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# The ether lipid ET-18-$OCH_3$ increases cytosolic $Ca^{2+}$ concentrations in Madin Darby canine kidney cells

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**1** The effect of the ether lipid 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine (ET-18-$OCH_3$) on the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in Madin Darby canine kidney (MDCK) cells was studied using fura-2 as the $Ca^{2+}$ probe. In $Ca^{2+}$ medium, ET-18-$OCH_3$ induced a significant rise in $[Ca^{2+}]_i$ at concentrations between 10–100 $\mu$M with a concentration-dependent delay of 45–175 s. The $[Ca^{2+}]_i$ signal was composed of a gradual rise and a sustained plateau.

**2** In $Ca^{2+}$-free medium, ET-18-$OCH_3$ (10–100 $\mu$M) induced a $Ca^{2+}$ release from internal $Ca^{2+}$ stores with a concentration-dependent delay of 45–175 s. This discharge of internal $Ca^{2+}$ triggered capacitative $Ca^{2+}$ entry in a concentration-dependent manner. This capacitative $Ca^{2+}$ entry was not inhibited by econazole (25 $\mu$M), 1-[$\beta$-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SKF96365; 50 $\mu$M), nifedipine (10 $\mu$M), verapamil (10 $\mu$M), diltiazem (10 $\mu$M) and cadmium (0.5 $\mu$M).

**3** Methyl 2-(phenylthio)ethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate (PCA-4248), a platelet-activating factor (PAF) receptor antagonist, inhibited 25 $\mu$M ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise in a concentration-dependent manner between 1–20 $\mu$M, with 20 $\mu$M exerting a complete block.

**4** The $[Ca^{2+}]_i$ rise induced by ET-18-$OCH_3$ (25 $\mu$M) was not altered when the production of inositol 1,4,5-trisphosphate ($IP_3$) was suppressed by the phospholipase C inhibitor U73122 (2 $\mu$M), but was partly inhibited by the phospholipase D inhibitor propranolol (0.1 mM) or the phospholipase $A_2$ inhibitor aristolochic acid (20–40 $\mu$M).

**5** In $Ca^{2+}$-free medium, pretreatment with 25 $\mu$M ET-18-$OCH_3$ completely depleted the endoplasmic reticulum $Ca^{2+}$ pump inhibitor thapsigargin-sensitive $Ca^{2+}$ store. In contrast, pretreatment with thapsigargin abolished 0.1 mM ATP-induced $[Ca^{2+}]_i$ rise without altering the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise. This suggests that ET-18-$OCH_3$ depleted thapsigargin-sensitive $Ca^{2+}$ stores and also released $Ca^{2+}$ from thapsigargin-insensitive stores. The thapsigargin-insensitive stores involve mitochondria because the mitochondria uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP; 2 $\mu$M) induced a release of mitochondrial $Ca^{2+}$ which was abolished by pretreatment with 25 $\mu$M ET-18-$OCH_3$.

**6** ET-18-$OCH_3$ (25 $\mu$M) induced a significant $Mn^{2+}$ quench of fura-2 fluorescence at 360 nm excitation wavelength confirming that ET-18-$OCH_3$ induced capacitative $Ca^{2+}$ entry. $La^{3+}$ (0.1 mM) or $Gd^{3+}$ (50 $\mu$M) abolished the ET-18-$OCH_3$-induced $Mn^{2+}$ quench and $[Ca^{2+}]_i$ rise.

**7** Our data imply that ET-18-$OCH_3$ induced a $[Ca^{2+}]_i$ rise in MDCK cells by activating PAF receptors leading to an internal $Ca^{2+}$ release followed by capacitative $Ca^{2+}$ entry. Phospholipase D and phospholipase $A_2$, but not phospholipase C, might be involved in mediating the capacitative $Ca^{2+}$ entry. $La^{3+}$ abolished the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise presumably by inhibiting PAF receptors.

**Keywords:** ET-18-$OCH_3$; MDCK cells; $Ca^{2+}$ signalling; capacitative $Ca^{2+}$ entry; $La^{3+}$

**Abbreviations:** ATP, (adenosine 5′-triphosphate); $[Ca^{2+}]_i$, intracellular free $Ca^{2+}$ concentration; DMEM, Dulbecco's modified Eagle medium); ER, endoplasmic reticulum; ET-18-$OCH_3$, 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine; fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N,N-tetraacetic acid pentaacetoxymethyl ester; $IP_3$, inositol 1,4,5-trisphosphate; MDCK cells, Madin Darby canine kidney cells; 2-O-methyl PAF, (±)1-O-hexadecyl-2-O-methylglycero-3-phosphorylcholine; PAF, platelet-activating factor; PCA-4248, methyl 2-(phenylthio)ethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate; SKF96365, 1-[$\beta$-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride; U73122, 1-(6-((17$\beta$-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione

## Introduction

A number of synthetic ether lipids with structure similar to platelet-activating factor (PAF) have been reported to be DNA-noninteractive anti-tumour drugs (Berdel, 1991). Among these drugs, 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine (ET-18-$OCH_3$) is the most commonly used for the investigation of effects of this category of either lipids at the cellular level (Mollinedo *et al*., 1993). The primary cellular target for ET-18-$OCH_3$ was previously thought to be the plasma membrane proteins (Paltauf, 1994; Boggs *et al*., 1995). For example, ET-18-$OCH_3$ inhibits $Na^+$-$K^+$-ATP pump in bovine brain (Zheng *et al*., 1990), protein kinase C in HL-60 cells

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(Berkovic *et al*., 1994), and phosphatylcholine synthesis in macrophage-like cells (Boggs *et al*., 1995). Additionally, ET-18-$OCH_3$ also increases intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in normal and tumour cells (Seewald *et al*., 1990; Lohmeyer & Workman, 1993; Bergmann *et al*., 1994; Brinkmeier *et al*., 1996; Alonso *et al*., 1997), and induces apoptosis in several human leukaemia cells (Mollinedo *et al*., 1993; 1997; Diomede *et al*., 1993). But how exactly ET-18-$OCH_3$ acts to trigger so many different cellular events is not clear at all.

Efforts have been exerted to investigate whether the effect of ET-18-$OCH_3$ on $[Ca^{2+}]_i$ is correlated to its effect on apoptosis. The idea is that a prolonged elevation in $[Ca^{2+}]_i$ could lead to apoptosis (McConkey & Orrenius, 1996). Two studies performed in several normal and tumour cells suggest that these two effects of ET-18-$OCH_3$ appear to be dissociated (Brinkmeier *et al*., 1996; Alonso *et al*., 1997), i.e. under the experimental conditions that the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise is suppressed, the apoptosis effect of this drug is not affected.

