Inhibition of Growth Factor-dependent Inositol Phosphate Ca²⁺ Signaling by Antitumor Ether Lipid Analogues


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ABSTRACT

Cytotoxic ether lipid analogues have been studied for their ability to inhibit growth factor-dependent [Ca²⁺]i signaling in Swiss 3T3 fibroblasts. 1-Octadecyl-2-methyl-rac-glycero-3-phosphocholine (ET-18-OCH3) inhibited Ca²⁺ uptake and inositol(1,4,5)trisphosphate-induced Ca²⁺ release in permeabilized cells with concentration producing 50% inhibition values of 55 and 360 μM, respectively. When cells were exposed to ET-18-OCH3 for 18 h before permeabilization there was selective inhibition of inositol(1,4,5)trisphosphate-induced Ca²⁺ release with a concentration producing 50% inhibition value of 20 μM, but no effect on Ca²⁺ uptake or on 45Ca²⁺ release by arachidonic acid. The concentration of ET-18-OCH3 with continuous exposure to inhibit cell growth was 19 μM. The ether lipid analogues 1-hexadecylthio-2-ethyl-rac-glycero-3-phosphocholine and 1-octadecyl-2-O-methylthiopropyl-3-N,N-dimethyl-γ-hydroxypropyl ammonium iodide had effects similar to those of ET-18-OCH3 but the noncytotoxic analogue 1-alkyl-2-hydroxy-sn-glycero-3-phosphocholine was without effect. Exposure of cells to 10 μM ET-18-OCH3 produced 81% inhibition of platelet-derived growth factor-stimulated inositol phosphate formation and 66% inhibition of fluorosulfonate anion-stimulated inositol phosphate formation. Addition of ET-18-OCH3 to cells in medium with 10% fetal calf serum gave a transient increase in [Ca²⁺]i without causing an increase in resting [Ca²⁺]i, while the addition of ET-18-OCH3 to cells in medium without serum gave a sustained increase in resting [Ca²⁺]i. Cells exposed to 5 μM ET-18-OCH3 for 18 h showed no increase in resting [Ca²⁺]i but there was 95% inhibition of the [Ca²⁺]i response to bradykinin, and 55% inhibition of the response to vasopressin. The block by ether lipid analogues of inositol phosphate-mediated [Ca²⁺]i signaling suggests a mechanism for preventing the action of growth factors that could contribute to the inhibition of cell proliferation by the agents.

INTRODUCTION

The ether lipid analogues of platelet activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) are a new class of DNA-noninteractive antitumor agents (1, 2). Their properties include activation of cytotoxic macrophages (3, 4), induction of cellular differentiation (5, 6), and direct cytotoxic or cytostatic activity (1, 7, 8). The mechanism of antitumor activity of the ether lipid analogues remains unknown. Considerable attention has focused on the actions of the ether lipid analogues on the cell plasma membrane. These actions include morphological damage to the cell plasma membrane (2, 9–11), altered physical properties of cell and model membranes (12), increased plasma membrane permeability (13), interference with phosphatidylcholine synthesis (14), and inhibition of plasma membrane-associated enzymes such as protein kinase C (15).

In addition to having actions on the cell plasma membrane we considered that the ether lipid analogues might also affect intracellular membranes and could modify intracellular signaling. An important function of the endoplasmic reticulum is to store Ca²⁺ that can be released by intracellular second messengers to increase the concentration of cytoplasmic-free Ca²⁺ (16–19). Resting [Ca²⁺]i in cells is very low, around 10,000-fold lower than the concentration of external Ca²⁺, and transient increases in [Ca²⁺]i, are used by cells as an intracellular signal that mediates the actions of a variety of hormones, neurotransmitters, and growth factors (16–20). Increases in [Ca²⁺]i, produced by growth factors and mitogens are predominantly due to the release of Ca²⁺ from stores in the endoplasmic reticulum or related structures (21–24). Stimulation of cell proliferation by some oncoproteins products may also be mediated by changes in [Ca²⁺]i, homeostasis (25). It is therefore possible that agents that block intracellular [Ca²⁺]i signaling could inhibit the actions of growth factors and mitogens on cell proliferation.

