Intracellular calcium mobilization and phospholipid degradation in sphingosylphosphorylcholine-stimulated human airway epithelial cells

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Extracellular sphingosylphosphorylcholine (SPC) caused a remarkable elevation in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in immortalized human airway epithelial cells (CFNP9o$^-$). An increase in total inositol phosphates formation was determined; however, the dose responses for $[Ca^{2+}]_i$ elevation and inositol phosphates production were slightly different and, furthermore, PMA and pertussis toxin almost completely inhibited $[Ca^{2+}]_i$ mobilization by SPC, whereas inositol phosphates production was only partially reduced. The possible direct interaction of SPC with Ca^{2+} channels of intracellular stores was determined by experiments with permeabilized cells, where SPC failed to evoke Ca^{2+} release, whereas lysophosphatidic acid was shown to be effective. The level of phosphatidic acid was increased by SPC only in the presence of $AACOCF_3$, a specific inhibitor of phospholipase A_2 (PLA_2) and blocked by both pertussis toxin and R59022, an inhibitor of diacylglycerol kinase. R59022 enhanced diacylglycerol production by SPC and also significantly reduced $[Ca^{2+}]_i$ mobilization. Only polyunsaturated diacylglycerol and phosphatidic acid were generated by SPC. Lastly, SPC caused stimulation of arachidonic acid release, indicating the involvement of PLA_2. Taken together, these data suggest that, after SPC stimulation, phospholipase C-derived diacylglycerol is phosphorylated by a diacylglycerol kinase to phosphatidic acid, which is further hydrolysed by PLA_2 activity to arachidonic and lysophosphatidic acids. We propose that lysophosphatidic acid might be the intracellular messenger able to release Ca^{2+} from internal stores.

INTRODUCTION

Sphingolipid derivatives have been recently reported to be involved in regulation of a variety of cellular functions [1]. In particular, sphingosine 1-phosphate (S-1-P) has been shown to be implicated as a second messenger, produced after stimulation with platelet-derived growth factor and serum in fibroblasts [2]. S-1-P has been reported to cause Ca^{2+} release from internal stores through a mechanism independent of the inositol 1,4,5-trisphosphate (IP_3) receptor and probably due to direct interaction with an unknown mechanism in the endoplasmic reticulum [3–5]. Another lysosphingolipid, sphingosylphosphorylcholine (SPC), has also been shown to release Ca^{2+} from intracellular stores [3,4]. The action of SPC has been suggested to occur inside the cell, probably as a consequence of its ability to penetrate the cell membrane. Subsequently, a new Ca^{2+} channel has been revealed by patch clamping in the endoplasmic reticulum and shown to be specifically gated by SPC, but not by S-1-P or sphingosine [6]. Molecular cloning has indicated that this sphingolipid-activated Ca^{2+}-release-mediating protein of the endoplasmic reticulum is a 181 amino acid protein with two putative membrane-spanning domains, unrelated to the intracellular IP_3 and ryanodine receptors [7].

In previous studies in different cellular systems, no significant production of inositol phosphates by SPC was measured, despite the fact that a significant elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was observed [8–10]. Conversely, Okajama and Kondo reported in HL60 leukaemia cells that Ca^{2+} mobilization induced by extracellular SPC was dependent on inositol phosphates production by phospholipase C (PLC) and mediated by a pertussis toxin-sensitive G protein [11]. Therefore, these authors suggested the existence of a G protein-linked receptor for SPC that was functionally coupled to PLC [11]. A similar suggestion was proposed by Van Koppen et al. [12], who also reported in human embryonic kidney cells that SPC, as well as S-1-P, increased $[Ca^{2+}]_i$ via a pertussis toxin-sensitive mechanism, although no indication was given as to the mechanism responsible for $[Ca^{2+}]_i$ mobilization.

In the present study, we have investigated $[Ca^{2+}]_i$ elevation by SPC in an immortalized-airway epithelial cell line [13]. We report that addition of SPC to human airway epithelial cells induced a rapid and transient elevation of $[Ca^{2+}]_i$. We have investigated whether SPC acts as an extracellular agonist, causing receptor-mediated stimulation of inositol phosphates production by PLC. The effect of compounds, such as pertussis toxin and the phorbol ester PMA, which are known to interact specifically and inactivate some components of the signal transduction pathway leading to inositol phosphates production and Ca^{2+} mobilization, has been determined. We observed that both these treatments significantly reduced Ca^{2+} mobilization, whereas inositol phosphates accumulation was only partially affected. Subsequently, we have determined whether Ca^{2+} mobilization by SPC, in addition to the inositol phosphates-mediated component, might depend on the presence of another intracellular mechanism, which might be due either to direct interaction of SPC with Ca^{2+}-selective channels in the endoplasmic reticulum or alternatively to formation of another second messenger, able to release Ca^{2+} directly from internal stores. It has been reported recently in permeabilized

Abbreviations used: SPC, sphingosylphosphorylcholine; S-1-P, sphingosine 1-phosphate; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PLA_2, phospholipase A_2; PLD, phospholipase D; IPn, total inositol phosphates; PKC, protein kinase C; DAG, diacylglycerol; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; AM, acetoxymethyl.

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Jurkat leukaemia cells that both phosphatidic acid and its metabolite lysophosphatidic acid were able to release Ca^{2+} from an intracellular store, which was distinct from that sensitive to IP_3 [14,15]. The role of these compounds in SPC signalling and the possible pathways responsible for their production have been investigated.

EXPERIMENTAL

Materials

Sulfinpyrazone, PMA, bradykinin, SPC and pertussis toxin were from Sigma (St. Louis, MO, U.S.A.), 4α-PMA and staurosporine were from Biomol Research Labs. (Plymouth Meeting, PA, U.S.A.), thapsigargin and R59022 were from Calbiochem (San Diego, CA, U.S.A.), fura-2/AM and fluo-3-free acid were from Molecular Probes (Eugene, OR, U.S.A.) and [2-^{3}H]*myo*-inositol, [1-^{14}C]palmitic acid and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid were from NEN Products (Stevenage, Herts, U.K.). SPC was dissolved in water.

Cell culture

CFNP9o$^-$ cells were derived from nasal epithelium [13]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 10 % fetal calf serum.

Measurement of $[Ca^{2+}]_i$

Trypsinized cells (4×10^6/ml) were loaded with 4 μM fura-2/AM (acetoxymethyl) for 30 min at 37 °C with continuous stirring in DMEM (pH 7.4) supplemented with 2 mg/ml BSA and 0.2 mM sulfinpyrazone. After washing, aliquots of 3×10^5 cells were incubated in a saline solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 5.5 mM D-glucose, 20 mM Na-Hepes, 1 mg/ml BSA and 0.2 mM sulphinpyrazone (pH 7.4). $[Ca^{2+}]_i$ was determined exactly as described in [16].

Measurement of Ca^{2+} release from permeabilized cells

Trypsinized cells (5×10^6/ml) were incubated at 37 °C within a stirred cuvette in a buffer containing 140 mM KCl, 10 mM NaCl, 10 mM Na-Hepes, 1.5 mM $MgCl_2$, 1.5 mM ATP, 5 μM rotenone, 7.5 mM phosphocreatine, 7.5 units/ml of creatine phosphokinase (pH 7.4) and 3 μM fluo-3 free acid. Excess divalent cations were removed from the buffer prior to addition of Mg-ATP by treatment with Chelex-100 resin. Before permeabilization, Ca^{2+} free in the medium was decreased by multiple additions of 2.5–5 μM EGTA. Permeabilization was obtained with 200 μg/ml digitonin. Calibration was performed by back-tritation of the Ca^{2+} traces with pulses of 2 μM EGTA. Fluorescence was measured at excitation and emission wavelengths set at 490 and 525 nm, respectively, with a Jasco spectrofluorimeter FP-770 (Tokyo, Japan).

