v/v) Triton X-100 and 0.5 % Nonidet P40 by ultrasonication. The soluble membrane extracts were obtained by re-centrifugation at 200000 *g* for 30 min. Samples were stored at –70 °C until required. The protein content of each fraction was determined as above. Protein of membrane fractions (20 μ g) was separated on SDS/PAGE (8 % gel) followed by immunoblotting with monoclonal mouse anti-PKC- α (IgG_{2b}), - γ (IgG₁) or - ϵ (IgG_{2a}) and visualized with the enhanced chemiluminescence detection system. Lysates from HeLa cells served as positive controls for α and ϵ isoforms, and cell lysates from Jurkat cells served as positive control for the γ isoform. Quantitative densitometric analyses were obtained with a high-resolution colour scanner, model PDI 3250e (Protein + DNA Imageware Systems, Huntington Station, NY, U.S.A.), using the ImageMaster scanning programme (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, U.S.A.).

RESULTS

Identification of proteins phosphorylated in response to PKC activation

The criteria used to identify proteins in cells whose phosphorylation was PKC-dependent were enhanced phosphorylation in response to stimulation with phorbol esters or diacylglycerol and inhibition of the induced phosphorylation by the specific PKC inhibitor Ro 31-8220 [10,18,19]. Studies were initially conducted to select concentrations and incubation times for stimulating the cells with the PKC agonists. Quiescent MCF-7 cells preincubated with [³²P]orthophosphate were stimulated with 1 μ M PMA for selected times (0–20 min), followed by analysis of the phosphorylation of proteins by one-dimensional PAGE. The results shown in Figure 1(A) revealed that the most prominent phosphorylation occurred on a protein(s) with an *M_r* of approx. 31000 (p31) and maximum phosphorylation was obtained after 10 min incubation. The effect of stimulating the cells with varying concentrations of PMA (0–1 μ M) for 10 min are shown in Figure 1(B). The phosphorylation of the p31 band was observed at all concentrations tested. In Figure 1(C) the results of studies to investigate the effect of selected DiC₈ concentrations on p31 phosphorylation, as well as the time course of phosphorylation, are presented. Phosphorylation of p31 was observed with DiC₈ concentrations of 10 μ M or greater, but this required a minimum incubation time of 5 min. As a consequence of the above studies, subsequent experiments were conducted with agonist concentrations of 1 μ M for PMA or 20 μ M for DiC₈, and stimulation was for 10 min. The phosphorylation of p31 appeared to be due to PKC activation, since preincubation of the cells with Ro 31-8220 (5 μ M) for 20 min before stimulation with PMA or DiC₈ abolished the phosphorylation (Figure 1D). The specificity of PMA-induced phosphorylation of p31 was confirmed when studies with the inactive phorbol ester 4- α -PMA showed that the latter was unable to cause the phosphorylation of p31 (Figure 2A, lane 2).

Figure 1 Characterization of PKC-dependent phosphorylation of p31 in MCF-7 cells

(A) Quiescent MCF-7 cells were prelabelled with [32 P]orthophosphate and stimulated with 1 μ M PMA (TPA) for 2, 5, 10 and 20 min, or left untreated as the control. Proteins in cell lysates were resolved on an 8% SDS gel and subjected to autoradiography. The cell treatments are indicated at the top of each lane. M_r values ($\times 10^{-3}$) are displayed on the left. The p31 band is indicated by the arrow. (B) [32 P]Orthophosphate-labelled quiescent cells were stimulated with 0, 0.05, 0.5 or 1 μ M PMA (TPA) for 10 min, and the cell lysates were processed for analysis of protein phosphorylation as described in (A). (C) [32 P]Orthophosphate-labelled cells were stimulated with the indicated concentrations of DiC₈ for the times indicated in the Figure. Cell lysates were processed for protein phosphorylation as indicated above (TPA = PMA). (D) [32 P]Orthophosphate-labelled cells were preincubated with 5 μ M Ro 31-8220 for the indicated times and the cells were stimulated with 1 μ M PMA (TPA) for 10 min. Analysis of phosphorylated proteins was as described above in (A).