In this study we report the effects of cytotoxic ether lipid analogues upon [Ca²⁺]i signaling in Swiss 3T3 fibroblasts and show that the analogues are inhibitors of growth factor-dependent inositol phosphate formation as well as selective inhibitors of inositol phosphate-mediated intracellular Ca²⁺ release. The ether lipid analogues are also potent inhibitors of growth factor-mediated [Ca²⁺]i signaling in intact cells. The results suggest that inhibition of [Ca²⁺]i signaling might contribute to the growth-inhibitory properties of the ether lipid analogues.

MATERIALS AND METHODS

The ether lipid analogues used were: ET-18-OCH3, provided by Dr. Wolfgang Berdel, Technical University of Munich, Federal Republic of Germany; SRI 62-834, provided by Dr. William Houlihan, Sandoz Research Institute, East Hanover, NJ (26); lyso-PAF, obtained from Avanti Polar Lipids, Birmingham, AL; ET-16S-OC2H5, synthesized by Dr. Susan Morris-Natschke, University of North Carolina, Chapel Hill, NC (27); and CP10, synthesized by Canio Marasco, University of North Carolina, Chapel Hill (28).

[Arg⁴] Vasopressin and bradykinin were purchased from Sigma Chemical Co. (St. Louis, MO). Platelet-derived growth factor B chain homodimer was obtained from Bachem Inc. (Torrance, CA). 4CaCl₂ (25 mCi/mg), (c-si₅)₃¹¹¹-PDFG (780 Ci/mmol) and myo[H]inositol (20 Ci/mmol) were obtained from the Amersham Corp. (Arlington Heights, IL). Aequorin was purchased from Dr. John Blinks, Mayo Clinic. Swiss 3T3 fibroblasts were provided by Dr. H. R. Herschmann, University of California (Los Angeles, CA), and maintained in DMEM that contained 10% fetal calf serum. The cells were harvested with 0.5% trypsin and 0.05 mM EDTA before becoming confluent and were used between passages 24 and 30. The inhibition of Swiss 3T3 cell proliferation by ether lipid analogues was measured as previously described (29).

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2 To whom requests for reprints should be addressed, at Department of Pharmacology, Mayo Clinic & Foundation, 200 First Street, S.W., Rochester, MN 55905.
"Ca" uptake and release by saponin-permeabilized Swiss 3T3 cells was measured by a modification of the method of Gill and Cheuh (30). Briefly, the cells in suspension at 2 x 10^6 cells/ml were permeabilized with medium containing 0.005% saponin for 20 min at 37°C with gentle stirring to give >98% trypan blue uptake. The cells were washed to remove saponin and incubated at 37°C at 2 x 10^6 cells/ml in medium containing 1 mM ATP (maintained by a generating system), 3% polyethylene glycol, 50 µM "Ca" (160 µCi/mmol), and sufficient ethylene-glycol bis(β-aminoethyl)ether-<i>N</i>,<i>N</i>′,<i>N</i>″,<i>N</i>‴-tetraacetic acid to buffer the free Ca" to a concentration of 10^{-7} M (31). 2,4-Dinitrophenol, antymycin A, and oligomycin were included in the medium as inhibitors of mitochondrial function. Aliquots (0.1 ml) of the incubated cell suspension were taken at 6 and 7 min and were filtered through GF/A glass microfiber filters (Whatman International, Ltd., Maidstone, England). The filters were washed 4 times with 5 ml of buffer containing 1 mM LaCl₃ and digested with 0.5 ml 1 N NaOH at 60°C for 1 h prior to liquid scintillation spectrometry. Preliminary studies showed that "Ca" uptake by Swiss 3T3 cells was maximal by 6 min. Agents known to release Ca" from stores in the endoplasmic reticulum were added at 6.25 min and the "Ca" remaining in the cells was measured at 7 min. All determinations were conducted in quintuplicate. "Ca" release was expressed as the percentage of release at 7 min compared to the 6-min value for each pair of determinations, corrected for "Ca" release in the absence of added agents. Ether lipid analogues were added to the incubations at 0 min. Alternately, cells in culture were exposed to the ether lipid analogues for up to 18 h before permeabilizing the cells. In these studies the ether lipid analogues were not added to the incubation medium during the "Ca" uptake and release studies.