Measurement of total inositol phosphates (IPn) production

IPn was determined exactly as described in [17]. IPn production in permeabilized cells was measured as described in [18].

Measurement of phosphatidic acid and diacylglycerol formation

Cells (3×10^5) were seeded in 36 mm multiwells (Nunc, Roskilde, Denmark). At confluence, cells were incubated for 18 h with DMEM supplemented with 10 % fetal calf serum and containing 1 μCi/ml [1-^{14}C]palmitic acid. The medium was aspirated and cells were rinsed and stimulated for 2 min with the indicated drugs, as detailed in Table and Figure legends. For phosphatidic acid determination, cells were then rinsed and lipids extracted with 2 ml of ice-cold methanol/HCl (100:1 by volume). Chloroform (1 ml) was added to the extract and phase separation was accomplished by addition of 1 ml of 1 M NaCl, followed by mixing and centrifugation. Lipids of the lower chloroform phase were separated by TLC on 60A TLC plates (Whatman, Maidstone, Kent, U.K.) developed with a solvent system consisting of the upper phase of a mixture of ethyl acetate 2,2,4-trimethylpentane, acetic acid and water (9:5:2:10, by vol.), to which 1 ml of acetic acid was added, as reported in [19]. Areas containing phosphatidic acid ($R_f = 0.15$), identified by co-chromatographed standards and visualized by iodine staining, were scraped, along with the remainder of each lane, and counted by liquid scintillation.

For diacylglycerol (DAG) determination, incubations were stopped with 2 ml of methanol and 2 ml of chloroform and lipids were extracted according to [20]. The DAG fraction was separated by TLC on silica-gel G plates (Merck, Darmstadt, Germany) using hexane/diethyl ether/formic acid (80:20:1.5, by vol.) as the solvent system. Spots were visualized under UV light by spraying with 2′7′-dichlorofluorescein (10 % in ethanol) and the DAG spots, identified by comparison with co-chromatographed standards, were scraped off and counted for radioactivity.

The fatty acid composition of phosphatidic acid and DAG was determined from the lipid fractions and the corresponding spots were scraped off, methyl-esterified according to [21] and gas chromatographed on a C. Erba HRCG model 5160 gas chromatograph (Carlo Erba, Milano, Italy) equipped with a capillary column (SP 2340, 0.10–0.15 μm film thickness) at a programmed temperature of 160–210 °C (with a 8 °C/min gradient), as reported previously [22].

Assay for arachidonic acid release

Cells, grown in six-well multiwells, were radiolabelled for 24 h with DMEM containing 10 % fetal calf serum and 0.25 μCi/ml [^{3}H]arachidonic acid, then washed three times with PBS at 37 °C. Radioactivity incorporated in cells was 50–60 % of total label. Cells were incubated for 5 min with different agents in 1 ml of saline solution containing 0.2 % BSA at 37 °C; the solution was aspirated and centrifuged for 20 s at 400 ***g*** in an Eppendorf 5248 centrifuge to remove floating cells, and aliquots of the resulting supernatants were counted for radioactivity.

Statistical analysis

Data are expressed as means $\pm$ S.D. Unless otherwise indicated, statistical analysis was performed using the Student's *t* test.

RESULTS

$[Ca^{2+}]_i$ mobilization and inositol phosphates production

Figure 1(A) shows a representative trace of $[Ca^{2+}]_i$ elevation induced by addition of 2 μM SPC to human airway epithelial cells incubated in Ca^{2+}-free solution containing 0.1 mM EGTA. $[Ca^{2+}]_i$ increased from the resting value of 88 ± 22 nM ($n = 14$) to 390 ± 54 nM ($n = 6$) after addition of SPC. Figure 1(B) shows $[Ca^{2+}]_i$ elevation induced by 0.1 μM bradykinin for comparison (peak value of 470 ± 67 nM, $n = 6$), which has been reported previously to activate PLC in airway epithelial cells, leading to IP_3 production and release of Ca^{2+} from internal stores [23]. It is

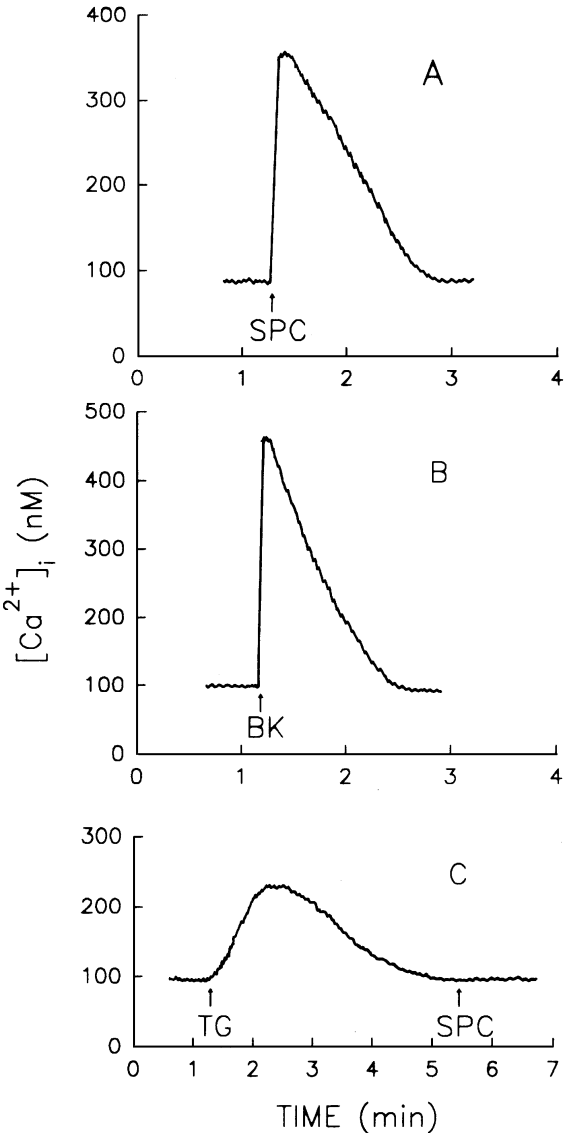

Figure 1 Effect of SPC, bradykinin and thapsigargin on $[Ca^{2+}]_i$ mobilization in human airway epithelial cells

Fura-2/AM-loaded cells were incubated in Ca^{2+}-free saline solution containing 0.1 mM EGTA. At the arrows: 2 μM SPC, 0. 1 μM bradykinin (BK) and 50 nM thapsigargin (TG) were added. Results are representative of 4–6 highly consistent experiments.