The mechanism underlying the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise is not completely understood. Alonso and coworkers (1997) showed that in human neutrophils ET-18-$OCH_3$ induces a robust $[Ca^{2+}]_i$ transient with a rapid rise and decline through stimulation of PAF receptors, because the $[Ca^{2+}]_i$ rise is suppressed by a PAF receptor antagonist. The authors also demonstrated that internal $Ca^{2+}$ release and external $Ca^{2+}$ influx both contribute to the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise, but neither the source of the internal $Ca^{2+}$ nor the identity of the $Ca^{2+}$ influx pathway was investigated. In contrast, in a neuroblastoma cell line Brinkmeier *et al*. (1996) observed that ET-18-$OCH_3$ induces a $Ca^{2+}$ signal with a delayed rise and a persistently elevated plateau, which is abolished by pretreatment with $La^{3+}$ or $Gd^{3+}$. Based on the lanthanide inhibition, the authors conclude that ET-18-$OCH_3$ induces a $[Ca^{2+}]_i$ rise by exclusively opening plasma membrane $Ca^{2+}$ channels. However, it is not known which types of $Ca^{2+}$ channels are involved. Further, the possibility that ET-18-$OCH_3$ might release internal $Ca^{2+}$ is not examined.

In the present study we investigated the effect of ET-18-$OCH_3$ on $[Ca^{2+}]_i$ in Madin Darby canine kidney (MDCK) cells, a non-tumour cell line, in more detail than the previous studies. We found that ET-18-$OCH_3$ induced a rise in $[Ca^{2+}]_i$ by activating PAF receptors leading to a release of $Ca^{2+}$ from both thapsigargin-sensitive and -insensitive internal $Ca^{2+}$ stores. This discharge of internal $Ca^{2+}$ triggered capacitative $Ca^{2+}$ entry (Putney & Bird, 1993) which was blocked by lanthanides. Lanthanides might also directly inhibit PAF receptors. Phospholipase D and phospholipase $A_2$, but not phospholipase C, might be involved in mediating the $[Ca^{2+}]_i$ rise.

## Methods

### *Cell culture*

MDCK cells obtained from American Type Culture Collection (CRL-6253, MD, U.S.A) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum, 100 U $ml^{-1}$ penicillin and 100 $\mu$g $ml^{-1}$ streptomycin at 37°C in 5% $CO_2$-containing humidified air.

### *Solutions*

$Ca^{2+}$ medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; HEPES 10; glucose 10. $Ca^{2+}$-free medium contained no $Ca^{2+}$ plus 1 mM EGTA. ET-18-$OCH_3$ was dissolved in dimethyl sulphoxide (DMSO) as a 10 mM stock solution. PCA-4248 and thapsigargin were dissolved in ethanol as a 20 mM stock solution. The experimental solution contained 0–1% of solvent (DMSO or ethanol) which did not affect $[Ca^{2+}]_i$ ($n=3$).

### *Fluorescence measurements*

Trypsinized cells ($10^6$ $ml^{-1}$) were loaded with 2 $\mu$M 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N,N-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) for 30 min at 25°C in DMEM. Cells were washed and resuspended in $Ca^{2+}$ medium and were washed every hour during experiments to minimize extracellular dye. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million of cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Japan) by continuously recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s intervals. Maximal and minimal fluorescence values were obtained by adding TX-100 (0.1%) and EGTA (20 mM) sequentially at the end of an experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously (Grynkiewicz *et al*., 1985). $Mn^{2+}$ quench experiments were performed in $Ca^{2+}$ medium containing $MnCl_2$ (50 $\mu$M) by recording excitation signal at 360 nm and emission signal at 510 nm continuously at 1-s intervals. Our previous studies have shown that trypsinized cells prepared by our protocol respond to stimulation with ATP (Jan *et al*., 1998a), bradykinin (Jan *et al*., 1998b) or thapsigargin (Jan *et al*., 1999c) similarly to cells attached to coverslips. We decided to use trypsinized cells because this procedure is easier and less time-consuming. All experiments were performed at room temperature (25°C).

### *Materials*

The reagents for cell culture were from Gibco (NY, U.S.A.). Fura-2/AM was from Molecular Probes (OR, U.S.A.). ET-18-$OCH_3$, (±)1-O-hexadecyl-2-O-methylglycero-3-phosphorylcholine (2-O-methyl PAF), and PCA-4248 were from Biomol (Plymouth Meeting, PA, U.S.A.). The other reagents were from Sigma (MO, U.S.A.).

### *Statistics*

All values are reported as means ± s.e.mean of 3–4 experiments. Statistical comparisons were determined by using the Student's paired *t*-test, and significance was accepted when $P<0.05$.

## Results

ET-18-$OCH_3$ at concentrations between 10–100 $\mu$M induced a delayed, gradual rise in $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$. A concentration of 1 $\mu$M had no effect. Representative traces are shown in Figure 1A. The concentration-response plot is shown in Figure 1B. The response did not saturate at 100 $\mu$M. Because the 100 $\mu$M ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise reached a peak value as great as ~1.5 $\mu$M followed by a sustained plateau, higher concentrations were not tested to avoid cell damage. The rise of the $Ca^{2+}$ signal was slower (i.e. delay time was shorter) in response to lower concentrations of