For studies of the formation of inositol phosphates the Swiss 3T3 cells were grown to confluence in 35-mm culture dishes and labeled for 18 h with myo[3H]inositol, 2 µCi/ml, in Medium 199 (Grand Island Biological Co., Grand Island, NY) containing 10 mM HEPES, pH 7.2, 20 mM L-glutamine, and 0.3% fetal calf serum, with or without 10 µM ET-18-OCH₃. The cells were washed and incubated for 30 min in Hank's balanced salt solution containing 10 mM HEPES, pH 7.2, 10 mM LiCl, and 0.1 mg/ml bovine serum albumin. PDGF, 3.3 x 10^{-6} M, or 10 µM AlCl₃ and 15 mM NaF were then added, with or without 10 µM ET-18-OCH₃, and after 1 h the cells were lysed with 0.5 ml 10% perchloric acid. To each sample 60 µl of 0.2 M HEPES, pH 7.4, and 50 µl of 5 M KOH were added and the mixture was centrifuged at 450 x g for 10 min. The supernatant was eluted through a 0.8 x 4-cm AG 1-X8 anion exchange column (Bio-Rad, Irvine, CA). The column was washed with 20 ml 60 mM sodium formate-5 mM sodium borate before total inositol phosphates were eluted with 5 ml 1.2 mM ammonium formate-100 mM formic acid. Radioactivity in the eluate was determined by liquid scintillation counting.

Changes in [Ca^{2+}] in intact Swiss 3T3 fibroblasts were measured with the Ca^{2+}-sensitive photoprotein aequorin (32). The cells were loaded with aequorin by a low-Ca^{2+} centrifugation technique and 10^6 cells were plated in a 35-mm culture dish in 2 ml of DMEM that contained 10% fetal calf serum. The cells were allowed to attach to the surface of the dish for 20 h at 37°C in 5% CO₂:95% air. The medium was replaced for 2 h with DMEM without fetal calf serum. The culture dish was then placed in a 37°C thermostated holder over a photomultiplier tube and the surface of the medium was flushed with humidified 5% CO₂:95% air. The illumination was used to increase [Ca^{2+}], were 3.3 x 10^{-6} M PDGF, 2 x 10^{-6} M bradykinin, and 10^{-7} M vasopressin, which have previously been shown to produce maximal [Ca^{2+}] responses in Swiss 3T3 cells (23). At the end of the study the cells were lysed with a solution of 1% Triton X-100 and 5 mM CaCl₂ and the total light signal was integrated. The mitogen-induced [Ca^{2+}] responses were integrated and normalized by dividing the total light signal obtained when the cells were lysed. An estimate of [Ca^{2+}] was obtained by using the calibration method for aequorin of Allen and Blinks (33).

The binding of (c-sis)-[^3H]PDGF to Swiss 3T3 fibroblasts was measured at 4°C for 4 h by the method of Bowen-Pope and Ross (34). Groups of data were analyzed by using Student's t test (35); p < 0.05 was considered significant.

**RESULTS**

"Ca" Uptake and Release in Permeabilized Cells. When added to preparations of permeabilized Swiss 3T3 fibroblasts, the ether lipid analogue ET-18-OCH₃ produced a concentration-dependent inhibition of the uptake of "Ca" into nonmitochondrial stores with an IC₅₀ of 55 µM (Fig. 1). There was also inhibition of the release of "Ca" by IP₃(1,4,5) with an IC₅₀ of 360 µM. These concentrations of ET-18-OCH₃ are considerably in excess of the IC₅₀ of 19 µM for growth inhibition of Swiss 3T3 cells by continuous exposure to ET-18-OCH₃. Other ether lipid analogues were tested for their ability to inhibit "Ca" uptake and IP₃(1,4,5)-dependent "Ca" release (Table 1). The cytotoxic analogues ET-165-OC₂H₅, CP10, and SRI 62-834 at a concentration of 50 µM all gave a significant inhibition of the uptake of "Ca". "Ca" release caused by IP₃(1,4,5) was significantly blocked by ET-165-OC₂H₅ and CP10, but not by SRI 62-834. Lyso-PAF which is noncytotoxic to Swiss 3T3 cells was without effect on "Ca" uptake or release.