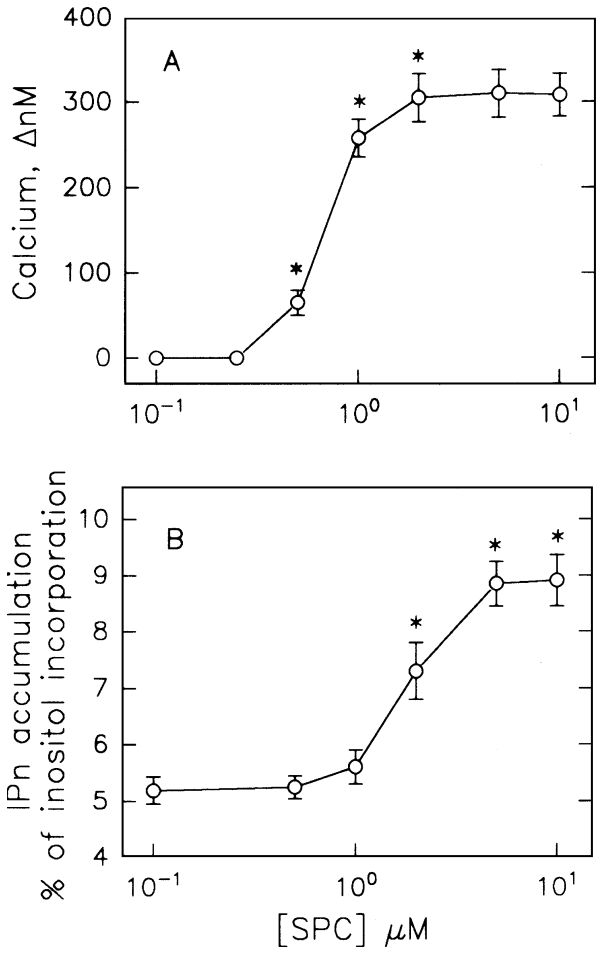

Figure 2 Dose response for SPC-induced $[Ca^{2+}]_i$ elevation and IPn production in human airway epithelial cells

(**A**) Data are expressed as absolute changes in $[Ca^{2+}]_i$ (Δ nM) over the resting value of 88 ± 22 nM, $n = 15$. Each data point is the mean $\pm$ S.D. from 3–6 independent experiments. (**B**) IPn production was determined after 10 min stimulation, as described in [17]. The radioactivity of the IPn fractions is expressed as percentage of total [2-^{3}H]*myo*-inositol incorporation, in terms of which no significant difference between basal and stimulated conditions was observed. Results are shown as mean $\pm$ S.D. for 3–5 determinations. IPn accumulation in control cells was 5.00 ± 0.25% ($n = 7$) of total inositol incorporation. In (**A**) and (**B**), *, significantly different from control ($P < 0.01$).

apparent that these two compounds showed a rather similar ability to increase $[Ca^{2+}]_i$. The transient elevation in $[Ca^{2+}]_i$ induced by SPC was the consequence of release from intracellular stores, since no Ca^{2+} was present in the extracellular medium. This finding is confirmed by the results illustrated in Figure 1(C), showing that pretreatment with thapsigargin, an irreversible inhibitor of endoplasmic reticulum Ca^{2+}-ATPase [24], completely abolished the Ca^{2+} response by SPC. Figure 2(A) reports the concentration dependence of SPC's effect on $[Ca^{2+}]_i$ elevation, showing that SPC increased $[Ca^{2+}]_i$ at concentrations of 0.5–1 μM and that the maximal effect was observed above 2 μM SPC.

It is well known that release of Ca^{2+} from internal stores is generally triggered by IP_3 generated by receptor-mediated activation of PLC [25]. We have therefore investigated whether SPC-induced $[Ca^{2+}]_i$ mobilization was dependent on PLC stimulation. As reported in Figure 2(B), addition of SPC to CFNP9o$^-$ cells induced a dose-dependent increase in the level of IPn, which was negligible at concentrations up to 1 μM, significant at 2 μM and maximal at 5 μM (1.72-fold over control). Incubation with 0.1 μM bradykinin, which mobilizes $[Ca^{2+}]_i$ in a way similar to SPC, increased IPn accumulation to a significantly higher extent (11.32 ± 0.3 %, $n = 4$, over total inositol incorporation, i.e. a 2.19-fold increase over control). These results indicate that SPC was indeed able to stimulate PLC and therefore $[Ca^{2+}]_i$ mobilization might be dependent on generation of inositol phosphates. However, the two processes exhibit slightly different dose responses.

In many cell types, receptor-mediated phosphoinositide hydrolysis is blocked by a brief treatment with PMA, which is known to activate protein kinase C (PKC) [26]. Figure 3(A) shows that a 2 min preincubation with 100 nM PMA significantly reduced (54 ± 11 %, $n = 4$) IPn production induced by 5 μM SPC, whereas incubation with the inactive phorbol ester ana-

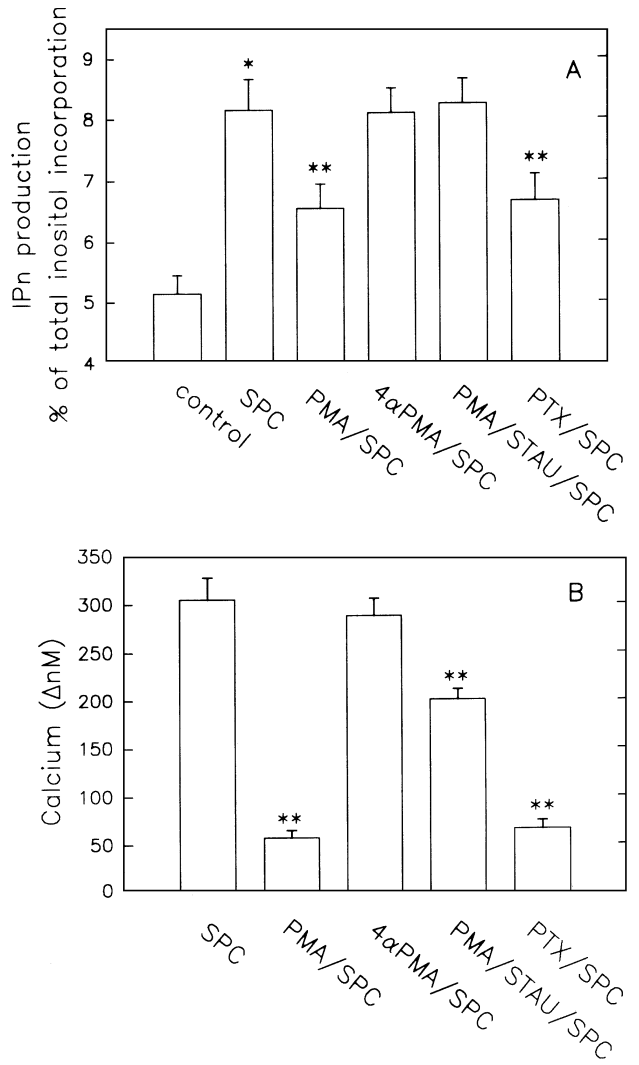

Figure 3 Effect of PMA, 4α-PMA, staurosporine and pertussis toxin on SPC-induced IPn accumulation (A) and Ca^{2+} mobilization (B)

Cells were stimulated in the absence or presence of 5 μM SPC for 10 min. Where indicated, 100 nM PMA and 100 nM 4α-PMA were added 2 min before SPC; 40 nM staurosporine (STAU) was added after PMA, followed after 5 min by SPC. Pertussis toxin (PTX; 400 ng/ml) was preincubated for 4 h during loading with [2-^{3}H]*myo*-inositol before stimulation with SPC. (**A**) Values are means ± S.D. of 3–6 determinations. **, Significantly different from control ($P < 0.01$); *, highly significantly different from control ($P < 0.001$). In (**B**), data are expressed as absolute changes in $[Ca^{2+}]_i$ (Δ nM) over the resting value of 88 ± 32 nM, $n = 18$, and are the mean ± S.D. from 3–6 independent experiments. **, Significantly different from SPC alone ($P < 0.01$).

