Effect of sphingosine derivatives on calcium fluxes in thyroid FRTL-5 cells

Kid TÖRNQUIST* and Elina EKOKOSKI

Department of Zoology, Division of Physiology, University of Helsinki, and the Minerva Foundation Institute for Medical Research, Helsinki, Finland

The effects of sphingosine derivatives on Ca^{2+} fluxes were investigated in thyroid FRTL-5 cells labelled with Fura 2. Addition of sphingosylphosphocholine (SPC) or sphingosine (SP) increased intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) in a dose-dependent manner. At the highest dose tested (30 μM), the response was biphasic: a rapid transient increase in $[\text{Ca}^{2+}]_i$, followed by a new, elevated, level of $[\text{Ca}^{2+}]_i$. Both phases of the SPC-evoked increase in $[\text{Ca}^{2+}]_i$ were dependent on extracellular Ca^{2+} , whereas only the SP-evoked elevated level of $[\text{Ca}^{2+}]_i$ was dependent on the influx of Ca^{2+} . Both compounds released sequestered Ca^{2+} from thapsigargin- and inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} pools. In addition, the increase in $[\text{Ca}^{2+}]_i$ in response to SPC, but not to SP, was attenuated in cells treated with phorbol myristate

acetate or with the putative Ca^{2+} -channel blocker SKF 96365, and in cells pretreated with pertussis toxin for 24 h. SPC did not activate the production of IP_3 . Furthermore, both SPC and SP released sequestered Ca^{2+} from permeabilized cells. We observed that SPC, but not SP, stimulated release of $[\text{H}^3]\text{arachidonate}$ from cells prelabelled with $[\text{H}^3]\text{arachidonate}$ for 24 h. Both SPC and SP stimulated the incorporation of $[\text{H}^3]\text{thymidine}$ into DNA in cells grown in the absence of thyroid-stimulating hormone (TSH). The results suggest that sphingosine derivatives are putative regulators of Ca^{2+} fluxes in FRTL-5 cells, and that SP and SPC may act on $[\text{Ca}^{2+}]_i$ via different mechanisms. Furthermore, both SP and SPC may be of importance in modulating thyroid-cell proliferation.

INTRODUCTION

Hydrophobic lipid-breakdown products, such as diacylglycerol, arachidonic acid and prostaglandins, are potent mediators and modulators of the signal transduction in several cell systems, including thyroid cells [1–3]. Substantial efforts have been made to investigate the mechanisms regulating Ca^{2+} influx as a result of activation of the $\text{PtdIns}(4,5)\text{P}_2/\text{Ins}(1,4,5)\text{P}_3$ pathway, or by direct emptying of intracellular Ca^{2+} pools [4–9]. We have shown that protein kinase C (PKC) has a powerful modulatory effect on Ca^{2+} fluxes in thyroid FRTL-5 cells [9,10]. A metabolite of the membrane lipid sphingomyelin, sphingosine (SP), has previously been shown to be a potent inhibitor of PKC, and it has been suggested that SP may be an endogenous inhibitor of PKC in cells [11,12]. Furthermore, SP and its derivatives have been shown to activate the release of sequestered Ca^{2+} [13–17]. In addition, SPs have been implicated as potent inhibitors of calmodulin-dependent enzymes [18], an interesting observation considering that calmodulin may be of importance in regulating Ca^{2+} fluxes in FRTL-5 cells [19].

SP derivatives are also potent mitogenic agents, and the mitogenicity may be linked to the formation of arachidonic acid [14,15]. Arachidonic acid and its metabolites may be important in the regulation of Ca^{2+} entry in FRTL-5 cells [20], and may be of significance in regulating the proliferation of FRTL-5 cells [21]. These observations prompted us to investigate the possible effects of SP derivatives on Ca^{2+} entry. Our results show that, in thyroid FRTL-5 cells, SP derivatives potently activate the release of sequestered Ca^{2+} and modulate Ca^{2+} entry. Furthermore, these compounds show a small, but significant, stimulatory effect on the proliferation of FRTL-5 cells.

MATERIALS AND METHODS

Materials

Culture medium, serum and hormones needed for cell culture

was purchased from Gibco (Grand Island, NY, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). Culture dishes were obtained from Falcon Plastics (Oxnard, CA, U.S.A.). SP, sphingosylphosphocholine (SPC), sphingomyelinase, sphingomyelin, ceramide, staurosporine, phorbol 12-myristate 13-acetate (PMA) and pertussis toxin were purchased from Sigma. Fura 2-AM was purchased from Molecular Probes (Eugene, OR, U.S.A.), thapsigargin and $\text{Ins}(1,4,5)\text{P}_3$ from LC Services Corp. (Woburn, MA, U.S.A.), $\text{myo}-[\text{H}^3]\text{inositol}$ (91 Ci/mmol), $[\text{H}^3]\text{arachidonic acid}$ (207 Ci/mmol) and $[\text{methyl}-^3\text{H}]\text{thymidine}$ (70–90 Ci/mmol) from Amersham International (Amersham, Bucks., U.K.). All other chemicals used were of reagent grade.