Exposing Swiss 3T3 cells in culture to ET-18-OCH₃ for 18 h before permeabilization had no significant effect on "Ca" uptake but produced a concentration-dependent inhibition of the release of "Ca" by IP₃(1,4,5) (Fig. 2). The same treatment had no effect on "Ca" release induced by either arachidonic acid or GTP (Table 2). An 18-h minimum exposure of the cells to ET-18-OCH₃ was required to give a significant block of IP₃(1,4,5)-induced "Ca" release (Fig. 3). A selective block of IP₃(1,4,5)-induced "Ca" release, but not of "Ca" release...
ether lipid analogue ET-18-OCH₃ on "Ca²⁺" uptake and release by saponin-permeabilized Swiss 3T3 fibroblasts. Cells were grown with ET-18-OCH₃ at the concentrations shown for 18 h before permeabilization. ET-18-OCH₃ was not added during the uptake and release studies. (C) "Ca²⁺" uptake measured over 6 min; (Φ) "Ca²⁺" release induced by IP₃₁,4,5) measured between 6.25 and 7.00 min. Points, mean of 5 studies expressed as a percentage of control values, which were "Ca²⁺" uptake, 551 ± 44 pmol/10⁶ cells and "Ca²⁺" release, 210 ± 27 pmol/10⁶ cells; bars, SD.

Table 2 "Ca²⁺" uptake and release by permeabilized Swiss 3T3 cells chronically exposed to ether lipid analogues

<table>
<thead>
<tr>
<th>Analogue</th>
<th>&quot;Ca²⁺&quot; uptake (pmol/10⁶ cells)</th>
<th>% of IP₃₁,4,5)</th>
<th>% of &quot;Ca²⁺&quot; release, C₂(M₃4.3</th>
<th>% of GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>592 ± 57</td>
<td>41.0 ± 5.7</td>
<td>34.3 ± 4.4</td>
<td>59.3 ± 6.1</td>
</tr>
<tr>
<td>ET-18-OCH₃</td>
<td>524 ± 32</td>
<td>23.0 ± 4.0</td>
<td>30.9 ± 5.1</td>
<td>54.4 ± 7.2</td>
</tr>
<tr>
<td>ET-165-OCH₃</td>
<td>510 ± 46</td>
<td>19.8 ± 4.4</td>
<td>35.6 ± 5.9</td>
<td>55.6 ± 5.9</td>
</tr>
<tr>
<td>CPI10</td>
<td>542 ± 61</td>
<td>27.3 ± 3.8</td>
<td>30.6 ± 5.5</td>
<td>61.7 ± 7.3</td>
</tr>
<tr>
<td>Lyso-PAF</td>
<td>539 ± 37</td>
<td>38.2 ± 5.0</td>
<td>33.7 ± 3.0</td>
<td>56.8 ± 6.4</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to the appropriate control value.