Effect of ET18-OCH₃ on PKC-dependent phosphorylation of endogenous proteins

Results of studies on the effect of ET18-OCH₃ on PMA-induced phosphorylation of p31 are shown in Figure 2. Although the

Figure 2 Enhanced phosphorylation of p31 in MCF-7 cells pretreated with ET18-OCH₃

(A) Quiescent MCF-7 cells prelabelled with [32 P]orthophosphate were incubated with or without ET18-OCH₃ (10 μ g/ml, 3 h) or Ro 31-8220 (5 μ M, 20 min), followed by stimulation with 1 μ M PMA (TPA) or 4- α -PMA (4- α -TPA) for 10 min, or left untreated as the control. Proteins in cell lysates were resolved on a 10% SDS gel and subjected to autoradiography. The cell treatments are indicated at the top of each lane. The p31 band is indicated by the arrow and M_r values ($\times 10^{-3}$) are displayed on the left. (B) [32 P]Orthophosphate-labelled cells were incubated with 20 μ M ET18-OCH₃ for the indicated times and the cell lysates were analysed for the phosphorylation of p31. Cells stimulated with PMA (TPA; 1 μ M, 10 min) served as the positive control.

PMA-stimulated phosphorylation of p31 was blocked by preincubation with Ro 31-8220, preincubation of the labelled quiescent cells with ET18-OCH₃ (10 μ g/ml) for 3 h before stimulation with PMA did not inhibit, but rather appeared to enhance, the phosphorylation of p31. Indeed, in cells preincubated with ET18-OCH₃ alone, an apparent phosphorylation of p31 was observed (Figure 2, lane 7). This led to an investigation of the time-course of enhancement of p31 phosphorylation by ET18-OCH₃. The results, displayed in Figure 2(B), show that ET18-OCH₃-enhanced phosphorylation of p31 in quiescent MCF-7 cells was a time-dependent event that was observed as early as 30 min after incubation with the compound, the maximum phosphorylation being observed after 2 h (Figure 2B).

The above observation suggested that ET18-OCH₃ could be activating PKC activity in intact cells, as one report had indicated [12]. This led us to investigate whether phosphorylation of endogenous proteins, other than p31, were also enhanced by preincubation of the cells with ET18-OCH₃. Because of the limited resolution of proteins obtained with one-dimensional gel electrophoresis, phosphorylated proteins were analysed by two-dimensional gel electrophoresis (Figure 3). Relative to unstimulated controls (Figure 3A), stimulation of the cells with

Figure 3 Two-dimensional gel analysis of phosphorylation of endogenous proteins in MCF-7 cells

Quiescent MCF-7 cells prelabelled with [32 P]orthophosphate were incubated with or without ET18-OCH₃ (10 μ g/ml, 3 h) or Ro 31-8220 (5 μ M, 20 min), followed by stimulation with 1 μ M PMA for 10 min, or left untreated as the control. Proteins in cell lysates were resolved on two-dimensional gels [IEF, pH 3–10, plus SDS/PAGE (12% gel)] and subjected to autoradiography. The polarity of the IEF is indicated at the top of each figure and the M_r values ($\times 10^{-3}$) are displayed on the left: (A) untreated control; (B) PMA stimulation (1 μ M, 10 min); (C) Ro 31-8220 treatment (5 μ M, 20 min); (D) Ro 31-8220 + PMA; (E) ET18-OCH₃ (10 μ g/ml, 3 h) + PMA; (F) ET18-OCH₃; (G) Ro 31-8220 + ET18-OCH₃. See text for explanation of arrowheads a–o.

Figure 4 Translocation of PKC isoforms from cytosol to membrane

(A) Quiescent MCF-7 cells were incubated with or without ET18-OCH₃ (10 μ g/ml, 3 h), followed by stimulation with PMA (TPA; 1 μ M, 10 min), or left untreated as the control. Proteins in the membrane fraction were resolved on 8% SDS gels, transferred to nitrocellulose membranes, probed with specific monoclonal anti-PKC antibodies, and visualized by the enhanced chemiluminescence detection system. (B) Densitometric analysis of the blots shown in (A).