Cell culture

Rat thyroid FRTL-5 cells were generously given by Dr. Leonard D. Kohn (NIH, Bethesda, MD, U.S.A.). The cells were grown in Coon's modified Ham's F12 medium, supplemented with 5% calf serum and six hormones [22] [insulin, 10 $\mu\text{g}/\text{ml}$; transferrin, 5 $\mu\text{g}/\text{ml}$; cortisol, 10 nM; the tripeptide Gly-L-His-L-Lys, 10 ng/ml; thyroid-stimulating hormone (TSH), 0.3 m-unit/ml; somatostatin, 10 ng/ml]. The cells were grown in a water-saturated atmosphere of air/ Ca^{2+} (19:1) at 37 °C. Before an experiment, cells from one donor culture dish were harvested with 0.25% trypsin solution and plated on plastic 100 mm-diam. culture dishes. The cells were grown for 7–8 days, with two or three changes of the culture medium. Fresh medium was always added 24 h before an experiment.

Measurement of intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$)

The medium was aspirated, and the cells were then harvested with buffered saline solution (BSS: NaCl, 118 mM; KCl, 4.6 mM; glucose, 10 mM; CaCl_2 , 1.0 mM; Hepes, 20 mM; pH 7.2) containing 0.02% EDTA and 0.1% trypsin. After washing the cells three times by pelleting, the cells were incubated

Abbreviations used: SP, sphingosine; SPC, sphingosylphosphocholine; PKC, protein kinase C; $[\text{Ca}^{2+}]_i$, intracellular $[\text{Ca}^{2+}]$; TSH, thyroid-stimulating hormone; PMA, phorbol 12-myristate 13-acetate.

* To whom all correspondence should be addressed, at: Minerva, Tukholmankatu 2, 00250 Helsinki, Finland.

with 1 μ M Fura 2-AM for 30 min at 37 °C. After the loading period, the cells were washed twice with BSS, incubated for at least 10 min at room temperature, and washed once again. The cells were added to a quartz cuvette, kept at 37 °C, and stirred throughout the experiment. Fluorescence was measured with a Hitachi F2000 fluorimeter. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM CaCl_2 and digitonin to obtain R_{max} . Chelating extracellular Ca^{2+} with 5 mM EGTA and addition of Tris base were used to elevate pH above 8.3 to obtain R_{min} . $[\text{Ca}^{2+}]_i$ was calculated as described by Grynkiewicz et al. [23], by using a computer program designed for the fluorimeter, with a K_d value of 224 nM for Fura 2.

Cell permeabilization and measurement of free $[\text{Ca}^{2+}]_i$

The cells were grown as described above. The cells were then harvested as described for intact cells, and were loaded with Fura 2-AM. After washing the cells once by centrifugation (80 g; 5 min) in buffer A (containing: KCl, 125 mM; Hepes, 25 mM; KH_2PO_4 , 2 mM; EGTA, 0.25 mM; and BSA, 1 mg/ml; pH 7.0), and once with buffer B (identical with buffer A, but containing no EGTA), the cells were added to the cuvette and allowed to equilibrate to 37 °C. The cells were then permeabilized with 5 μ M digitonin. After a stabilization period (3–4 min), the test compounds were added. The excitation and emission wavelengths and the calibration procedure were identical with those used for the $[\text{Ca}^{2+}]_i$ measurements. The efficiency of the permeabilization was assessed by Trypan Blue uptake. By this protocol, more than 94% of the cells were permeabilized.

Incubation with pertussis toxin

The cells were treated with pertussis toxin (50 ng/ml) for 24 h [24], and then harvested and loaded with Fura 2 as described above.

Measurement of phosphoinositides in FRTL-5 cells

The cells were incubated with *myo*- $[\text{^3H}]$ inositol (6 μ Ci/plate) for 48 h, and then harvested with trypsin as mentioned above. The cells were incubated for 10 min at 37 °C in BSS buffer, and for 10 min at 37 °C in BSS buffer containing 10 mM LiCl. The cells were then stimulated with 100 μ M ATP for 10 min at 37 °C. Inositol phosphates were extracted with 20% HClO_4 , and the different inositol phosphates were separated by using Amprep (SAX) mini-columns [25].