<table>
<thead>
<tr>
<th>Study 1</th>
<th>No treatment, % of control</th>
<th>ET-18-OCH₃-treated, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 23</td>
<td>56 ± 18⁰</td>
</tr>
<tr>
<td>PDGF</td>
<td>1218 ± 51</td>
<td>265 ± 11⁰</td>
</tr>
<tr>
<td>[AlF₄]⁻</td>
<td>231 ± 8</td>
<td>100 ± 24b</td>
</tr>
<tr>
<td>Study 2</td>
<td>Cells grown with ET-18-OCH₃</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 14</td>
<td>102 ± 21</td>
</tr>
<tr>
<td>PDGF</td>
<td>1954 ± 304</td>
<td>175 ± 11⁰</td>
</tr>
<tr>
<td>[AlF₄]⁻</td>
<td>316 ± 78</td>
<td>157 ± 15⁰</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to nontreated control.  
⁰P < 0.01 compared to nontreated control.
ETHER LIPID INHIBITION OF Ca\textsuperscript{2+} SIGNALING

Serum lipid analogues might be explained either by the inhibition of phospholipase C by depletion of phosphatidylinositol-4,5-bisphosphate as a substrate for phospholipase C or increased breakdown of IP\textsubscript{3}(1,4,5). We have not distinguished between these possibilities in this study.

In permeabilized cells chronically exposed to ether lipid analogues there was a selective inhibition of the IP\textsubscript{3}(1,4,5)-mediated release of \( ^{40} \text{Ca}^{2+} \) from nonmitochondrial stores. \( ^{40} \text{Ca}^{2+} \) release by arachidonic acid and GTP was not affected. IP\textsubscript{3}(1,4,5) is thought to act on a specific receptor located on the endoplasmic reticulum to release stored \( \text{Ca}^{2+} \) (35). Inhibitors of \( \text{Ca}^{2+} \) release such as heparin (41), dextran sulfate (42), and suramin (43) can compete with the binding of IP\textsubscript{3}(1,4,5) to this receptor. The mechanism for the block of IP\textsubscript{3}(1,4,5)-induced \( ^{40} \text{Ca}^{2+} \) release by the ether lipid analogues is not known but probably does not involve a direct interaction with the IP\textsubscript{3}(1,4,5) receptor. Although the ether lipid analogues can acutely inhibit the release of \( ^{40} \text{Ca}^{2+} \) by IP\textsubscript{3}(1,4,5) this requires a high concentration of the ether lipid analogue (IC\textsubscript{50}, 360 \( \mu \text{M} \)) which also...

DISCUSSION

An increase in \([\text{Ca}^{2+}]_i\) due to the release of \( \text{Ca}^{2+} \) from stores associated with the endoplasmic reticulum is an important intracellular signaling mechanism that mediates the actions of a variety of hormones, neurotransmitters, and growth factors (17, 18). A widely studied intracellular second messenger that releases \( \text{Ca}^{2+} \) from endoplasmic reticulum stores is IP\textsubscript{3}(1,4,5), formed by the ligand-activated, phospholipase C-catalyzed, hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate (19, 20). There may be other second messengers that release endoplasmic reticulum \( \text{Ca}^{2+} \), including arachidonic acid (36) and GTP (37). GTP also facilitates the release of \( \text{Ca}^{2+} \) by IP\textsubscript{3}(1,4,5) (38).

We found that the ether lipid analogue ET-18-OCH\textsubscript{3} is an inhibitor of inositol phosphate formation induced by PDGF and [AIF\textsubscript{4}]\textsuperscript{1-}. This occurred when the ET-18-OCH\textsubscript{3} was incubated with the cells for 18 h or when it was added to cells acutely. Binding of PDGF to the PDGF receptor is known to lead to the receptor-mediated tyrosine-specific phosphorylation of phospholipase C, resulting in an increase in phospholipase C activity and inositol phosphate formation (39). [AIF\textsubscript{4}]\textsuperscript{1-} stimulates inositol phosphate formation through activation of phospholipase C mediated by guanine nucleotide regulatory proteins (40). Ether lipid analogues inhibit both the PDGF and [AIF\textsubscript{4}]\textsuperscript{1-}-induced formation of inositol phosphates. The effect of ether lipid analogues might be explained either by the inhibition of phospholipase C by depletion of phosphatidylinositol-4,5-bisphosphate as a substrate for phospholipase C or increased breakdown of IP\textsubscript{3}(1,4,5). We have not distinguished between these possibilities in this study.