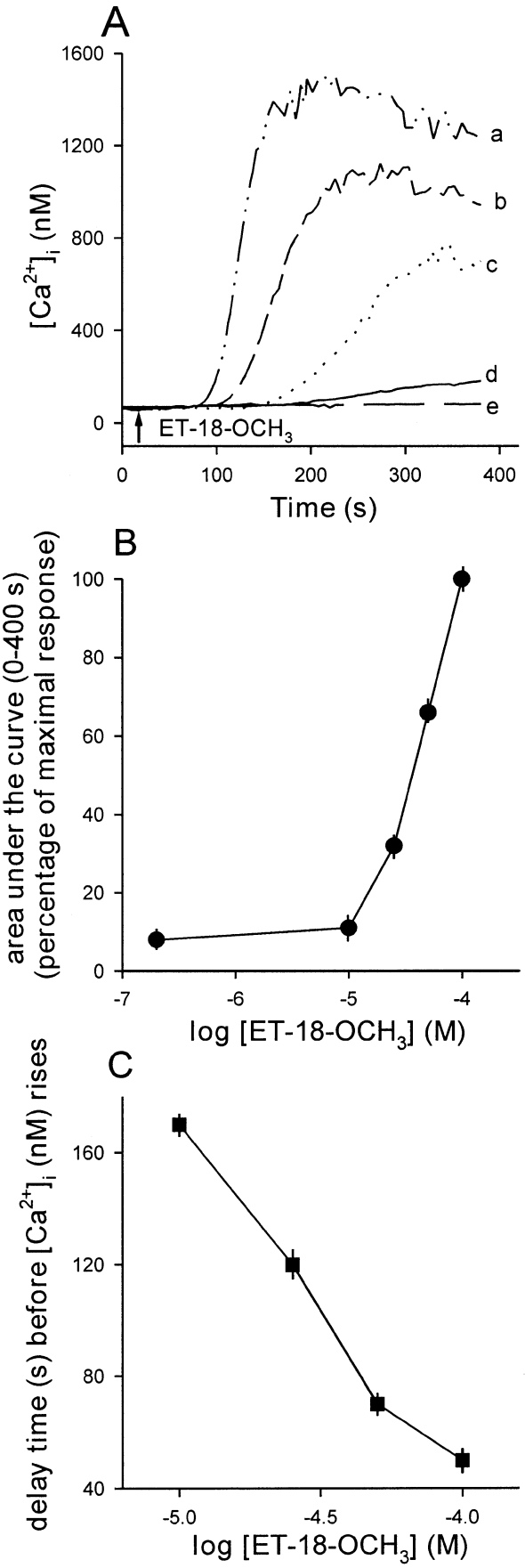


**Figure 1** (A) Concentration-dependent effects of ET-18-OCH$_3$ on $[Ca^{2+}]_i$. Concentration of ET-18-OCH$_3$ was 100 $\mu$M in trace a, 50 $\mu$M in trace b, 25 $\mu$M in trace c, 10 $\mu$M in trace d and zero in trace e. ET-18-OCH$_3$ was applied at 30 s. The experiments were performed in $Ca^{2+}$ medium. Traces are typical of 3–4 experiments. (B) A concentration-response plot of the ET-18-OCH$_3$-induced $Ca^{2+}$ signals shown in (A). The y axis is the area under the curve (percentage of maximum response). The x axis is the concentration of ET-18-OCH$_3$ in logarithmic scale. The data are means±s.e.mean of 3–4 experiments. (C) Relationship between the delay time (s) before $[Ca^{2+}]_i$ (nM) rises. The data are means±s.e.mean of 3–4 experiments.

ET-18-OCH$_3$, and the relationship between concentration and the delay time before $[Ca^{2+}]_i$ rises is plotted in Figure 1C. For example, in the $[Ca^{2+}]_i$ rise induced by 100 $\mu$M ET-18-OCH$_3$, the delay time was 48±3 s ($n=3$; $P<0.05$), while that induced by 10 $\mu$M ET-18-OCH$_3$ had a delay time of 171±4 s ($n=3$; $P<0.05$). At a concentration of 25 $\mu$M, 2-O-methyl PAF, another PAF agonist, induced a rise in $[Ca^{2+}]_i$ with a similar shape and magnitude as that induced by 50 $\mu$M ET-18-OCH$_3$ ($n=5$; data not shown).

We next examined whether ET-18-OCH$_3$ releases $Ca^{2+}$ from internal stores. Figure 2A shows that in $Ca^{2+}$-free medium (no added $Ca^{2+}$ plus 1 mM EGTA) 10–100 $\mu$M ET-18-OCH$_3$ induced significant $[Ca^{2+}]_i$ rises. In MDCK cells, a release of $Ca^{2+}$ from the endoplasmic reticulum (ER) store often induces capacitative $Ca^{2+}$ entry (Jan *et al*., 1998a–c; 1999a–d). Thus, we next examined whether ET-18-OCH$_3$ triggers capacitative $Ca^{2+}$ entry. Figure 2A shows that $CaCl_2$ (3 mM) added at the time point of 360 s when the internal $Ca^{2+}$ stores had been substantially discharged by ET-18-OCH$_3$ pretreatment induced significant capacitative $Ca^{2+}$ entry with a magnitude proportionally correlating to the concentration of ET-18-OCH$_3$, i.e. a larger concentration of ET-18-OCH$_3$ induces a larger capacitative $Ca^{2+}$ entry. We examined the effects of several $Ca^{2+}$ channel blockers on the ET-18-OCH$_3$-induced capacitative $Ca^{2+}$ entry. The blocker was added 30 s prior to $CaCl_2$. These blockers were 10 $\mu$M of nifedipine, diltiazem and verapamil, 25 $\mu$M econazole, 50 $\mu$M SKF96365, and 0.5 mM cadmium. None of the drugs tested had any inhibition ($n=3$; data not shown). The effect of $La^{3+}$ (0.1 mM) on capacitative $Ca^{2+}$ entry could not be tested using this experimental protocol because $La^{3+}$ is chelated by EGTA. Addition of EGTA (1 mM) is needed for measuring capacitative $Ca^{2+}$ entry because the ET-18-OCH$_3$-induced $[Ca^{2+}]_i$ rise remained elevated at ~400 nM for a sustained period of time (>6 min) in nominally $Ca^{2+}$-free (no EGTA) medium, thus addition of 3 mM $CaCl_2$ did not induce a further rise in $[Ca^{2+}]_i$ (data not shown). The concentration-response plot of the capacitative $Ca^{2+}$ entry suggests that the response saturates at a concentration of 50 $\mu$M (Figure 2B) with an $EC_{50}$ of about 32 $\mu$M. Note that the $[Ca^{2+}]_i$ rises induced by all concentrations of ET-18-OCH$_3$ have a similar magnitude in terms of the area under the curve and the net peak value (~40 nM). However, these responses are different in the delay time before $[Ca^{2+}]_i$ rises. Thus, similar to the responses observed in the presence of extracellular $Ca^{2+}$, in the absence of extracellular $Ca^{2+}$ the ET-18-OCH$_3$-induced internal $Ca^{2+}$ release also has a delay time depending on the concentration of ET-18-OCH$_3$, i.e. a larger concentration of ET-18-OCH$_3$ induces a $Ca^{2+}$ release with a shorter delay time. The relationship between delay time and concentration is plotted in Figure 2C.

Because ET-18-OCH$_3$ is a synthetic analogue of platelet-activating factor (PAF), we tested whether the ET-18-OCH$_3$-induced $[Ca^{2+}]_i$ rise is downstream to activation of PAF receptors. We used PCA-4248, a PAF receptor antagonist (Ortega *et al*., 1990), to see if it could affect the ET-18-OCH$_3$-induced $[Ca^{2+}]_i$ rise. Figure 3A shows that pretreatment with