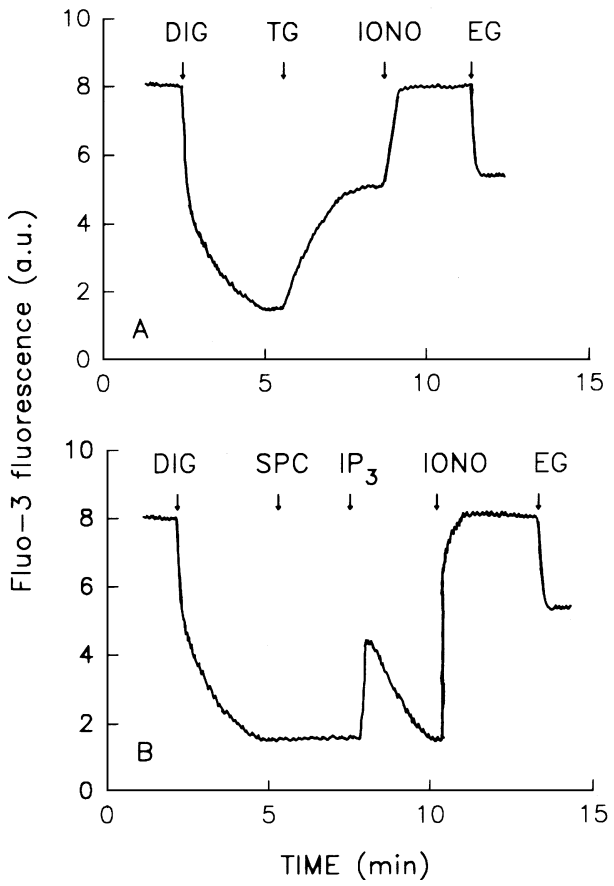

Figure 4 SPC fails to evoke Ca^{2+} release from permeabilized cells

Cells were incubated in the permeabilization medium containing 3 μM fluo-3, as described in the Experimental section. Where indicated: 200 μg/ml digitonin (DIG), 2 μM thapsigargin (TG), 2 μM ionomycin (IONO), 2 μM EGTA (EG), 20 μM SPC and 2 μM IP_3 were added. Traces are representative of 3–6 highly consistent experiments.

logue, 4α-PMA (100 nM), which does not stimulate PKC, had no effect. The addition of 40 nM staurosporine, an inhibitor of PKC, after a 2 min incubation with PMA, completely restored the SPC-induced IPn response (Figure 3A). Figure 3(B) shows the effect of the same compounds on $[Ca^{2+}]_i$ mobilization: 4α-PMA was ineffective, whereas 100 nM PMA significantly inhibited (79 ± 9 %, $n = 7$) SPC-induced $[Ca^{2+}]_i$ elevation. The effect of PMA was only partially restored by the subsequent addition of staurosporine. Calphostin C, another inhibitor of PKC, at a concentration of 100 nM gave similar results (not shown). Finally, pretreatment with 400 ng/ml of pertussis toxin, which causes ADP-ribosylation of the α subunit of certain G proteins, inhibited both the stimulation of IPn accumulation and $[Ca^{2+}]_i$ elevation induced by SPC by 49 ± 11 %, $n = 4$, and 82 ± 5 %, $n = 6$, respectively (Figure 3). These data suggest that $[Ca^{2+}]_i$ mobilization by SPC is mediated by an unknown membrane receptor, functionally coupled through a G_i type of G protein to PLC and under control of PKC. However, the fact that Ca^{2+} mobilization was more effectively reduced by PMA and pertussis toxin than IPn production, together with the difference reported in the dose responses for SPC, suggest that another mechanism might contribute to $[Ca^{2+}]_i$ elevation.

Ca^{2+} release from permeabilized cells

SPC has been reported to release Ca^{2+} from internal stores of various cell lines by directly interacting with Ca^{2+} channels within the endoplasmic reticulum [4,6,7]. It is therefore possible that Ca^{2+} mobilization by SPC in CFNP9o$^-$ cells might be due to an intracellular effect of this compound. To test this hypothesis, experiments with digitonin-permeabilized cells have been performed. Addition of digitonin to cells incubated in the permeabilization medium promoted a large decrease of fluo-3 fluorescence, due to Ca^{2+} uptake into the endoplasmic reticulum. In fact, the accumulated Ca^{2+} was largely released by addition of 2 μM thapsigargin, as illustrated in Figure 4(A). The subsequent addition of the Ca^{2+} ionophore ionomycin allowed release of further Ca^{2+} stored, probably from other intracellular organelles.

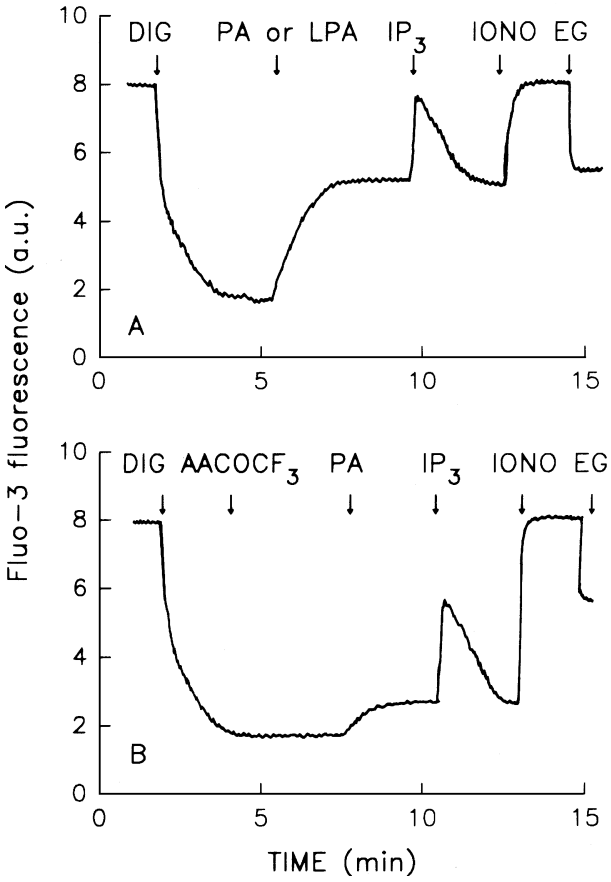

Figure 5 Lysophosphatidic acid releases Ca^{2+} from permeabilized cells

Experimental conditions as in Figure 4. Where indicated: 200 μg/ml digitonin (DIG), 10 μM $AACOCF_3$, 10 μM phosphatidic acid (PA), 10 μM lysophosphatidic acid (LPA), 2 μM IP_3, 2 μM ionomycin (IONO) and 2 μM EGTA (EG) were added. Traces are representative of 3–6 highly consistent experiments.

Addition of 20 μM SPC failed to induce Ca^{2+} mobilization, whereas the subsequent addition of 2 μM IP_3 evoked a rapid release of Ca^{2+}, which returned quickly to its initial level (Figure 4B). These results indicate clearly that SPC is unable to directly gate a Ca^{2+} channel within the membrane of internal stores in CFNP9o$^-$ cells.