In permeabilized cells chronically exposed to ether lipid analogues there was a selective inhibition of the IP\textsubscript{3}(1,4,5)-mediated release of \( ^{40} \text{Ca}^{2+} \) from nonmitochondrial stores. \( ^{40} \text{Ca}^{2+} \) release by arachidonic acid and GTP was not affected. IP\textsubscript{3}(1,4,5) is thought to act on a specific receptor located on the endoplasmic reticulum to release stored \( \text{Ca}^{2+} \) (35). Inhibitors of \( \text{Ca}^{2+} \) release such as heparin (41), dextran sulfate (42), and suramin (43) can compete with the binding of IP\textsubscript{3}(1,4,5) to this receptor. The mechanism for the block of IP\textsubscript{3}(1,4,5)-induced \( ^{40} \text{Ca}^{2+} \) release by the ether lipid analogues is not known but probably does not involve a direct interaction with the IP\textsubscript{3}(1,4,5) receptor. Although the ether lipid analogues can acutely inhibit the release of \( ^{40} \text{Ca}^{2+} \) by IP\textsubscript{3}(1,4,5) this requires a high concentration of the ether lipid analogue (IC\textsubscript{50}, 360 \( \mu \text{M} \)) which also...

**Table 4 Inhibition of mitogen-induced [Ca\textsuperscript{2+}], responses by ET-18-OCH\textsubscript{3}**

Swiss 3T3 cells were loaded with the Ca\textsuperscript{2+}-sensitive photoprotein aequorin and cultured for 18 h in DMEM containing 10% fetal calf serum, with or without 5 \( \mu \text{M} \) ET-18-OCH\textsubscript{3}, and then were serum deprived for 3 h before the addition of 3.3 \( \times \) 10\textsuperscript{-7} M PDGF, 10\textsuperscript{-7} M vasopressin (VP) or 2 \( \times \) 10\textsuperscript{-7} M bradykinin (BK). Light emission was measured and peak areas were integrated and normalized for the total light signal when the cells were lysed with 1% Triton X-100 and 5 mM CaCl\textsubscript{2}. Values are the mean ± SE of \( n \) preparations.

<table>
<thead>
<tr>
<th></th>
<th>PDGF</th>
<th>[AIF\textsubscript{4}]\textsuperscript{1-}</th>
<th>Vasopressin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response, relative area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF</td>
<td>12</td>
<td>41.6 ± 6.5</td>
<td>2.2 ± 0.6*</td>
</tr>
<tr>
<td>[AIF\textsubscript{4}]\textsuperscript{1-}</td>
<td>12</td>
<td>47.9 ± 5.3</td>
<td>17.7 ± 3.3*</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>13</td>
<td>9.9 ± 1.8</td>
<td>4.5 ± 1.1*</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \) compared to control value.
causes inhibition of $^{45}$Ca$^{2+}$ uptake. This suggests a nonspecific effect of the ether lipid analogues on membrane function. Exposure of cells to low concentrations (1 to 5 μM) of ether lipid analogue for 18 h selectively inhibits IP$_3$(1,4,5)-mediated $^{45}$Ca$^{2+}$ release. The mechanism responsible for this effect is not known. Metabolism of the ether lipid analogues or incorporation into cell membranes could lead to altered activity of the IP$_3$(1,4,5) receptor or altered coupling to the Ca$^{2+}$ release channel. Ether lipid analogues are known to be incorporated into cell membranes (44, 45).