Measurement of $[\text{^3H}]$ thymidine incorporation in FRTL-5 cells

The cells were plated on 35 mm-diam. dishes and grown in 6 H (complete cell culture medium) for 3 days. The cells were then washed twice with PBS (NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4 , 8 mM; KH_2PO_4 , 1.5 mM; pH 7.4) and grown in 5 H (medium without TSH) for 2 days. The medium was then changed to 5 H containing the appropriate concentrations of SP or SPC, and $[\text{^3H}]$ thymidine (0.8 μ Ci/ml), and incubated for 24 h [26]. The cells were washed four times with cold PBS, and then with cold 5% trichloroacetic acid. The trichloroacetic acid-insoluble precipitate was dissolved in 0.1 M NaOH, and the radioactivity was measured.

$[\text{^3H}]$ Arachidonic acid release

The cells were incubated with $[\text{^3H}]$ arachidonic acid (0.1 μ Ci/ml) for 24 h [27] in 35 mm-diam. plastic dishes. The procedure is a modification of that described previously [28]. After the incubation the cells were washed three times (5 min intervals) with release buffer (NaCl, 134 mM; KCl, 4.7 mM; glucose, 5 mM; CaCl_2 , 2.0 mM; MgSO_4 , 1.2 mM; KH_2PO_4 , 1.2 mM; NaHCO_3 , 2.5 mM; Hepes, 10 mM; pH 7.2; and 0.1% BSA). The cells were then incubated in 750 μ l of the release buffer for the times indicated with the appropriate agents. Then a 600 μ l portion was removed and counted for radioactivity in a 1214 Rackbeta liquid-scintillation spectrophotometer.

Statistics

The results are expressed as means $\pm$ S.E.M. The traces shown are representative traces of at least five independent experiments. Statistical analysis was done by Student's *t* test for paired observations. Analysis of variance was used for testing three or more means.

RESULTS

Effects of SP and SPC on $[\text{Ca}^{2+}]_i$ in FRTL-5 cells

Recent investigations have suggested a role for SP derivatives in regulating Ca^{2+} fluxes and Ca^{2+} release in both intact and permeabilized cells [13–17]. Addition of 30 μ M SP to FRTL-5 cells induced a modest increase in $[\text{Ca}^{2+}]_i$ (115 ± 13 nM), which then stabilized to a new plateau phase slightly (83 ± 11 nM) above pre-stimulatory $[\text{Ca}^{2+}]_i$ levels (Figure 1). The initial increase in $[\text{Ca}^{2+}]_i$ was entirely dependent on release of sequestered Ca^{2+} , as the increase was of similar magnitude in cells incubated in a nominally Ca^{2+} -free buffer containing 100 μ M EGTA (108 ± 14 nM), or in the presence of the putative Ca^{2+} -channel blocker SKF 96365 (142 ± 32 nM). The last two experimental designs totally abolished the plateau phase of $[\text{Ca}^{2+}]_i$ in response to SP (Figure 1).

Figure 1 Effect of SP and SPC on $[\text{Ca}^{2+}]_i$ in FRTL-5 cells

The cells were harvested and loaded with Fura-2 as described in the Materials and methods section. Upper traces (from left to right): cells stimulated with 30 μ M SP in Ca^{2+} -containing buffer, in the presence of 10 μ M SKF 96365, or in a nominally Ca^{2+} -free buffer containing 100 μ M EGTA. Lower traces (from left to right): cells stimulated with 30 μ M SPC in Ca^{2+} -containing buffer, in a nominally Ca^{2+} -free buffer containing 100 μ M EGTA, and in the presence of 10 μ M SKF 96365. The horizontal bar denotes 1 min.

Figure 2 Dose-dependent effect of SPC on $[Ca^{2+}]_i$ in FRTL-5 cells

The cells were stimulated with increasing doses of SPC (□), and after 3 min the cells were stimulated with 100 μ M ATP (■). Each point is the mean $\pm$ S.E.M. of 5–9 separate experiments.

Figure 3 Effects of SPC and thapsigargin on $[Ca^{2+}]_i$ in FRTL-5 cells

Left trace: the cells were stimulated first with 30 μ M SPC, and then thapsigargin (Tg, 2 μ M) was added. Middle trace: vehicle (Veh; 0.2% ethanol) was first added, and then Tg was added. Right trace: the cells were first stimulated with Tg, and then SPC was added. The horizontal bar denotes 1 min.

Figure 4 Effect of SPs on $[Ca^{2+}]_i$ in permeabilized FRTL-5 cells

The cells were loaded with Fura 2 and permeabilized as described in the Materials and methods section. The cells were then stimulated with 10 μ M SPC (upper trace), 10 μ M SP (middle trace), or with 10 μ M IP_3 (lower trace). The horizontal bar denotes 1 min.