In permeabilized Jurkat leukaemia cells, it has been recently reported that both phosphatidic acid and its metabolite lysophosphatidic acid were able to release Ca^{2+} from an intracellular store, which was distinct from that sensitive to IP_3, suggesting that different kinds of Ca^{2+} stores exist in Jurkat cells [14,15]. Figure 5(A) shows that also in permeabilized human airway epithelial cells the addition of phosphatidic acid (10 μM) caused a significant Ca^{2+} release; this is superimposed on that obtained after addition of 10 μM lysophosphatidic acid. Ca^{2+} was released by these two compounds more slowly than by IP_3 and never returned to the initial value, with a behaviour similar to that of thapsigargin (see Figure 4A). Since lysophosphatidic acid can be generated by phosphatidic acid via activation of phospholipase A_2 (PLA_2), the effect of the inhibitor of PLA_2 activity, $AACOCF_3$ [27], was determined. Addition of 10 μM $AACOCF_3$ alone did not influence Ca^{2+} steady-state, but strongly attenuated the effect of phosphatidic acid on Ca^{2+} release (Figure 5B). This result suggests that generation of lysophosphatidic acid contributes to

Table 1 Phosphatidic acid formation in human airway epithelial cells

Cells, loaded with [1-^{14}C]palmitic acid for 18 h as described in the Experimental section, were stimulated for 2 min in the absence or presence of 5 μM SPC or 100 nM PMA. $AACOCF_3$ (10 μM), 20 μM R59022 and 400 ng/ml pertussis toxin were preincubated for 5 min, 10 min and 4 h, respectively, before stimulation. Numbers of experiments (n) are shown in parentheses. *, Significantly different from control, $P < 0.001$.

	Phosphatidic acid formation (c.p.m.)	
Additions	Control	SPC (5 μM)
None	535 ± 51 (6)	612 ± 64 (7)
$AACOCF_3$	497 ± 51 (3)	1071 ± 89 (6)*
R59022	535 ± 25 (3)	561 ± 38 (4)
$AACOCF_3$ + R59022	446 ± 38 (3)	535 ± 25 (3)
Pertussis toxin	535 ± 25 (3)	548 ± 25 (3)
Pertussis toxin + $AACOCF_3$	446 ± 38 (3)	586 ± 25 (3)
PMA	1096 ± 76 (3)*	–

SPC-induced Ca^{2+} mobilization from internal stores in this cell line. Addition of arachidonic acid (10 μM), the other product of PLA_2 activity, did not cause any significant release of Ca^{2+} from permeabilized cells (results not shown).

To verify whether Ca^{2+} release from permeabilized cells might result from IPn formation due to direct stimulation of PLC activity by intracellular lysophosphatidic acid, CFNP9o$^-$ cells were prelabelled with [^{3}H]inositol for 24 h, washed and permeabilized as described in Figures 4 and 5. Aliquots of permeabilized cells were incubated for 5 min at 37 °C with 10 μM GTP, to monitor the extent of G protein-stimulated PLC activity, or with 10 μM lysophosphatidic acid, following the protocol described in [18]. IPn production was similar in control and lysophosphatidic acid-treated cells (4560 ± 220 c.p.m., $n = 3$, and 4478 ± 302 c.p.m., $n = 3$, respectively), whereas a two-fold increase was induced by incubation with GTP (8936 ± 478 c.p.m., $n = 3$).

Formation of phosphatidic acid and diacylglycerol, and release of arachidonic acid

In Table 1 it is reported that stimulation of cells with 5 μM SPC alone failed to change the phosphatidic acid level, whereas preincubation with 10 μM $AACOCF_3$ increased phosphatidic acid formation by SPC two-fold. This finding indicates that SPC could cause production of phosphatidic acid, provided that PLA_2 activity is blocked. This suggests that phosphatidic acid might be hydrolysed subsequently by PLA_2 to lysophosphatidic acid and arachidonic acid. One of the major routes of phosphatidic acid generation is from DAG via activation of DAG kinase [28]. To examine the involvement of DAG kinase, cells were preincubated with the inhibitor of this enzyme, R59022 (20 μM) [29]. R59022 abolished the increase in phosphatidic acid level caused by $AACOCF_3$ + SPC. In Table 1 it is also shown that preincubation with pertussis toxin blocked phosphatidic acid formation triggered by SPC + $AACOCF_3$. Another pathway for phosphatidic acid formation involves phosphatidylcholine hydrolysis by phospholipase D (PLD), which is well known to be regulated by PKC in most mammalian cells [30]. Table 1 reports that 100 nM PMA stimulated phosphatidic acid formation as much as $AACOCF_3$ + SPC.

It is well known that polyunsaturated phosphatidates are generated by inositol phospholipid hydrolysis, whereas saturated or monounsaturated phosphatidates are the product of PLD

Table 2 Phosphatidic acid fatty acid composition of unstimulated cells (control) or cells stimulated with 5 μM SPC + 10 μM AACOCF$_3$ or with 100 nM PMA

The fatty acid analysis (as methyl esters) was performed in the conditions reported in the Experimental section. Values are means ± S.D. of 4 determinations. Statistical analysis was performed by one-way analysis of variance (*, at least $P < 0.05$ in comparison with control cells).

	Phosphatidic acid fatty acid composition (mol/100 mol)		
Fatty acid	Control	SPC + AACOCF$_3$	PMA
16:0	24.64 ± 1.18	22.32 ± 1.03*	25.41 ± 1.06
16:1	1.78 ± 0.64	1.06 ± 0.76	1.26 ± 0.41
18:0	23.69 ± 1.03	26.28 ± 0.95*	24.12 ± 1.38
18:1	19.50 ± 1.12	16.31 ± 0.99*	23.82 ± 1.02*
18:2 n-6	14.49 ± 0.98	15.24 ± 0.76	15.17 ± 0.83
20:4 n-6	7.47 ± 1.01	12.86 ± 0.84*	5.84 ± 0.64*
20:5 n-3	1.37 ± 0.36	1.21 ± 0.35	1.03 ± 0.23
22:4 n-6	2.60 ± 0.18	2.12 ± 0.41	1.03 ± 0.23*
22:5 n-6	1.21 ± 0.57	0.71 ± 0.42	1.18 ± 0.31
22:5 n-3	0.16 ± 0.08	0.12 ± 0.08	0.10 ± 0.05
22:6 n-3	2.16 ± 0.15	1.95 ± 0.21	1.00 ± 0.17*

activation [26]. To assess the source of phosphatidic acid generated by SPC in the presence of AACOCF$_3$, alteration of the fatty acid content of the purified lipid fraction was determined. A significant decrease in palmitic and oleic acids' relative molar content and a relevant increase in stearic and arachidonic acids' relative molar content were detected (Table 2). Conversely, in PMA-stimulated cells, an increase in the relative molar content of oleic acid and a decrease in that of arachidonic and docosatetraenoic acids, the direct elongation products of arachidonic acid, were observed (Table 2).

Stimulation of PLC by SPC caused a significant elevation of DAG level (2.2-fold over control), which was further increased (2.95-fold over control) by preincubation with R59022 (Table 3). This finding confirms that a significant amount of DAG produced by PLC is further phosphorylated by a DAG kinase to phosphatidic acid. Short-term preincubation with PMA and long-term treatment with pertussis toxin significantly reduced DAG formation. As shown for IPn production, addition of staurosporine after a short-term preincubation with PMA, almost completely restored DAG production (Table 3). The fatty acid composition of DAG derived from control and SPC-stimulated cells is reported in Table 4. SPC stimulation caused a decrease in the relative molar content of palmitic and oleic acids and an increase in the relative molar content of stearic and arachidonic acids.

Table 3 DAG formation in human airway epithelial cells

Cells, loaded with [1-^{14}C]palmitic acid for 18 h as described in the Experimental section, were stimulated for 2 min in the absence or presence of 5 μM SPC. R59022, PMA and pertussis toxin were preincubated for 5 min, 2 min and 4 h, respectively, before stimulation. Data are from 4 determinations.