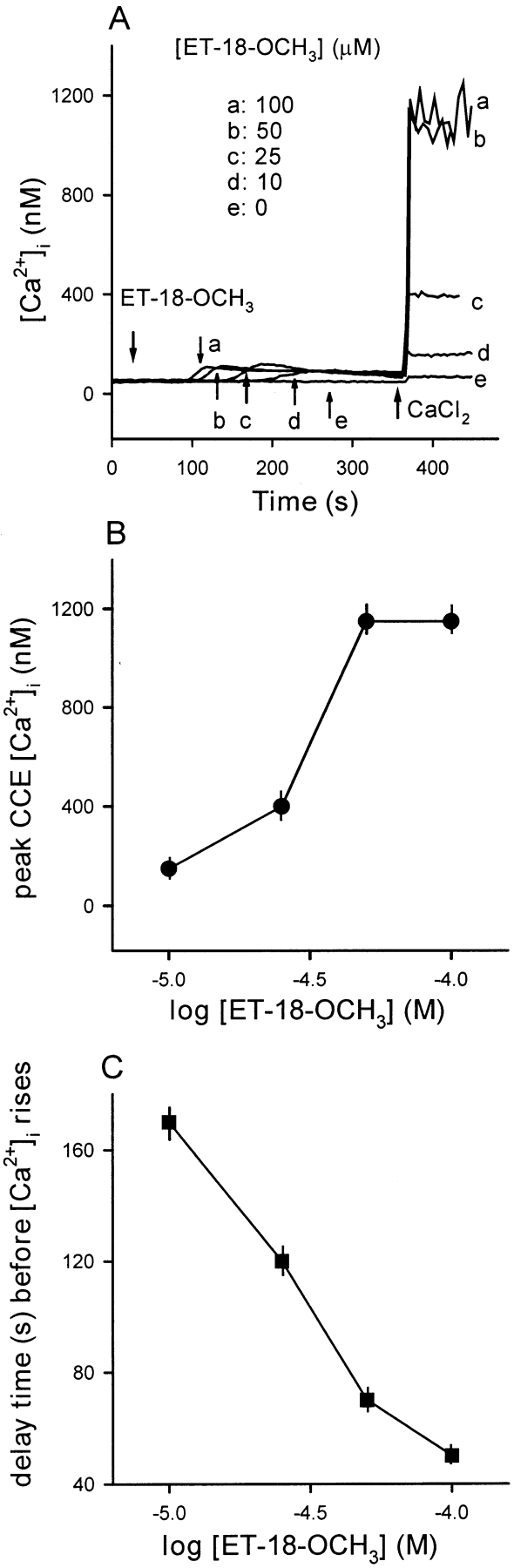


**Figure 2** (A) ET-18-OCH$_3$-induced capacitative $Ca^{2+}$ entry. These experiments were performed in $Ca^{2+}$-free medium. Capacitative $Ca^{2+}$ entry was induced by depleting intracellular $Ca^{2+}$ stores with different concentrations of ET-18-OCH$_3$ (10–100 $\mu$M) in $Ca^{2+}$-free medium (no added $Ca^{2+}$ plus 1 mM EGTA) followed by addition of 3 mM $CaCl_2$. Trace e is the control $CaCl_2$ effect without ET-18-OCH$_3$ pretreatment. (B) A concentration-response plot of the ET-18-OCH$_3$-induced capacitative $Ca^{2+}$ entry shown in (A). The y axis is the peak height of the $[Ca^{2+}]_i$ rise induced by addition of 3 mM $CaCl_2$. The x axis is the concentration of ET-18-OCH$_3$ in logarithmic scale. The data are means$\pm$s.e.mean of 3–4 experiments. (C) Relationship between delay time (s) before $[Ca^{2+}]_i$ rises and the concentration of ET-18-OCH$_3$. The data are means$\pm$s.e.mean of 3–4 experiments.

PCA-4248 for 30 s inhibited the 25 $\mu$M ET-18-OCH$_3$-induced $[Ca^{2+}]_i$ rise in a concentration-dependent manner between 1–20 $\mu$M. Figure 3B shows the concentration-inhibition plot of the PCA-4248 effect. The areas under the curves of the five traces in Figure 3A were computed and compared. Maximum inhibition occurs at 20 $\mu$M PCA-4248 which inhibited 95$\pm$5% ($n=3$; $P<0.05$) of the control ET-18-OCH$_3$ response (in the absence of PCA-4248). The plot indicates an $IC_{50}$ of about 2.5 $\mu$M.

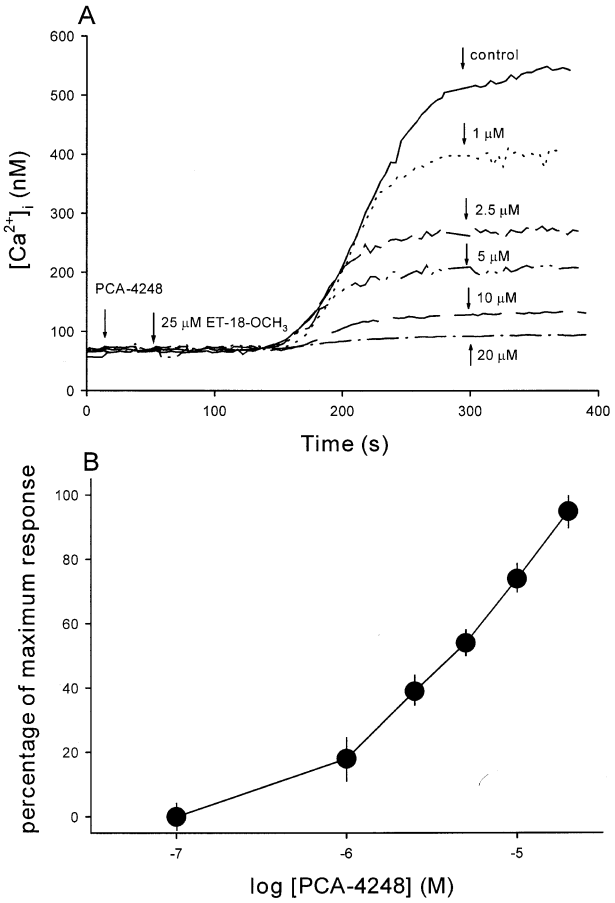


**Figure 3** Effect of PCA-4248 on the ET-18-OCH$_3$-induced $[Ca^{2+}]_i$ rise. (A) ET-18-OCH$_3$ (25 $\mu$M) was added at 50 s. PCA-4248 at a concentration between 1–20 $\mu$M was added at 20 s. Control trace represents the ET-18-OCH$_3$ response without PCA-4248 pretreatment. The experiments were performed in $Ca^{2+}$ medium. Traces are typical of 3–4 experiments. (B) A concentration-response plot of the PCA-4248 inhibition of the ET-18-OCH$_3$-induced $Ca^{2+}$ signal shown in (A). The y axis is the percentage of maximum inhibition which is exerted by 20 $\mu$M PCA-4248 (95$\pm$5% inhibition of control ET-18-OCH$_3$ response in terms of the area under the curve). The x axis is the concentration of PCA-4248 in logarithmic scale. The data are means$\pm$s.e.mean of 3–4 experiments.