There has been speculation regarding the mechanism by which growth factors such as PDGF cause an increase in [Ca$^{2+}$]. Work by Nanberg and Rozengurt (46) utilizing Swiss 3T3 fibroblasts indicates that the increase in [Ca$^{2+}$], caused by PDGF precedes the increase in IP$_3$(1,4,5), and it has been suggested that other inositol phosphates may modulate the ability of PDGF to increase [Ca$^{2+}$], (20, 47). Vasopressin and bombesin, on the other hand, cause an increase in [Ca$^{2+}$], that coincides with the increase in IP$_3$(1,4,5) (46). The increase in [Ca$^{2+}$], caused by bradykinin in Swiss 3T3 fibroblasts may be mediated through the formation of arachidonic acid (48). It is of interest, therefore, that in intact cells the ether lipid analogues gave a much greater inhibition of PDGF-dependent increases in [Ca$^{2+}$], than the increases caused by vasopressin or bradykinin. This suggests that the effects of PDGF on [Ca$^{2+}$], are mediated through the inositol phosphate pathway signaling, although this does not necessarily implicate IP$_3$(1,4,5) as the Ca$^{2+}$-releasing agent. An influx of external Ca$^{2+}$ has previously been found to contribute about one-half of the increase in [Ca$^{2+}$], produced by PDGF in Swiss 3T3 fibroblasts but not to contribute to the [Ca$^{2+}$], increases caused by bradykinin or vasopressin (23). Inositol (1,3,4,5)tetrakisphosphate may act along with IP$_3$(1,4,5) to cause an influx of external Ca$^{2+}$ in some (49) although not all (50) cells. Since the increase in [Ca$^{2+}$], caused by PDGF is almost completely blocked by exposure to the ether lipid analogues it is possible that the ether lipid analogues can prevent the influx of external Ca$^{2+}$, perhaps by inhibiting inositol (1,3,4,5)tetrakisphosphate formation, as well as blocking the release of Ca$^{2+}$ from the endoplasmic reticulum.

Ether lipid analogues have been reported to increase cell membrane permeability and to cause an influx of external Ca$^{2+}$ resulting in a sustained increase in [Ca$^{2+}$], (13, 51, 52). In our studies we saw a sustained increase in [Ca$^{2+}$], with ET-18-OCH$_3$, only when cells were incubated in medium without fetal calf serum. This could be because ET-18-OCH$_3$ binds to serum proteins thus reducing its free concentration. Plasma protein binding would not be surprising for hydrophobic compounds such as the ether lipid analogues. Indirect evidence for extensive binding of ET-18-OCH$_3$ to serum proteins is the observation that the $K_{	ext{diss}}$ of ET-18-OCH$_3$ for inhibition of Swiss 3T3 cell growth following 1-h exposure to the agent in the presence of 10% fetal calf serum is 75 μM but only 1.2 μM in the absence of serum proteins. We do not believe that a sustained increase in [Ca$^{2+}$], contributes to the cytotoxicity of the ether lipid analogues since significant sustained increases in [Ca$^{2+}$], were seen only in the absence of serum when the free ether lipid concentrations were higher than cytotoxic concentrations. In the presence but not in the absence of serum proteins, ET-18-OCH$_3$ caused a transient increase in [Ca$^{2+}$], similar to that caused by growth factors. This could be because ET-18-OCH$_3$ displaces factors normally bound to plasma protein that are capable of increasing [Ca$^{2+}$].

ET-18-OCH$_3$ at relatively high concentrations has been reported to decrease the number of epidermal growth factor receptors in human breast cancer cells (53). However, inhibition of the binding of PDGF to its receptor does not appear to account for the growth-inhibitory action of ET-18-OCH$_3$ since we saw no effect of ET-18-OCH$_3$ at low concentrations, and only weak inhibition at high ET-18-OCH$_3$ concentrations, on specific PDGF binding to Swiss 3T3 cells.

A number of growth factors and mitogens cause an increase in [Ca$^{2+}$], which has been linked to the stimulation of cell proliferation (19, 54, 55). It is tempting to speculate that the block of some growth factor- and mitogen-induced increases in [Ca$^{2+}$], caused by the ether lipid analogues is related to their growth-inhibitory effects. In the present study only the growth-inhibitory ether lipid analogues were found to produce inhibition of [Ca$^{2+}$], responses. The ether lipid analogues block intracellular signaling at several sites, including the inhibition of inositol phosphate formation, the inhibition of the release of intracellular Ca$^{2+}$ by IP$_3$(1,4,5), as well as inhibition of protein kinase C (15). This multiple action might provide a very effective block of the action of some growth factors and mitogens and could contribute to the growth-inhibitory effects of the ether lipid analogues.

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