	DAG formation (c.p.m.)	
Additions	Control	SPC (5 μM)
None	1280 ± 235	2893 ± 179
R59022 (20 μM)	1297 ± 238	3776 ± 115
PMA (100 nM)	1276 ± 234	1946 ± 64
PMA + staurosporine (40 nM)	1281 ± 233	2688 ± 38
Pertussis toxin (400 ng/ml)	1294 ± 240	1702 ± 51

Table 4 DAG fatty acid composition of unstimulated cells (control) and of cells stimulated with 5 μM SPC

The fatty acid analysis (as methyl esters) was performed in the conditions reported in the Experimental section. Values are means ± S.D. of 4 determinations. Statistical analysis was performed by one-way analysis of variance (*, at least $P < 0.05$ in comparison with control cells).

	DAG fatty acid composition (mol/100 mol)	
Fatty acid	Control	SPC (5 μM)
16:0	26.55 ± 1.15	22.50 ± 1.24*
16:1	2.68 ± 1.06	2.51 ± 0.73
18:0	22.71 ± 2.17	26.86 ± 1.03*
18:1	14.90 ± 1.88	11.51 ± 0.55*
18:2 n-6	10.34 ± 0.85	11.06 ± 0.59
20:4 n-6	13.42 ± 1.02	17.89 ± 1.07*
20:5 n-3	2.82 ± 0.48	2.65 ± 0.85
22:4 n-6	2.00 ± 0.92	1.12 ± 0.83
22:5 n-6	2.52 ± 1.08	1.76 ± 1.00
22:5 n-3	0.02 ± 0.01	0.05 ± 0.02
22:6 n-3	2.02 ± 0.01	2.07 ± 0.02

Table 5 Effect of SPC and PMA + ionomycin on [^{3}H]arachidonic acid release by human airway epithelial cells

Cells, loaded with [^{3}H]arachidonic acid for 24 h as described in the Experimental section, were incubated in the presence of the indicated stimuli for 5 min. Results represent means ± S.D., with n in parentheses. *, Significantly different from control ($P < 0.001$).

Additions	[^{3}H]Arachidonic acid released (c.p.m.)	Activity (%)
None	1863 ± 117 (8)	100
SPC (5 μM)	3446 ± 225 (8)*	185
AACOCF$_3$ (10 μM)	1791 ± 151 (3)	96
AACOCF$_3$ (10 μM) + SPC (5 μM)	1890 ± 168 (3)	101
PMA (100 nM) + ionomycin (1 μM)	4602 ± 151 (4)*	247

Desai et al. reported previously that in 3T3 fibroblasts SPC caused the production of arachidonic acid, probably due to stimulation of PLA$_2$ activity [31]. Table 5 reports that addition of SPC to CFNP9o$^-$ cells stimulated arachidonic acid release of 80% in comparison with the control, this effect being abolished by preincubation with AACOCF$_3$. Activation of PKC and $[Ca^{2+}]_i$ elevation are the major mechanisms regulating 85 kDa cytosolic PLA$_2$, which is thought to play a central role in the release of arachidonic acid triggered by hormones and growth factors [32]. Accordingly, PMA + ionomycin are generally used to obtain the maximal activation of PLA$_2$ activity. PMA (100 nM) + ionomycin (1 μM) stimulated arachidonic acid release of 147% over the control, which is approximately twice the SPC-induced stimulation.

Figure 6(A) shows that pretreatment with R59022 significantly reduced (51 ± 7%, $n = 5$) $[Ca^{2+}]_i$ elevation evoked by SPC, the maximal effect being observed at 20 μM R59022 (Figure 6B). It seems therefore that at least a portion of $[Ca^{2+}]_i$ elevation might derive from a DAG kinase-dependent step. AACOCF$_3$ also inhibited $[Ca^{2+}]_i$ elevation by SPC; however the extent of inhibition could not be evaluated, since the PLA$_2$ inhibitor caused a slow leakage of fura-2 from cells (result not shown).

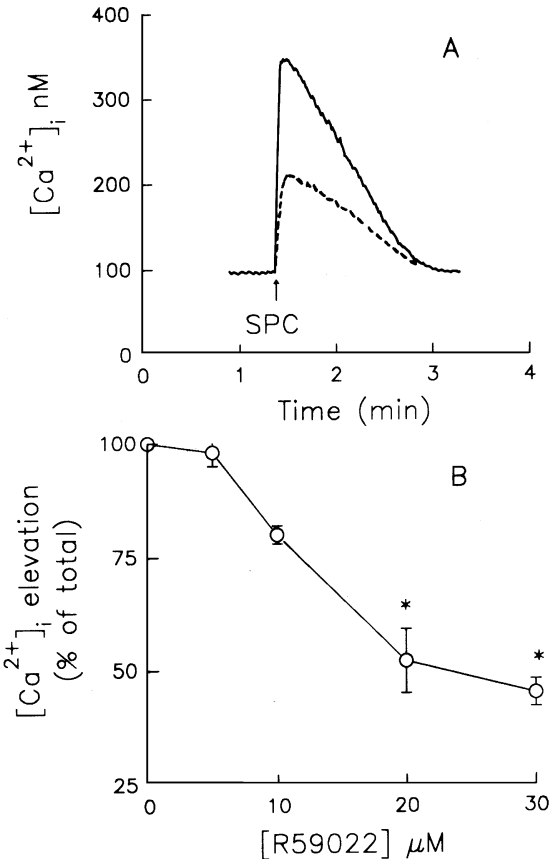

Figure 6 Effect of the DAG kinase inhibitor R59022 on Ca^{2+} mobilization by SPC

Fura-2-loaded cells were incubated in Ca^{2+}-free saline solution containing 0.1 mM EGTA. (**A**) Cells were either stimulated with 2 μM SPC (solid line) or preincubated with 20 μM R59022 for 10 min, after which 2 μM SPC was added (dashed line). (**B**) Dose response for R59022 inhibition of maximal $[Ca^{2+}]_i$ elevation induced by SPC. The 100% value was the absolute change of $[Ca^{2+}]_i$ elevation caused by SPC alone (323 ± 47 Δ nM, $n = 4$). Each data point is the mean $\pm$ S.D. of 4 determinations.

DISCUSSION

In the present paper we report that exogenous SPC induced a remarkable $[Ca^{2+}]_i$ elevation in CFNP9o$^-$ cells which was, at least in part, associated with IPn formation by PLC. Short-term preincubation with PMA significantly inhibited the effects of SPC in CFNP9o$^-$ cells, suggesting a regulatory role for PKC. This is confirmed by the lack of effect of the inactive PMA analogue 4α-PMA and, furthermore, by the efficacy of the PKC inhibitors staurosporine and calphostin to reverse PMA inhibition. Taken together, these data indicate that both IPn formation and $[Ca^{2+}]_i$ mobilization by SPC are under control of PKC. It is well known that PMA causes desensitization of many receptors coupled to phosphoinositide hydrolysis. The desensitization may be associated with phosphorylation of the receptor itself, as shown for α_1 adrenergic receptor in cultured smooth-muscle cells [33], or of the phosphoinositide-specific PLC [34]. Although it is not possible to define the molecular target of PKC action, this result suggests the existence of a receptor-mediated mechanism involved in SPC-induced response. This suggestion is strongly supported by the finding that both IPn production and $[Ca^{2+}]_i$ mobilization were significantly reduced by pertussis toxin treatment, indicating the involvement of a G_i type of G protein. Our data are consistent with those reported previously in HL60 cells by Okajama and Kondo [11], who showed that SPC-induced $[Ca^{2+}]_i$ elevation was associated with PLC activation and mediated by a pertussis toxin-sensitive G protein. However, in this latter study, a concentration of 30 μM SPC was required to obtain the maximal IPn production, which was rather small (only 40% increase over control) and, furthermore, concentrations as low as those used in the present study were poorly effective. Conversely, our results are in disagreement from those reported in 3T3 fibroblasts [8] and HL60 cells [10], where it was shown that SPC evoked $[Ca^{2+}]_i$ mobilization without increasing the levels of inositol phosphates. The reason for this difference is unknown, but it may be due to differences in the cell types, which may either express different amounts of receptors or distinct subtypes of receptors with different affinities for the lyso compound or coupled to distinct G proteins.