We tested the effect of SPC, a more soluble SP derivative, on $[Ca^{2+}]_i$ in FRTL-5 cells. Addition of 30 μ M SPC to the cells transiently increased $[Ca^{2+}]_i$ by 487 ± 25 nM, which then stabilized at a new plateau level, 76 ± 8 nM above the pre-stimulation

$[Ca^{2+}]_i$ level (Figure 1). Both the initial increase in $[Ca^{2+}]_i$ and the plateau level of $[Ca^{2+}]_i$ in response to SPC were dependent on influx of extracellular Ca^{2+} (Figure 1). Addition of Ca^{2+} to cells stimulated with SPC in a Ca^{2+} -free buffer resulted in an influx of Ca^{2+} (191 ± 22 nM, compared with 63 ± 22 nM in control cells; $P < 0.05$; results not shown). Neither SP nor SPC induced an uptake of Trypan Blue in the cells, suggesting that the effects of SP and SPC on $[Ca^{2+}]_i$ were not the result of a non-specific permeabilization of the cell membrane.

Intracellular Ca^{2+} pools mobilized by SP and SPC in FRTL-5 cells

We wanted to investigate the source of the SP- and SPC-induced Ca^{2+} release. Addition of increasing doses of SP (0.1–30 μ M) before stimulating the cells with ATP had no effect on the subsequent ATP-evoked increase in $[Ca^{2+}]_i$ (results not shown). Furthermore, stimulating the cells with 100 μ M ATP had no effect on the SP-induced increase in $[Ca^{2+}]_i$ (results not shown). However, in a Ca^{2+} -free buffer, addition of 100 μ M ATP after SP resulted in a decreased transient in $[Ca^{2+}]_i$ (43 ± 17 nM), compared with control cells (279 ± 38 nM; $P < 0.05$; results not shown). If the cells were stimulated with ATP before SP, the SP-evoked increase in $[Ca^{2+}]_i$ was also attenuated (32 ± 10 nM), in comparison with control cells (108 ± 14 nM; $P < 0.05$; results not shown).

Addition of increasing doses of SPC resulted in a dose-dependent attenuation of the ATP-induced increase in $[Ca^{2+}]_i$ when ATP was added after SPC (Figure 2). However, addition of ATP before SPC had no effect on the SPC-induced increase in $[Ca^{2+}]_i$ in cells incubated in a Ca^{2+} -containing buffer (574 ± 36 nM versus 487 ± 25 nM in control cells; results not shown). In a Ca^{2+} -free buffer, addition of SPC before ATP almost totally abolished the ATP-evoked increase in $[Ca^{2+}]_i$, and stimulating the cells with ATP before addition of SPC totally abolished the SPC-evoked change in $[Ca^{2+}]_i$ (results not shown). The results thus suggested that both SP and SPC released sequestered Ca^{2+} from the same intracellular Ca^{2+} pool as did ATP in FRTL-5 cells.

SPC did not attenuate the thapsigargin-evoked changes in $[Ca^{2+}]_i$ (Figure 3). However, in cells stimulated with thapsigargin, addition of SPC evoked a very modest change in $[Ca^{2+}]_i$ (Figure 3). In a Ca^{2+} -free buffer pretreatment with thapsigargin completely abolished the SPC-evoked increase in $[Ca^{2+}]_i$ (results not shown). SPC decreased slightly, but significantly, the thapsigargin-evoked increase in $[Ca^{2+}]_i$ in cells in a Ca^{2+} -free buffer (77 ± 5 nM, in comparison with 119 ± 6 nM in control cells; $P < 0.05$; results not shown). SP had no effect on the thapsigargin-evoked change in $[Ca^{2+}]_i$, but thapsigargin totally abolished the SP-evoked increase in $[Ca^{2+}]_i$ (results not shown). Thus SPC (and SP) apparently released sequestered Ca^{2+} from a thapsigargin-sensitive intracellular Ca^{2+} pool.

We investigated further the SP- and SPC-evoked release of sequestered Ca^{2+} in permeabilized FRTL-5 cells. The results in Figure 4 show that both SP and SPC released Ca^{2+} from permeabilized FRTL-5 cells. However, both the SP- and SPC-evoked increase in $[Ca^{2+}]_i$ appeared to be slower compared with the release obtained in intact cells.

Mechanism of action of SP and SPC

Sphingomyelinase, the enzyme metabolizing sphingomyelin to sphingolipids, had no effect on $[Ca^{2+}]_i$ or on the ATP-evoked increase in $[Ca^{2+}]_i$ (results not shown). Furthermore, sphingomyelin or ceramide had only marginal effects on $[Ca^{2+}]_i$ and did not affect the ATP-evoked increase in $[Ca^{2+}]_i$ (results not shown).

Figure 5 Dependence on temperature of the effects of SP and SPC on $[Ca^{2+}]_i$ in FRTL-5 cells

Upper panel: the cells were incubated at 37 °C (a) or at 22 °C (b), and then stimulated with 30 μ M SP. Lower panel: the cells were incubated at 37 °C (a) or at 22 °C (b), and then stimulated with 30 μ M SPC. The horizontal bar denotes 1 min.