We next examined whether ET-18-OCH$_3$ releases internal $Ca^{2+}$ by first elevating cytosolic levels of $IP_3$. We have previously shown that the phospholipase C inhibitor U73122 blocks $IP_3$ production leading to an inhibition of ATP- or bradykinin-induced release of $Ca^{2+}$ from thapsigargin-sensitive ER $Ca^{2+}$ stores (Jan *et al*., 1998c). Because U73122 induces significant $Ca^{2+}$ influx (Jan *et al*., 1998c), the following experiments were performed in $Ca^{2+}$-free medium. Shown in Figure 4A is the $[Ca^{2+}]_i$ transient induced by ATP (0.1 mM). Figure 4B (solid trace) shows that pretreatment with U73122 (2 $\mu$M) for 320 s did not alter the resting $[Ca^{2+}]_i$, but substantially reduced the ATP-induced $[Ca^{2+}]_i$ rise by $91\pm5\%$ in net peak height ($31\pm5$ vs $350\pm16$ nM; $n=3$; $P<0.05$). This result most likely suggests that the $IP_3$ production *via* phospholipase C was significantly inhibited under this condition. When ET-18-OCH$_3$ (25 $\mu$M) was added subsequently at 440 s there occurred a $[Ca^{2+}]_i$ rise which was indistinguishable from the control ET-18-OCH$_3$ response (without U73122/ATP pretreatment; dashed trace). We also examined whether phospholipase D and phospholipase $A_2$ are involved in mediating the ET-18-OCH$_3$-induced $[Ca^{2+}]_i$ rise. We used propranolol to inhibit phospholipase D (Billah, 1989) and aristolochic acid to inhibit phospholipase $A_2$ (Rosenthal *et al*., 1989). Table 1 shows that in the presence of extracellular $Ca^{2+}$, propranolol (0.1 mM) and aristolochic acid (20–40 $\mu$M) both significantly inhibited the $[Ca^{2+}]_i$ rise induced by 25 $\mu$M ET-18-OCH$_3$. However, these two inhibitors did not alter the $[Ca^{2+}]_i$ rise induced by ET-18-OCH$_3$ in the absence of extracellular $Ca^{2+}$ ($n=3$; data not shown).

**Table 1** Effects of propranolol and aristolochic acid on the $[Ca^{2+}]_i$ rise induced by ET-18-OCH$_3$ (25 $\mu$M)

| *Inhibitor* | *Incubation time* (s) | *Inhibition* (% of control) |
|---|---|---|
| Propranolol (0.1 mM) | 260 | $13\pm4$* |
| Aristolochic acid (20 $\mu$M) | 400 | $26\pm5$* |
| Aristolochic acid (40 $\mu$M) | 270 | $38\pm7$* |

The experiments were performed in $Ca^{2+}$ medium. The data are presented as percentage of control response in peak height and are the mean$\pm$s.e.mean of four experiments. *$P<0.05$.

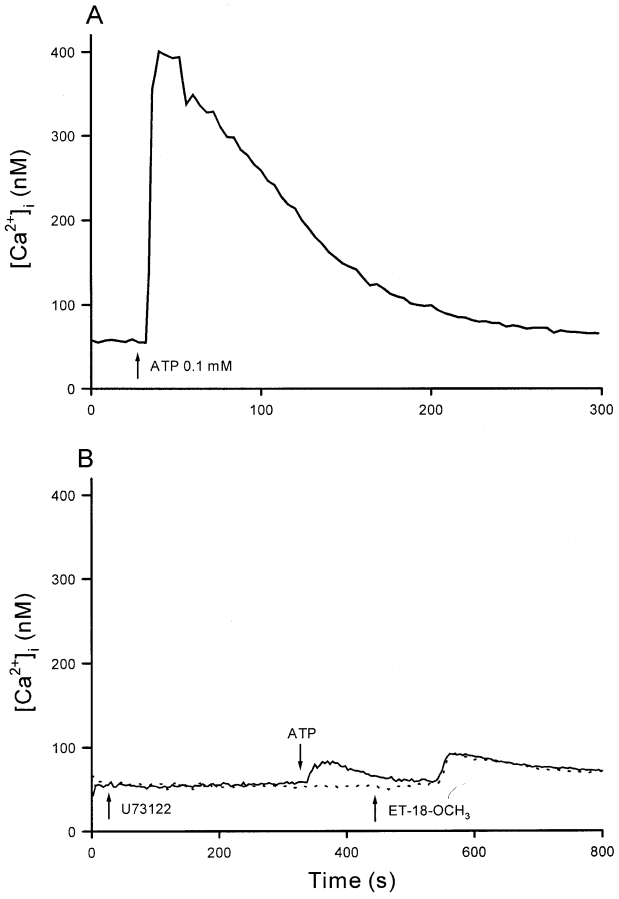


**Figure 4** Effect of inhibition of $IP_3$ production on the ET-18-OCH$_3$-induced internal $Ca^{2+}$ release. (A) 0.1 mM ATP was added at 30 s. (B) U73122 (2 $\mu$M) was added at 30 s followed by ATP (0.1 mM) and ET-18-OCH$_3$ (25 $\mu$M) at 340 s and 440 s, respectively. These experiments were performed in $Ca^{2+}$-free medium. Traces are typical of 3–4 experiments.

We went on to investigate whether ET-18-OCH$_3$ releases $Ca^{2+}$ from the thapsigargin-sensitive ER store, the major internal $Ca^{2+}$ store in MDCK cells (Jan et al. 1998a–c; 1999a–d). Thapsigargin is a substance known to cause a passive leak of $Ca^{2+}$ from the ER store by inhibiting the ER $Ca^{2+}$ pump (Thastrup *et al*., 1990). Figure 5A shows that in the absence of extracellular $Ca^{2+}$, after pretreatment with 25 $\mu$M ET-18-OCH$_3$ for 320 s thapsigargin hardly induced a rise in $[Ca^{2+}]_i$ (see Figure 5B for control thapsigargin response). This suggests that ET-18-OCH$_3$ completely discharged the thapsigargin-sensitive ER $Ca^{2+}$ store. However, other $Ca^{2+}$ stores also contribute to the ET-18-OCH$_3$ response because, as shown in Figure 5B, after thapsigargin had completely depleted the thapsigargin-sensitive ER $Ca^{2+}$ store (evidenced by the observation that 0.1 mM ATP added after thapsigargin did not increase $[Ca^{2+}]_i$), ET-18-OCH$_3$ still induced a rise in $[Ca^{2+}]_i$ normally (compared to Figure 5A).

The thapsigargin-insensitive $Ca^{2+}$ store in MDCK cells which could be examined by pharmacological tools is mitochondria. We have previously shown that the mitochondrial uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP; 2 $\mu$M) induced a rise in $[Ca^{2+}]_i$ (Jan *et al*., 1998c; 1999b), suggesting that in this cell mitochondria contain a significant amount of $Ca^{2+}$ which could be released when mitochondria are uncoupled by CCCP. Thus, we investigated whether ET-18-OCH$_3$ releases $Ca^{2+}$ from mitochondria. Figure 6A shows that in the absence of extracellular $Ca^{2+}$, pretreatment with 25 $\mu$M ET-18-OCH$_3$ prevented CCCP (2 $\mu$M) from inducing a rise in $[Ca^{2+}]_i$. However Figure 6B clearly demonstrates that CCCP induced a significant rise in $[Ca^{2+}]_i$ with a net peak height of $51\pm7$ nM ($n=4$; $P<0.05$). Thus, these results suggest that ET-18-OCH$_3$ depletes the mitochondrial $Ca^{2+}$ store. Figure 6B also shows that the ET-18-OCH$_3$-induced rise in $[Ca^{2+}]_i$ was not much altered after the mitochondrial $Ca^{2+}$ had been depleted.