In this respect, Bünemann et al. [35] first proposed a pharmacological classification of the putative sphingolipid receptor family into three classes based on the selectivity toward SPC and S-1-P. According to this classification, the sphingolipid receptor of CFNP9o$^-$ cells would belong to the class that is characterized by a comparable sensitivity to both SPC and S-1-P, although the two compounds are effective at different concentrations and operate through different signal-transduction mechanisms [36]. Recently, two closely related G protein-coupled receptors, rat H218 and human edg-3, have been indicated as functional receptors for S-1-P and SPC. Overexpression of these proteins in Jurkat cells activated serum-response-elements-driven transcriptional reporter gene in response to both S-1-P and SPC [37]. Edg-1, another related G protein-coupled receptor, has been shown to be the receptor for S-1-P [38,39]. Studies are in progress to detect the presence of edg-3/edg-1 messengers in CFNP9o$^-$ cells.

It is noteworthy that the dose responses reported in the present study for Ca^{2+} elevation and IPn production (Figure 2) are slightly different, maximal $[Ca^{2+}]_i$ elevation being observed at SPC concentrations lower than that required for IPn accumulation. Furthermore, stimulation of IPn production by SPC was significantly weaker than that caused in the same cell line by other agonists, such as bradykinin or histamine [17]. Lastly, PMA and pertussis toxin only partially reduced IPn accumulation but almost completely inhibited $[Ca^{2+}]_i$ mobilization by SPC. A possible explanation for this finding could be that, in addition to its receptor-mediated effect, SPC might permeate the plasma membrane and directly release Ca^{2+} from intracellular stores. Exogenous SPC is readily taken up by Swiss 3T3 fibroblasts and metabolized relatively slowly after uptake [31]. However, we observed that SPC failed to evoke any significant Ca^{2+} release from permeabilized CFNP9o$^-$ cells, which allowed direct access to the Ca^{2+} release sites of intracellular stores. This is in contrast with the data reported in [6,7], where SPC was shown to induce release of Ca^{2+} from IP_3-insensitive stores in permeabilized cells. In this respect, it has to be noticed that in rat pancreatic acinar cells, SPC was effective at a concentration of 5 μM [40], whereas much higher concentrations (40–60 μM) were required in leukaemia [6] and endothelial [41] cells. We cannot rule out the possibility that SPC might also be effective in airway cells at these very high concentrations; however, we believe that the physiological relevance of this effect remains questionable. Also, the other sphingosine derivative S-1-P was unable to directly activate Ca^{2+} release in permeabilized cells (S. Orlati and M. Rugolo, unpublished work). The reason for the lack of effect of SPC and S-1-P on Ca^{2+} release from permeabilized cells is unknown, but it may be related to the different origins of the various cell lines

that may or may not express the sphingolipid-gated Ca^{2+} channel in the endoplasmic reticulum.

It is likely therefore that another molecular mechanism should operate in addition to IPn production, leading to generation of a different intracellular metabolite also able to release Ca^{2+} from internal stores. We observed that both phosphatidic and lysophosphatidic acid were able to release Ca^{2+} from internal stores in permeabilized airway epithelial cells. This result is in agreement with data reported previously in Jurkat T cells, where the existence of a phosphatidic acid/lysophosphatidic acid-sensitive intracellular Ca^{2+} store has been reported [14,15]. In the present study we were able to show that the effect of phosphatidic acid was strongly reduced by $AACOCF_3$, and this clearly indicates lysophosphatidic acid as the effective metabolite. In this connection, it was important to exclude the possibility that lysophosphatidic acid might release Ca^{2+} from internal stores by increasing IPn formation through direct activation of PLC in permeabilized cells. This possibility could be ruled out by the lack of stimulation of IPn production by lysophosphatidic acid in permeabilized cells.

It is noteworthy that the level of phosphatidic acid in CFNP9o$^-$ cells was increased by SPC through the sequential action of PLC and DAG kinase only when the PLA_2 activity was inhibited. Therefore, it can be proposed that, after SPC stimulation, PLC-derived DAG is phosphorylated by DAG kinase to phosphatidic acid, which is further hydrolysed by PLA_2 activity to lysophosphatidic acid and arachidonic acid. The occurrence of this pathway is supported by the finding that phosphatidic acid generation was inhibited by pertussis toxin, and furthermore by the analysis of the fatty acid composition of phosphatidic acid, which clearly resembles DAG composition, indicating the involvement of DAG kinase. This result also rules out the possible role for PLD in SPC-induced phosphatidic acid generation. It is of interest that the phosphatidic acid composition of PMA-stimulated cells, in which PLD is stimulated, is completely different, the 1-palmitoyl (or stearoyl) and 2-oleoyl species being the most representative species, in agreement with data reported previously [26,42].

The observation that R59022, which blocks phosphatidic acid formation, also significantly reduced $[Ca^{2+}]_i$ elevation caused by SPC, further suggests a crucial role for DAG kinase on generation of an intracellular messenger able to release Ca^{2+} from internal stores. This is in accordance with the widely accepted notion that DAG kinase has two important functions: first, to limit cellular level of DAG, and second, to generate additional second messengers. Recently, a number of DAG kinase isoforms have been detected and shown to differ from each other with respect to molecular masses, enzymic properties, activators, substrate specificity and tissue- and cell-specific distribution [43,44]. In Jurkat T cells, the presence of four different isoforms of DAG kinase have been reported, of which two in the cytosol are activated by sphingosine [45]. Thus, the effect of sphingosine and/or other sphingolipid metabolites, such as SPC, could be different depending on the type of DAG kinase isozyme present in a particular cell line.

Stimulation of arachidonic acid release caused by SPC and its inhibition by $AACOCF_3$ provide more evidence for the involvement of PLA_2. This is consistent with data reported previously in 3T3 fibroblasts, although in these cells SPC was effective at higher concentrations and longer times [8]. Arachidonic acid, however, was not able to directly release Ca^{2+} from internal stores, as indicated by experiments on permeabilized CFNP9o$^-$ cells. Although the significance of the arachidonic acid release during SPC stimulation is not clear, the increase in $[Ca^{2+}]_i$ as well as DAG and arachidonic acid levels might potentiate PKC activation and contribute to signal transduction through the PKC pathway [26].

Although arachidonic acid could be produced from DAG by the action of DAG lipase, it was shown that it is derived mainly from phospholipids by the activation of PLA_2 [26]. We could not present direct evidence that arachidonic acid released by PLA_2 after stimulation with SPC does indeed derive from phosphatidic acid generated by DAG kinase. However, we showed that the levels of polyunsaturated DAG and phosphatidic acid species increased after SPC stimulation, as reported by others [25,46], and this suggests that it is the polyunsaturated DAG that is converted predominantly to phosphatidic acid by some isoforms of DAG kinase [44,47]. Since the pertussis toxin-sensitive phosphatidic acid production by SPC in CFNP9o$^-$ cells was revealed only in the presence of $AACOCF_3$, this suggests that arachidonic acid might be released from DAG kinase-generated phosphatidic acid, producing lysophosphatidic acid, which seems therefore to be the intracellular messenger able to release Ca^{2+} from internal stores. Lysophosphatidic acid has been widely characterized as a lipid mediator involved in many biological activities, including cell proliferation, platelet aggregation, neurite retraction and cell motility [48]. Here we propose that lysophosphatidic acid belongs to a novel class of lipid mediators that act both extracellularly, as ligands for cell-surface receptors, and intracellularly, as second messengers.