Figure 6 Effect of activation of PKC on the SPC-induced change in $[Ca^{2+}]_i$ in FRTL-5 cells

Upper traces: stimulating the cells with 30 μ M SPC (left trace), or addition of 200 nM PMA 2 min before stimulating the cells with SPC (right trace). The horizontal bar denotes 1 min. Lower panel: effect of 100 μ M staurosporine (Sts), 200 nM PMA, or depolarization with 50 mM K^+ on the SPC-evoked transient increase in $[Ca^{2+}]_i$ (■) and the plateau level of $[Ca^{2+}]_i$ (▨): * $P < 0.05$ compared with control cells stimulated with SPC only.

The effects of SP and its derivatives on $[Ca^{2+}]_i$ have been suggested to occur via the production of a metabolite, SP 1-phosphate [13,14]. Stimulating FRTL-5 cells with 30 μ M SP at 22 °C, instead of at 37 °C, resulted in a very slow onset of the

effect of SP (Figure 5). However, at 22 °C, the onset of the effect of SPC was not different from that observed at 37 °C, although the overall kinetics were different (Figure 5).

The conversion of SP into SP 1-phosphate is mediated by a kinase, SP 1-kinase, which can be inhibited by DL-threo-SP [29]. However, incubating FRTL-5 cells with 10 μ M DL-threo-SP for 30 min did not inhibit the effect of SP or SPC on $[Ca^{2+}]_i$ (results not shown). The results suggested that the effects of SP and SPC on $[Ca^{2+}]_i$ were not mediated via conversion into a 1-phosphate metabolite.

Previous studies have shown that neither SP nor SPC activated the formation of $InsP_3$ in cells [15–17]. Stimulating FRTL-5 cells loaded with myo-[3H]inositol for 10 min with 30 μ M SPC did not induce an increase in the formation of inositol metabolites (2100 ± 580 c.p.m., compared with 3050 ± 280 c.p.m. in control cells), thus excluding an effect of SPC mediated via mobilization of $InsP_3$.

PKC and the effects of SP and SPC on $[Ca^{2+}]_i$ in FRTL-5 cells

We have shown recently that both the ATP- and the thapsigargin-evoked changes in $[Ca^{2+}]_i$ can be modified by PKC [9,10]. It was thus decided to investigate whether modulation of PKC activity could affect the Ca^{2+} responses observed with either SP or SPC. Addition of 200 nM PMA before stimulating the cells with SP did not affect the SP-evoked changes in $[Ca^{2+}]_i$ (93 ± 13 nM, compared with 107 ± 12 nM in control cells; results not shown). However, the SPC-evoked change in $[Ca^{2+}]_i$ was attenuated (300 ± 25 nM and 48 ± 7 nM for the transient in $[Ca^{2+}]_i$ and the new elevated level of $[Ca^{2+}]_i$, compared with 487 ± 25 nM and 76 ± 8 nM in control cells; $P < 0.05$, Figure 6). The decrease in the SPC-evoked transient in $[Ca^{2+}]_i$ was not due to the PMA-induced depolarization of the cells [9], as the initial increase in $[Ca^{2+}]_i$ was 425 ± 45 nM in cells depolarized with 50 mM K^+ . The plateau phase was decreased in depolarized cells (41 ± 7 nM; $P < 0.05$; Figure 6) probably due to the decreased electrochemical gradient for Ca^{2+} .

Down-regulation of PKC with 200 nM PMA for 24 h [30], or pretreatment of the cells with 100 μ M staurosporine, had no effect on the SPC-evoked increase in $[Ca^{2+}]_i$ (results not shown). Stimulating PKC-down-regulated cells first with SPC and then with ATP resulted in an increase in the ATP-evoked change in $[Ca^{2+}]_i$ (296 ± 26 nM, compared with 120 ± 18 nM in control cells; $P < 0.05$; results not shown). The increase was entirely due to an enhanced influx of extracellular Ca^{2+} . Similar results on the effect of PKC down-regulation on ATP-evoked changes in $[Ca^{2+}]_i$ have been reported in FRTL-5 cells [10]. Down-regulation of PKC or inhibition of PKC with staurosporine had no effects on the SP-evoked change in $[Ca^{2+}]_i$ (results not shown).