PMA resulted in increased phosphorylation of a number of proteins, the major ones being spots a, d, e, f, g, h, j, k and l (Figure 3B). The results in Figures 3(A) and 3(B) revealed that a series of spots with M_r approx. 31000 (Figures 3B–3G) are phosphorylated in unstimulated and stimulated cells with spots d–g being most prominently phosphorylated in PMA-stimulated cells. The phosphorylation of spots b and c in controls disappeared in PMA-stimulated cells, whereas phosphorylation of d, e, f and g were increased. Although this apparent shift of the phosphorylation pattern to the positive side (right) following PMA stimulation may indicate that the spots could be different phosphorylated forms of the same protein, such an inference is tempered by results obtained with ET18-OCH₃ (see below) that suggests that they may be distinct proteins. Preincubation of the cells with Ro 31-8220 for 20 min (Figure 3C) resulted in decreased phosphorylation relative to the controls of spots a–e and l. Preincubation of cells with Ro 31-8220 for 20 min and subsequent stimulation by PMA for 10 min (Figure 3D) inhibited the phosphorylation of the spots observed in response to PMA stimulation, i.e. spots a, d, f, g, h, j, k and l. The ability of Ro 31-8220 to inhibit the phosphorylation of these proteins identified the phosphorylation of these spots as being PKC-dependent.

The results obtained from cells pretreated with ET18-OCH₃ before stimulation with PMA are shown in Figure 3(E). ET18-OCH₃ was equally effective as Ro 31-8220 in inhibiting the

PMA-induced phosphorylation of spots a, d, e, h, i, j and l. ET18-OCH₃ decreased the PMA-stimulated phosphorylation of spot k down to the unstimulated control levels (Figure 3A), but not to the levels observed with Ro 31-8220-treated cells (Figure 3D). In addition to the above inhibition of phosphorylation, ET18-OCH₃ enhanced the incorporation of ³²P in spots f, g, m, n and o. An enhanced incorporation of ³²P into these same spots was observed when the cells were preincubated with ET18-OCH₃ alone (Figure 3F), indicating that the enhanced phosphorylations were a consequence of incubation with the ALP. The phosphorylation of these spots was not abolished by the presence of Ro 31-8220 during incubation with ET18-OCH₃ (Figure 3G). The fact that ET18-OCH₃ stimulated the phosphorylation of spots f and g, whereas it had little effect on the phosphorylation of other p31 proteins such as d and e, suggests that the panel of p31 proteins could be distinct proteins and may not represent different phosphorylated forms of the same protein. Interestingly, spots m, n and o were not observed in controls or PMA-treated cells.

Effect of ET18-OCH₃ on PKC translocation

To investigate what effect, if any, ET18-OCH₃ had on the translocation of selected PKC species, cells were preincubated with the ALP under identical conditions to those used for the phosphorylation studies, and stimulated with PMA for selected periods (0–10 min), followed by rapid washing and separation of membrane fractions from the cytosol as described in the Materials and methods section. The association of α , γ and ϵ PKC species with membranes was assessed by Western blot analysis with specific isotype antibodies. The results obtained are displayed in Figure 4(A), and the densitometric analysis in Figure 4(B). In quiescent unstimulated MCF-7 cells, PKC- α , γ and ϵ were not associated with the membrane, but following stimulation with PMA, we observed the association of PKC- α , γ and ϵ with the membrane fraction in a time-dependent fashion. Pretreatment of cells with ET18-OCH₃ alone appeared to cause a slight increase in membrane-associated PKC- α in some experiments but not in others, but no redistribution of PKC- γ or PKC- ϵ were observed in any experiments. After correcting for any ET18-OCH₃-induced increase in PKC membrane association, the plot of the densitometric analysis of the Western blots revealed a slight inhibitory effect of the drug on PMA-induced translocation of PKC- α . It is worth noting that there was no difference in the total amount of membrane-associated PKC- α in the ALP-treated and control cells after 10 min stimulation with PMA. Slight differences were observed in the kinetics of PMA-induced translocation of PKC- γ and ϵ to the membrane between the control and ET18-OCH₃-treated cells. After 10 min stimulation with PMA, the amount of membrane-associated PKC- γ was similar in the control and ALP-treated cells, whereas a slight inhibition of translocation of PKC- ϵ was apparent in the drug-treated cells relative to controls.

DISCUSSION

Although there is sufficient evidence to indicate that ET18-OCH₃ inhibits PKC activity *in vitro*, its effect on the activity of the enzyme in intact cells and the severity of any such inhibition remains to be established. In this study we have circumvented problems associated with *in vitro* assays of PKC by examining the effect of ET18-OCH₃ on the phosphorylation of endogenous proteins in intact cells in response to PKC activators. Although previous studies have used a similar approach, the relationship between the ET18-OCH₃ incubation conditions used and what effect these had on inhibition of cell proliferation were not clarified, although it was reported that cell viability was