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REFERENCES

1 Spiegel, S. and Milstien, S. (1995) J. Membr. Biol. **146**, 225–237
2 Olivera, A. and Spiegel, S. (1993) Nature (London) **365**, 557–560
3 Gosh, T. K., Bian, J. and Gill, D. L. (1990) Science **248**, 1653–1656
4 Gosh, T. K., Bian, J. and Gill, D. L. (1995) J. Biol. Chem. **269**, 22628–22635
5 Mattie, M., Brooker, G. and Spiegel, S. (1994) J. Biol. Chem. **269**, 3181–3188
6 Kindman, L., Kim, S., McDonal, T. and Gardner, P. (1994) J. Biol. Chem. **269**, 13088–13091
7 Mao, C., Kim, S. H., Almenoff, J. S., Rudner, X. L., Kearney, D. M. and Kindman, L. A. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 1993–1996
8 Desai, N. N., Carlson, R. O., Mattie, M. E., Olivera, A., Buckley, N. E., Seki, T., Brooker, G. and Spiegel, S. (1993) J. Cell Biol. **121**, 1385–1395
9 Torquist, K. and Ekokoski, E. (1994) Biochem. J. **299**, 213–218
10 Van Koppen, C. J., Heringdorf, D. M., Zhang, C., Laser, K. T. and Jacobs, K. H. (1996) Mol. Pharmacol. **49**, 956–961
11 Okajama, F. and Kondo, Y. (1995) J. Biol. Chem. **270**, 26332–26340
12 Van Koppen, C. J., Heringdorf, D. M., Laser, K. T., Zhang, C., Jacobs, K. H., Bünemann, M. and Pott, L. (1996) J. Biol. Chem. **271**, 2082–2087
13 Cozens, A. L., Yezzi, M. J., Chin, L., Simon, E. L. M., Finkbeiner, W. E., Wagner, J. A. and Gruenert, D. C. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 5171–5172
14 Sakano, S., Takemura, H., Yamada, K., Imoto, K. and Ohshika, H. (1996) J. Biol. Chem. **271**, 11148–11155
15 Takemura, H., Imoto, K., Sakano, S., Kaneko, M. and Ohshika, H. (1996) Biochem. J. **319**, 393–397
16 Rugolo, M., Mastrocola, T., Wöhrle, C. W., Rasola, A., Gruenert, D. C., Romeo, G. and Galietta, L. J. V. (1993) J. Biol. Chem. **268**, 24779–24784
17 Rugolo, M., Barzanti, F., Gruenert, D. C. and Hrelia, S. (1996) Am. J. Physiol. **271**, L665–L671
18 Cockcroft, S., Thomas, G. M. H., Cunningham, E. and Ball, A. (1994) Methods Enzymol. **238**, 154–168
19 Ella, K. M., Meier, G. P., Bradshaw, C. D., Huffman, K. M., Spivey, E. C. and Meier, K. E. (1994) Anal. Biochem. **218**, 136–142
20 Folch, J., Lees, M. and Sloane Stanley, G. H. (1957) J. Biol. Chem. **226**, 497–509
21 Stoffel, W., Chu, F. and Ahrens, E. H. (1959) Anal. Chem. **31**, 307–311
22 Bordoni, A., Lopez Jimenez, J. A., Spanò, C., Biagi, P. L., Horrobin, D. F. and Hrelia, S. (1996) Mol. Cell. Biochem. **157**, 217–222
23 Denning, G. M. and Welsh, M. J. (1991) J. Biol. Chem. **266**, 12932–12938

24 Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2466–2470
25 Berridge, M. J. (1994) Nature (London) **361**, 315–325
26 Nishizuka, Y. (1992) Science **258**, 607–615
27 Bartoli, F., Lin, H.-K., Gromashchi, F., Gelb, M. H., Jain, M. K. and Apitz-Castro, M. (1994) J. Biol. Chem. **269**, 15625–15630
28 Kanoh, H., Yamada, K. and Sakane, F. (1990) Trends Biochem. Sci. **15**, 47–50
29 De Chaffoy de Courcelles, D., Roevens, P. and Van Belle, H. (1985) J. Biol. Chem. **260**, 15762–15770
30 Exton, J. H. (1997) J. Biol. Chem. **272**, 15579–15582
31 Desai, N. N., Zhang, H., Olivera, A., Mattie, M. E. and Spiegel, S. (1992) J. Biol. Chem. **267**, 23122–23128
32 Lin, L.-L., Lin, A. Y. and Knopf, J. L. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6147–6151
33 Leeb-Lundberg, L. M. F., Cotecchia, S., De Blasi, A., Caron, M. G. and Lefkowitz, R. J. (1987) J. Biol. Chem. **262**, 3098–3105
34 Murray, R. K., Bennet, C. F., Fluharty, S. J. and Kotlikoff, M. I. (1989) Am. J. Physiol. **257**, L209–L216
35 Bünemann, M., Liliom, K., Brandts, B. K., Pott, L., Tseng, J-L., Desiderio, D. M., Sun, G., Miller, D. and Tagyi, G. (1996) EMBO J. **15**, 5527–5534
36 Orlati, S., Porcelli, A. M., Hrelia, S. and Rugolo, M. (1998) Cell Calcium **23**, 387–394
37 An, S., Bleu, T., Huang, W., Hallmark, O. G., Couglin, S. R. and Goetzl, E. J. (1997) FEBS Lett. **417**, 279–282
38 Lee, M.-J., Van Brocklyn, J. R., Shobha, T., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S. and Hla, T. (1998) Science **279**, 1552–1555
39 Zondag, G. C. M., Postma, F. R., van Etten, I., Verlan, I. and Moolenaar, W. H. (1998) Biochem. J. **330**, 605–609
40 Yule, D. I., Wu, D., Essington, T. E., Sayman, J. A. and Williams, J. A. (1993) J. Biol. Chem. **268**, 12353–12358
41 Kim, S., Lakhani, V., Costa, D. J., Sharara, A. I., Fitz, J. G., Huang, L.-W., Peters, K. and Kindmann, L. A. (1995) J. Biol. Chem. **270**, 5266–5269
42 Petitt, T. R., Martin, A., Horton, T., Liossis, C., Lord, J. M. and Wakelam, M. J. O. (1997) J. Biol. Chem. **272**, 17354–17359
43 Sakane, F., Imai, S., Kai, M., Wada, I. and Kanoh, H. (1996) J. Biol. Chem. **271**, 8394–8401
44 Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M. and Prescott, S. M. (1996) J. Biol. Chem. **271**, 10237–10241
45 Yamada, K., Sakane, F., Imai, S. I. and Takemura, H. (1993) Biochim. Biophys. Acta **1169**, 217–224
46 Pettitt, T. R. and Wakelam, M. J. O. (1993) Biochem. J. **289**, 487–495
47 Hodgkin, M. N., Gardner, S. D., Rose, S., Paterson, A., Martin, A. and Wakelam, M. J. O. (1997) Biochem. J. **322**, 529–534
48 Moolenaar, W. H. (1995) J. Biol. Chem. **270**, 12949–12952

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