Pertussis toxin and the effects of SP and SPC on $[Ca^{2+}]_i$ in FRTL-5 cells

The above results suggested that SPC, at least in part, induced changes in $[Ca^{2+}]_i$ in a manner similar to that observed with ATP and hormones activating the $InsP_3$ pathway. We incubated the cells with pertussis toxin (50 ng/ml for 24 h [7]). Stimulating these cells with ATP increased $[Ca^{2+}]_i$ by 227 ± 14 nM, which was roughly half of the increase in $[Ca^{2+}]_i$ observed in control cells (419 ± 34 nM; $P < 0.05$; Figure 7). Pertussis toxin pretreatment had no effect on the SP-evoked increase in $[Ca^{2+}]_i$ (98 ± 16 nM, compared with 107 ± 12 nM in control cells; results not shown).

The SPC-evoked increase in $[Ca^{2+}]_i$ was attenuated (358 ± 21 nM, compared with 487 ± 25 nM in control cells; $P < 0.05$; Figure 7) in cells pretreated with pertussis toxin. No

Figure 7 Effect of pretreatment with pertussis toxin on the SPC- and ATP-induced changes in $[Ca^{2+}]_i$ in FRTL-5 cells

The cells were pretreated with pertussis toxin (50 ng/ml) for 24 h. Upper traces: control cells stimulated with 30 μ M SPC (left trace); cells treated with pertussis toxin and stimulation with SPC (right trace). The horizontal bar denotes 1 min. Lower panel: summary of the effects of 100 μ M ATP and 30 μ M SPC on $[Ca^{2+}]_i$ in control cells (■) or cells treated with pertussis toxin (▨): * $P < 0.05$ compared with control cells.

Figure 8 Effects of SP or SPC on DNA synthesis in FRTL-5 cells

The incorporation of $[^3H]$ thymidine was assayed as described in the Materials and methods section. The cells were grown for 2 days in medium lacking TSH and then stimulated with the appropriate dose of SP or SPC, or with 0.3 m-unit of TSH for 24 h. Each bar gives the mean $\pm$ S.E.M. of triplicate dishes: * $P < 0.05$ compared with control cells.

effect of pertussis toxin was seen on the ATP-evoked increase in $[Ca^{2+}]_i$, when ATP was added to the cells after stimulation with SPC (Figure 7). Furthermore, pertussis toxin had no effect on the SP- or SPC-induced plateau phase of $[Ca^{2+}]_i$. The results thus suggested that the effect of SPC on $[Ca^{2+}]_i$ may in part be mediated via a mechanism dependent on a G-protein.

Both SP and SPC are mitogens in FRTL-5 cells

SP and SPC have been implicated to have potent mitogenic activity [14,15]. We compared the mitogenic effects of SP and

SPC with the effect of TSH, which is vital for the growth of FRTL-5 cells. Both SP and SPC stimulated mitogenesis in FRTL-5 cells, as measured by the incorporation of $[^3H]$ thymidine into DNA, in the absence of TSH (Figure 8).

SPC stimulates release of $[^3H]$ arachidonate in FRTL-5 cells

Several agents that activate Ca^{2+} influx in FRTL-5 cells, or have mitogenic properties, stimulate $[^3H]$ arachidonate release [20,21,28,31]. We observed that SPC potently stimulated the release of $[^3H]$ arachidonate in FRTL-5 cells (888 ± 5 c.p.m./well, compared with 469 ± 10 c.p.m./well in control cells; $P < 0.05$). This release was also observed in a $[Ca^{2+}]_i$ -free buffer (606 ± 24 c.p.m./well, compared with 293 ± 40 c.p.m./well in control cells; $P < 0.05$), suggesting that SPC may activate phospholipase A_2 via a mechanism independent of Ca^{2+} entry only. SP had no effect on the release of $[^3H]$ arachidonate in FRTL-5 cells (results not shown).

DISCUSSION

In the present paper we show that SP, and especially SPC, mobilized sequestered Ca^{2+} , and activated capacitative entry of Ca^{2+} in thyroid FRTL-5 cells. Both SP and SPC appeared to release Ca^{2+} from $InsP_3$ - and thapsigargin-sensitive intracellular Ca^{2+} pools. The mechanism of action of these compounds still remains unknown. SP has been shown in previous studies to activate [32] or to have no effect on [17] the $PtdInsP_2/InsP_3$ pathway. We did not investigate the effect of SP, but observed no effect of SPC on the $PtdInsP_2/InsP_3$ pathway, a result similar to that of previous studies [15,16]. However, the inhibitory effects of PMA and pertussis toxin on the SPC-evoked changes in $[Ca^{2+}]_i$ were similar to the effects of these compounds on the ATP-evoked changes in $[Ca^{2+}]_i$ [7,10], suggesting some similarities between the mechanisms regulating the $PtdInsP_2/InsP_3$ pathway and the SPC signal-transduction pathway.