unaffected [20]. These studies with leukaemic cells also reported that although addition of ET18-OCH₃ (75 μ M) for 40 min was sufficient to inhibit phosphorylation induced by 20 nM PMA, no inhibitory effect of the ALP was observed when 200 nM PMA was used to stimulate HL60 or K569 cells [21]. In the present study, the preincubation conditions with ET18-OCH₃, 10 μ g/ml ($\approx$ 20 μ M) for 3 h, inhibited the stimulatory effects of 1 μ M PMA. Under these conditions proliferation of MCF-7 cells was completely inhibited after re-addition of the growth medium, without any effect on the viability of the cells [6]. The ability of ET18-OCH₃ to effectively inhibit the phosphorylation of endogenous proteins by PKC to the degree observed with the specific PKC inhibitor Ro 31-8220 [10,18,19] provides evidence that ET18-OCH₃ inhibits PKC activity in intact MCF-7 cells. The complete inhibition observed in our studies contrasts with results from studies based on *in vitro* measurements of PKC activity in cell fractions that reported only moderate inhibition of PKC activity [13,14]. This could well be due to the approach used, which does not discriminate between the effect of the compound on the activated PKC fraction and that on the total PKC fraction. However, since leukaemic cells were used by Salari et al. [13], Berkovic et al. [14] and Kiss et al. [20], whereas an epithelial cell line was used in the current study, we cannot rule out the possibility that this may be due to the differences between the two cell types. We have previously noted differences in the effects of antitumour ether lipids on various parameters of the two cell types and have in fact suggested that the mechanism of inhibition of cell growth could well differ between them [22,23].

One of the novel and unexpected findings of this study was the observation that ET18-OCH₃ stimulates the enhanced incorporation of ³²P into selected proteins in MCF-7 cells. In contrast, no effect on basal phosphorylation was observed in similar studies with HL60 or K562 leukaemic cell lines [20]. We do not know if the observed enhanced phosphorylations in MCF-7 cells are due to inhibition of phosphatases or activation of kinase(s) by ET18-OCH₃. If it is the latter, the inability of Ro 31-8220 to inhibit the enhanced phosphorylation suggests that it is a PKC-independent phenomenon. We were also unable to observe an increase in protein kinase A activity in cells pretreated with ET18-OCH₃ [6]. The enhanced phosphorylation of proteins by ET18-OCH₃ leaves open the possibility that inappropriate phosphorylation of proteins could contribute to the antiproliferative effects of the compound.

Both Salari et al. [13] and Berkovic et al. [14] reported that the antitumour ether lipids had no effect on the translocation of PKC. Our studies on the translocation of selected isotopes of PKC, on the whole, tend to support this view. What effects ET18-OCH₃ had on PKC translocation were minimal and unlikely to be responsible for the inhibition of PKC phosphorylation by the ALP. This suggests that the effect of the compound on PKC activity is probably due to its competition with phosphatidylserine [9,21], but an irreversible effect of the compounds on PKC activity cannot be ruled out [14].

Whether the inhibition of PKC by ET18-OCH₃ in MCF-7 cells is a prerequisite for the antiproliferative action of the compound still remains to be established. In leukaemic cells inhibition of PKC activity does not appear to be required for the antiproliferative action of antitumour ether lipids [13,14]. The most convincing evidence involved the use of anomers of an ether lipid with differential effects on cell proliferation but identical effects on PKC activity [13]. Unfortunately the differential effect seen with leukaemic cells was not observed with a number of epithelial

cells, including MCF-7 cells [22], and therefore we are unable to use this approach to determine whether inhibition of PKC activation is a prerequisite to the inhibitory action of the compounds. In view of our result that ET18-OCH₃ effectively inhibits PKC activity in intact MCF-7 cells under conditions where cell proliferation is inhibited, we believe the relevant issue in these cells hinges on whether PKC activation is essential for the proliferation of these cells. Since activation of the mitogen-activated protein kinase cascade may be sufficient to initiate cell proliferation [24], and we have demonstrated that this pathway is inhibited by ET18-OCH₃ [6], the inhibition of PKC activation by ET18-OCH₃ would be superfluous with respect to inhibiting cell proliferation, unless PKC activation itself is required for signal transduction via the mitogen-activated protein kinase cascade, or is sufficient to overcome the block imposed by inhibition of Raf-1 activation. It is worth noting that PKC has been reported to activate Raf-1 in some cells [20,24,25]. Studies to address these issues should provide a definitive answer to the question of the role of inhibition of PKC in the mechanism of growth inhibition by ET18-OCH₃.

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