To confirm that ET-18-OCH$_3$ induced capacitative $Ca^{2+}$ entry, we next measured whether ET-18-OCH$_3$ caused $Mn^{2+}$ quench of fura-2 fluorescence. $Mn^{2+}$ enters cells through similar pathways as $Ca^{2+}$, but quenches fura-2 fluorescence at all excitation wavelengths (Merritt *et al*., 1989). Thus, $Ca^{2+}$ influx can be estimated by $Mn^{2+}$ quench of fura-2 fluorescence at the $Ca^{2+}$-insensitive excitation wavelength of 360 nm and emission wavelength of 510 nm. Figure 7A illustrates that 25 $\mu$M ET-18-OCH$_3$ induced a significant $Mn^{2+}$ quench of fura-2 fluorescence after a delay of $120\pm8$ s ($n=3$; $P<0.05$) (control; solid trace) which closely matches the delay time observed in Figure 1C. This result suggests that ET-18-OCH$_3$ induced considerable $Ca^{2+}$ influx, in keeping with our data in Figure 2 that ET-18-OCH$_3$ induced significant capacitative $Ca^{2+}$ entry. Because we have observed that $La^{3+}$ is a potent

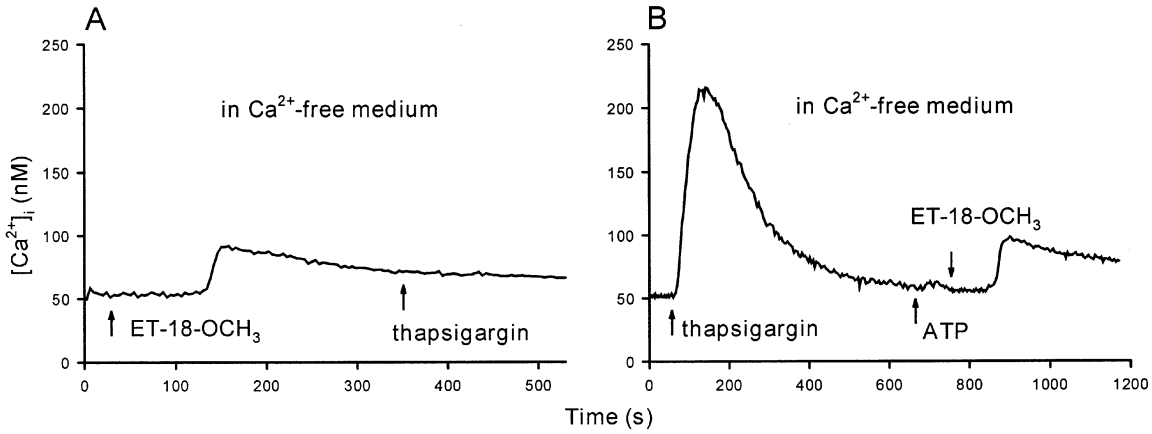


**Figure 5** Effect of ET-18-OCH$_3$ on internal $Ca^{2+}$ stores. (A) ET-18-OCH$_3$ (25 $\mu$M) was added at 30 s followed by thapsigargin (1 $\mu$M) at 350 s. (B) Thapsigargin (1 $\mu$M) was added at 30 s, followed by ATP (0.1 mM) and ET-18-CH$_3$ (25 $\mu$M) at 630 s and 760 s, respectively. These experiments were performed in $Ca^{2+}$-free medium. Traces are typical of 3–4 experiments.

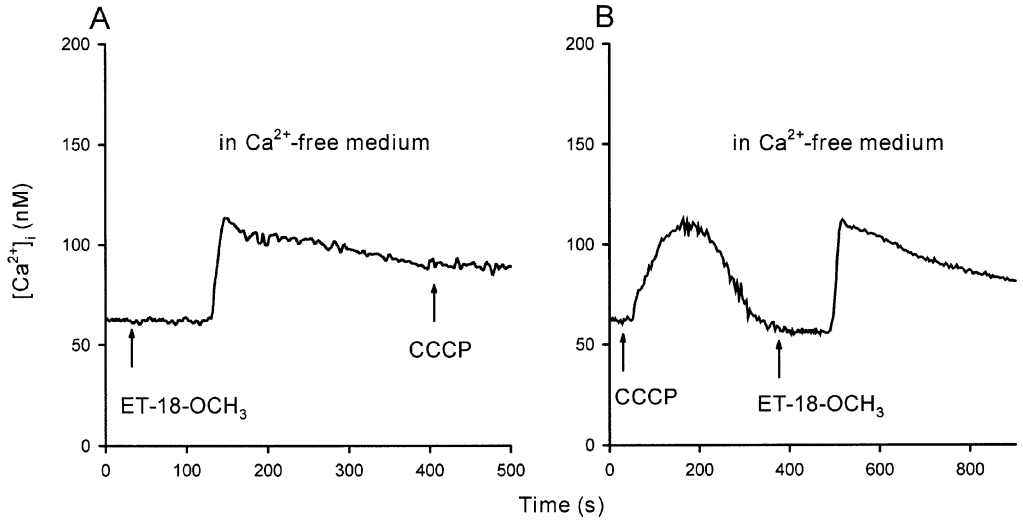


**Figure 6** Effect of ET-18-OCH$_3$ on mitochondrial $Ca^{2+}$ stores. (A) In $Ca^{2+}$-free medium, ET-18-OCH$_3$ (25 $\mu$M) was added at 30 s followed by CCCP (2 $\mu$M) at 410 s. (B) Similar to (A) except that CCCP was added at 30 s followed by ET-18-OCH$_3$ at 390 s. Traces are typical of 3–4 experiments.

blocker of capacitative $Ca^{2+}$ entry in MDCK cells (Jan *et al.*, 1998a, c; 1999c, d), we next examined the effect of $La^{3+}$ on the ET-18-OCH$_3$-induced $Mn^{2+}$ quench of fura-2 fluorescence. Figure 7A shows that the ET-18-OCH$_3$-induced $Mn^{2+}$ quench of fura-2 fluorescence was abolished by pretreatment with 0.1 mM $La^{3+}$ (dashed trace). Consistently, Figure 7B shows that pretreatment with 0.1 mM $La^{3+}$ for 20 s totally suppressed 25 $\mu$M ET-18-OCH$_3$-induced $[Ca^{2+}]_i$ rise (trace f). $La^{3+}$ (0.1 mM) also abolished the $[Ca^{2+}]_i$ rise induced by 25 $\mu$M 2-O-methyl PAF ($n=3$; data not shown). When $La^{3+}$ is added during the ET-18-OCH$_3$ response, the inhibition of $La^{3+}$ depends on the time it is added. Thus, when added at time points of 180 s (trace e), 205 s (trace d), 220 s (trace c) and 265 s (trace b), $La^{3+}$ immediately stopped the rise of $[Ca^{2+}]_i$ and maintained the $[Ca^{2+}]_i$ at a persistently elevated phase. However, when added at the late phase of the $[Ca^{2+}]_i$ rise, for example at 475 s, $La^{3+}$ had little effect on the $[Ca^{2+}]_i$ rise (trace a). Substitution of 50 $\mu$M $Gd^{3+}$ for 0.1 mM $La^{3+}$ produced identical results (data not shown).