One possible mediator of the effect of both SP and SPC is SP 1-phosphate. This lipid metabolite, produced from SP by the action of a SP 1-kinase, has been shown to be the mediator of SP-evoked changes in $[Ca^{2+}]_i$ in 3T3 cells [14]. However, pretreatment of the FRTL-5 cells with DL-threo-SP, an inhibitor of the SP 1-kinase [29], had no effect on either the SPC- or the SP-evoked changes in $[Ca^{2+}]_i$. Thus SP 1-phosphate appears to be of no or very modest importance in mediating the effect of SP and SPC on $[Ca^{2+}]_i$ in FRTL-5 cells. We were also unable to see any effects of treating the cells with ceramide, sphingomyelin or sphingomyelinase. We cannot, however, exclude the possibility that the formation of some metabolite of SP may be the mediator of the observed effects on $[Ca^{2+}]_i$. When the incubation temperature of the cell suspension was 22 $^{\circ}C$ instead of the normal 37 $^{\circ}C$, the onset of the response was clearly delayed. In contrast with this observation, no effect of the incubation temperature was observed on the onset of the SPC-evoked change in $[Ca^{2+}]_i$, although the kinetics of the change were different from that seen at 37 $^{\circ}C$. In permeabilized cells, addition of SP or SPC to FRTL-5 cells also increased $[Ca^{2+}]_i$. Thus an intact plasma membrane is not a prerequisite for the SPs to mobilize Ca^{2+} . A possible metabolite may, however, occur at the endoplasmic reticulum (see [13]).

SPs are reported to be potent inhibitors of PKC activity in cells [11]. We have shown previously that PKC has a potent effect on regulating Ca^{2+} entry in FRTL-5 cells, and that inhibition of PKC enhances Ca^{2+} entry [9,10]. We could not observe an enhanced response in $[Ca^{2+}]_i$ when cells were stimulated with ATP added after SP, although the cells were incubated up to 30 min with SP (results not shown). However, SP may inhibit a

subset of PKC enzymes not involved in regulating Ca^{2+} entry. Interestingly, activation of PKC with PMA decreased the SPC-evoked increase in $[\text{Ca}^{2+}]_i$, whereas inhibition of PKC was without effect. This is an intriguing observation, especially when considering that, in cells pretreated with pertussis toxin, the effect of SPC was decreased, but that of SP was not. Recently, Wong and co-workers were unable to observe an effect of pertussis toxin on the SP-evoked changes in $[\text{Ca}^{2+}]_i$ [17]. The results thus suggested that the effects of SPC and SP on $[\text{Ca}^{2+}]_i$ may be mediated via separate pathways. Similar results have been obtained in 3T3 cells [15], although no information on the nature of these putative pathways is at present available.

SPs have been shown to inhibit calmodulin-dependent enzymes [18]. We have observed that the calmodulin inhibitor W-7 depleted intracellular Ca^{2+} pools and decreased Ca^{2+} influx in FRTL-5 cells [19]. A possible role for calmodulin in regulating Ca^{2+} fluxes has been suggested in some recent reports [33,34]. However, both SP and SPC enhanced Ca^{2+} entry in our cells, which is not expected if the effects of these compounds were the result of inhibition of calmodulin. However, we cannot exclude the possibility that part of the effect of SPC on the ATP- and thapsigargin-evoked changes in $[\text{Ca}^{2+}]_i$ could be due to inhibition of calmodulin. This aspect is now the object of further studies.

Both SP and SPC have been reported to be potent stimulators of mitogenesis in cells [14,15]. In FRTL-5 cells, TSH is necessary to stimulate proliferation [22]. In addition, the TSH-induced stimulation of phospholipase A_2 may be of importance in this mitogenic response [21]. We observed that both SP and SPC enhanced the incorporation of $[\text{H}]$ thymidine in FRTL-5 cells deprived of TSH, although the effect was not as potent as that seen with TSH. We also observed that SPC, but not SP, stimulated the release of $[\text{H}]$ arachidonate, and that this stimulatory effect of SPC was evident also in a Ca^{2+} -free buffer. The activation of phospholipase A_2 and the release of $[\text{H}]$ arachidonate is not observed in FRTL-5 cells in a Ca^{2+} -free buffer in response to Ca^{2+} -mobilizing agents ([20,31]; K. Törnquist and E. Ekokoski, unpublished work). Thus our results suggest that SPC activates phospholipase A_2 via some Ca^{2+} -independent mechanism. In addition, the arachidonic acid produced may participate in the mitogenesis induced by SPC [21]. Further studies are, however, needed on the mitogenic effects of SP and SPC in FRTL-5 cells.

In conclusion, we have shown that SP, and especially SPC, are potent modulators of Ca^{2+} fluxes in FRTL-5 cells. Furthermore, both SP and SPC have mitogenic effects in these cells. It is thus possible that endogenous sphingolipids may have an important regulatory function in FRTL-5 cells.

This study was supported by the Sigrid Juselius Foundation, the Nordisk Insulin Foundation Committee, and the Academy of Finland.

REFERENCES

- Brenner-Gati, L., Berg, K. A. and Gershengorn, M. C. (1988) *J. Clin. Invest.* **82**, 1144–1148
- Lombardi, A., Veneziani, B. M., Tramontano, D. and Ingbar, S. H. (1988) *Endocrinology* (Baltimore) **123**, 1544–1552
- Vassart, G. and Dumont, J. E. (1992) *Endocr. Rev.* **13**, 596–611
- Field, J. B., Ealey, P. A., Marshall, N. J. and Cockcroft, S. (1987) *Biochem. J.* **247**, 519–524
- Corda, D., Marocci, R., Kohn, L. D., Axelrod, J. and Luini, A. (1985) *J. Biol. Chem.* **260**, 9230–9236
- Mockel, J., Delcroix, C., Rodesch, F. and Dumont, J. E. (1987) *Mol. Cell. Endocrinol.* **51**, 95–104
- Okajima, F., Sho, K. and Kondo, Y. (1988) *Endocrinology* (Baltimore) **123**, 1035–1043
- Törnquist, K. (1992) *J. Cell. Physiol.* **150**, 90–98
- Törnquist, K. (1993) *Biochem. J.* **290**, 443–447
- Törnquist, K. (1993) *Mol. Cell. Endocrinol.* **93**, 17–21
- Hannun, Y. A. and Bell, R. M. (1989) *Science* **243**, 500–506
- Merril, A. H. and Stevens, V. L. (1989) *Biochim. Biophys. Acta* **1010**, 131–139
- Ghosh, T., Bian, J. and Gill, D. L. (1990) *Science* **248**, 1653–1656
- Zhang, H., Desai, N. N., Olivera, A., Seki, T., Brooker, G. and Spiegel, S. (1992) *J. Cell Biol.* **114**, 155–167
- Desai, N. N., Carlson, R. O., Mattie, M. E., Olivera, N., Buckley, N. E., Seki, T., Brooker, G. and Spiegel, S. (1993) *J. Cell Biol.* **121**, 1385–1395
- Yule, D. I., Wu, D., Essington, T. E., Shayman, J. A. and Williams, J. A. (1993) *J. Biol. Chem.* **268**, 12353–12358
- Wong, K. and Kwan-Yeung, L. (1993) *Cell Calcium* **14**, 493–505
- Jefferson, A. B. and Schulman, H. (1988) *J. Biol. Chem.* **263**, 15241–15244
- Törnquist, K. (1993) *Biochem. Biophys. Res. Commun.* **190**, 37–41
- Törnquist, K., Ekokoski, E., Forss, L. and Mattsson, M. (1994) *Cell Calcium*, in the press
- Tahara, K., Grollman, E. F., Saji, M. and Kohn, L. D. (1991) *J. Biol. Chem.* **266**, 440–448
- Ambesi-Impiombato, F. S., Parks, L. A. M. and Coon, H. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3455–3459
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Sho, K., Okajima, F., Majid, M. A. and Kondo, Y. (1991) *J. Biol. Chem.* **266**, 12180–12184
- Oldham, K. G. (1990) in *Receptor-Effector Coupling: A Practical Approach* (Hume, E. C., ed.), pp. 99–117, Oxford University Press, Oxford, New York and Tokyo
- Valente, W. A., Vitti, P., Kohn, L. D., Brandi, M., Toccafondi, R., Tramontano, D., Azou, S. M. and Ambesi-Impiombato, F. S. (1983) *Endocrinology* (Baltimore) **112**, 71–79
- Di Girolamo, M., A'Arcangelo, D., Bizzarri, C. and Corda, D. (1991) *Acta Endocrinol. (Copenhagen)* **125**, 192–200
- Okajima, F., Sato, K., Nazarea, M., Sho, K. and Kondo, Y. (1989) *J. Biol. Chem.* **264**, 13029–13037
- Buehrer, B. M. and Bell, R. M. (1992) *J. Biol. Chem.* **267**, 3154–3159
- Jaken, S., Tashjian, A. H., Jr. and Blumberg, P. M. (1981) *Cancer Res.* **41**, 2175–2181
- Burch, R. M., Luini, A., Mais, D. E., Corda, D., Vanderhoeck, J. Y., Kohn, L. D. and Axelrod, J. (1986) *J. Biol. Chem.* **261**, 11236–11241
- Zhang, H., Desai, N. N., Murphey, J. M. and Spiegel, S. (1990) *J. Biol. Chem.* **265**, 21309–21316
- Alvarez, J., Montero, M. and Garcia-Sanchos, J. (1992) *FASEB J.* **6**, 786–792
- Mason, M., Mayer, B. and Hymel, L. J. (1993) *Am. J. Physiol.* **264**, C654–C662