## Discussion

Although ET-18-OCH$_3$ has been found to cause a rise in $[Ca^{2+}]_i$ in a number of cells, its effect on $Ca^{2+}$ signalling is not fully characterized and the precise underlying mechanisms are not completely clear. In the present study we investigated how ET-18-OCH$_3$ affects $[Ca^{2+}]_i$ in MDCK cells. Our results suggest the following mechanism for the ET-18-OCH$_3$-induced $[Ca^{2+}]_i$ rise: ET-18-OCH$_3$ activates PAF receptors leading to a release of $Ca^{2+}$ from both thapsigargin-sensitive and -insensitive $Ca^{2+}$ stores. This discharge of internal $Ca^{2+}$ subsequently activates capacitative $Ca^{2+}$ entry which is blocked by lanthanides. Lanthanides might also directly inhibit PAF receptors. Phospholipase D and phospholipase $A_2$, but not phospholipase C, might be involved in triggering the $[Ca^{2+}]_i$ rise.

We have found that ET-18-OCH$_3$ induces a significant rise in $[Ca^{2+}]_i$ between 10–100 $\mu$M. A similar effective concentration range was found for a neuroblastoma cell line (Brinkmeier

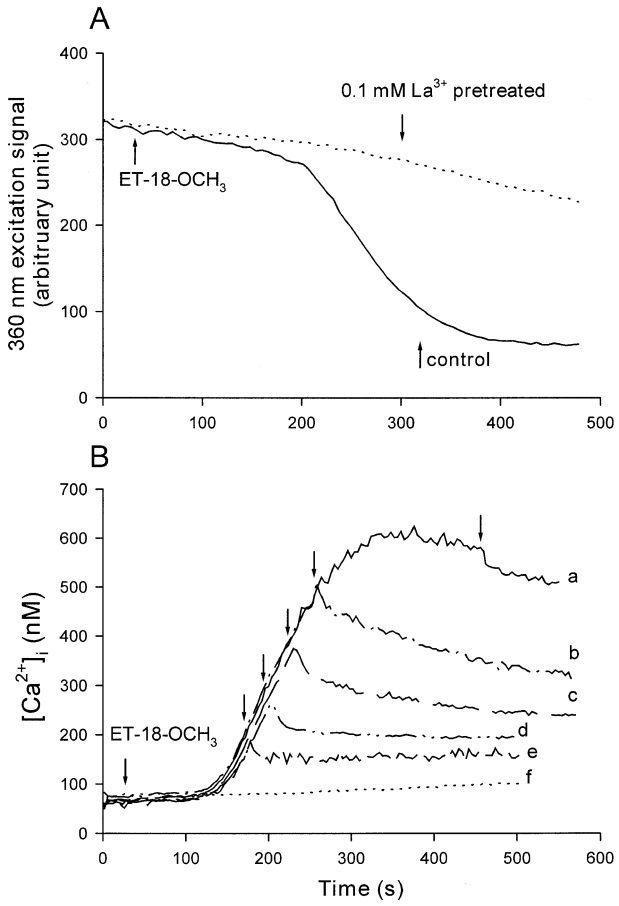


**Figure 7** (A) The ET-18-$OCH_3$-induced $Ca^{2+}$ influx detected by $Mn^{2+}$ quench measurements. The experiments were performed in $Ca^{2+}$ medium plus 50 $\mu$M $MnCl_2$. Solid trace: control response of ET-18-$OCH_3$ (25 $\mu$M), applied at 30 s. Dashed trace: $La^{3+}$ (0.1 mM) was added at 0 s followed by ET-18-$OCH_3$ (25 $\mu$M) at 30 s. Excitation signal at 360 nm and emission signal at 510 nm were recorded. (B) Effect of $La^{3+}$ on the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise. ET-18-$OCH_3$ was added at 30 s in traces a–f and $La^{3+}$ (0.1 mM) was added at 475 s in trace a, 265 s in trace b, 220 s in trace c, 205 s in trace d, 180 s in trace e and 0 s in trace f. Traces are typical of 3–4 experiments.

*et al*., 1996) but a lower range was effective in human neutrophils (10 nM–10 $\mu$M) (Alonso *et al*., 1997). This discrepancy might be due to differences in cell type. We propose that ET-18-$OCH_3$ acts through activating PAF receptors because the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise is inhibited by PCA-4248, a PAF receptor antagonist, in a concentration-dependent manner. Our results are supported by the observations that 2-O-methyl PAF, another PAF receptor agonist, also induces a $[Ca^{2+}]_i$ rise in MDCK cells similarly to ET-18-$OCH_3$ (data not shown), and that in U937 cells and human neutrophils WEB-2170, a PAF receptor antagonist (Heuer *et al*., 1990), also blocks the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise (Alonso *et al*., 1997).

One of the unique characteristics of the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise in MDCK cells is that it has a concentration-dependent delay before the $[Ca^{2+}]_i$ starts to rise, which is not observed in the $[Ca^{2+}]_i$ responses induced by other agents such as ATP (Jan *et al*., 1998a), bradykinin (Jan *et al*., 1998b), the phospholipase C inhibitor U73122 (Jan *et al*., 1998c), econazole (Jan *et al*., 1999a), SKF96365 (Jan *et al*., 1999b) and thapsigargin (Jan *et al*., 1998c) in MDCK cells. This delay time is not altered by removal of extracellular $Ca^{2+}$ (compare Figure 1C to Figure 2C), implying that this delay time is needed for $Ca^{2+}$ to be released from internal stores in response to ET-18-$OCH_3$ stimulation. Because the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise is blocked by lanthanides and PCA-4248, the possibility that ET-18-$OCH_3$ induces a $[Ca^{2+}]_i$ rise by entering the cell and directly acting on intracellular sites is unlikely.

Our findings suggest that activation of phospholipase D and phospholipase $A_2$, but not phospholipase C, might be involved in mediating the $[Ca^{2+}]_i$ rise induced by ET-18-$OCH_3$ because the $[Ca^{2+}]_i$ rise is partly inhibited by the phospholipase D inhibitor propranolol and the phospholipase $A_2$ inhibitor aristolochic acid, but not the phospholipase C inhibitor U73122. Indeed, in Swiss 3T3 fibroblasts ET-18-$OCH_3$ was found to inhibit the $IP_3$ production induced by platelet-derived growth factor (PDGF) and $[AlF_4]^{1-}$ (Seewald *et al*., 1990). We found that phospholipase D and phospholipase $A_2$ appear to be involved in the regulation of the ET-18-$OCH_3$-induced extracellular $Ca^{2+}$ influx instead of intracellular $Ca^{2+}$ release because propranolol and aristolochic acid only inhibit the $[Ca^{2+}]_i$ rise induced by ET-18-$OCH_3$ in the presence, but not in the absence, of extracellular $Ca^{2+}$. How ET-18-$OCH_3$ releases internal $Ca^{2+}$ is not clear.

We have previously shown that U73122 (Jan *et al*., 1998c), econazole (Jan *et al*., 1999a) and SKF96365 (Jan *et al*., 1999b) all release $Ca^{2+}$ from the thapsigargin-sensitive ER $Ca^{2+}$ store presumably *via* inhibition of the ER $Ca^{2+}$ pump. However, the action of ET-18-$OCH_3$ differs from that of these three substances at least in two aspects. First, the latter three substances are not known to act through a receptor on the plasma membrane. Second, ET-18-$OCH_3$ not only depletes the thapsigargin-sensitive ER $Ca^{2+}$ store but also releases $Ca^{2+}$ from mitochondria and possibly other stores, because in the absence of extracellular $Ca^{2+}$, pretreatment with ET-18-$OCH_3$ prevents thapsigargin or CCCP from releasing $Ca^{2+}$. Consistently, the $[Ca^{2+}]_i$ rise induced by ET-18-$OCH_3$ is not altered by pretreatment with either thapsigargin or CCCP, suggesting that both the thapsigargin-sensitive ER store and the CCCP-sensitive mitochondrial store contribute to the internal $Ca^{2+}$ release induced by ET-18-$OCH_3$. The involvement of other stores cannot be excluded.

It is interesting that econazole (25 $\mu$M) and SKF96365 (50 $\mu$M) do not inhibit the capacitative $Ca^{2+}$ entry induced by ET-18-$OCH_3$. We have recently shown that econazole partly inhibits the capacitative $Ca^{2+}$ entry induced by thapsigargin (Jan *et al*., 1999c), cyclopiazonic acid (Jan *et al*., 1999a) and U73122 (Jan *et al*., 1998c); and that SKF96365 partly inhibited the capacitative $Ca^{2+}$ entry induced by thapsigargin (Jan *et al*., 1999c) and 2,5-di-tert-butylhydroquinone (Jan *et al*., 1999d). Thus, the capacitative $Ca^{2+}$ entry induced by ET-18-$OCH_3$ and that induced by the other agents appear to be different in nature.

Brinkmeier and coworkers (1996) also found a 10–25 s delay time before $[Ca^{2+}]_i$ starts to rise in response to ET-18-$OCH_3$ in a neuroblastoma cell line, but whether this delay depends on concentration is not shown. However, there is no delay time in the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise in other cells such as HL-60 cells, U937 cells and human neutrophils (Alonso *et al*., 1997). This discrepancy may be caused by differences in cell type. Brinkmeier and coworkers (1996) proposed that the delay time is required for ET-18-$OCH_3$ or its derivative(s) to integrate into the plasma membrane and open a $Ca^{2+}$ channel based on the fact that $La^{3+}$ and $Gd^{3+}$ abolish the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise. However, the authors did not investigate whether ET-18-$OCH_3$ could release internal $Ca^{2+}$ by removing extracellular $Ca^{2+}$ as we did in this study.

As we previously discussed, $La^{3+}$ is unlikely to enter MDCK cells (Jan *et al*., 1998a), and because we have demonstrated that $La^{3+}$ abolishes the capacitative $Ca^{2+}$ entry induced by ATP (Jan *et al*., 1998a), thapsigargin (Jan *et al*., 1999c), 2,5-di-tert-butylhydroquinone (Jan *et al*., 1999d) and U73122 (Jan *et al*., 1998c) in MDCK cells, it is reasonable to believe that $La^{3+}$ might also abolish the ET-18-$OCH_3$-induced capacitative $Ca^{2+}$ entry. But this cannot explain why $La^{3+}$ completely inhibits the ET-18-$CH_3$-induced $Mn^{2+}$ entry and $[Ca^{2+}]_i$ rise because $La^{3+}$ would not be expected to inhibit internal $Ca^{2+}$ release. The inhibition of $La^{3+}$ is not specific for ET-18-$OCH_3$ alone because $La^{3+}$ also abolishes the $[Ca^{2+}]_i$ rise induced by another PAF agonist, 2-O-methyl PAF (data not shown). Thus, one possible interpretation is that $La^{3+}$ might inhibit PAF receptors. This hypothesis gains support from the fact that $La^{3+}$ might also inhibit ATP receptors in MDCK cells (Jan *et al*., 1998a).

Figure 7B shows that the $La^{3+}$-sensitive capacitative $Ca^{2+}$ entry is triggered early during the ET-18-$OCH_3$-induced $Ca^{2+}$ signal because when added 40 s after the $[Ca^{2+}]_i$ starts to rise, $La^{3+}$ is able to inhibit the rise of the signal. We have found a similar effect of $La^{3+}$ on the phospholipase C inhibitor U73122-induced $[Ca^{2+}]_i$ rise in MDCK cells (Jan *et al*., 1999c). These results suggest that capacitative $Ca^{2+}$ entry is initiated when internal $Ca^{2+}$ stores are only partly depleted.

In HL-60 cells, Lohmeyer & Workman (1993) proposed that the ether lipid (ET-18-$OCH_3$ and its analogue SRI 62-834)-induced $[Ca^{2+}]_i$ rise should be transient (i.e. with an initial peak followed by a fast decline), and the previously reported elevated plateau of the $[Ca^{2+}]_i$ rise induced by SRI 62-834 (Lazenby *et al*., 1990) is due to external $Ca^{2+}$ influx and leakage of internal quin-2 caused by extensive cell damage. In view of our results, it is unlikely that the elevated plateau in the 25 $\mu$M ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise in MDCK cells could be attributed to cell damage-induced fura-2 leakage and external $Ca^{2+}$ influx, because if it were the case, the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise would not be abolished by pretreatment with $La^{3+}$ or PCA-4248.

Taken together, we have found several unique characteristics about the ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise which have not been reported previously: (1) The delay time before the $[Ca^{2+}]_i$ rise is dependent on the concentration of ET-18-$OCH_3$. A higher concentration needs a shorter delay time. (2) ET-18-$OCH_3$ releases internal $Ca^{2+}$ from both thapsigargin-sensitive ER stores and thapsigargin-insensitive, CCCP-sensitive mitochondrial stores. (3) The ET-18-$OCH_3$-induced $[Ca^{2+}]_i$ rise in the presence of external $Ca^{2+}$ is composed of two sources. First, an $IP_3$-independent internal $Ca^{2+}$ release followed by econazole- and SKF96365-insensitive capacitative $Ca^{2+}$ entry. This capacitative $Ca^{2+}$ entry might be modulated by phospholipase D and phospholipase $A_2$, but not by phospholipase C. And lastly, we suspect that lanthanides might directly inhibit PAF receptors. What remains to be answered is how ET-18-$OCH_3$ activation of PAF receptors causes a release of internal $Ca^{2+}$ *via* a mechanism independent of the activities of phospholipases C, D and $A_2$. The possibility that lanthanides might inhibit PAF receptors needs further investigation.

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