Ceramide 1-phosphate increases intracellular free calcium concentrations in thyroid FRTL-5 cells: evidence for an effect mediated by inositol 1,4,5-trisphosphate and intracellular sphingosine 1-phosphate

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Sphingolipid (SP) derivatives have diverse effects on the regulation of intracellular free calcium concentrations ($[Ca^{2+}]_i$) in a multitude of non-excitabile cells. In the present investigation, the effect of C_2 -ceramide 1-phosphate (C1P) on $[Ca^{2+}]_i$ was investigated in thyroid FRTL-5 cells. C1P evoked a concentration-dependent increase in $[Ca^{2+}]_i$, both in a calcium-containing and a calcium-free buffer. A substantial part of the C1P-evoked increase in $[Ca^{2+}]_i$ was due to calcium entry. The effect of C1P was attenuated by overnight pretreatment of the cells with pertussis toxin. Similar results were obtained with C_8 -ceramide 1-phosphate, although the magnitude of the responses was smaller than with C1P. The phospholipase C inhibitor U73122 attenuated the effect of C1P. C1P invoked a small, but significant, increase in inositol 1,4,5-trisphosphate (IP_3). How-

ever, the effect of C1P on $[Ca^{2+}]_i$ was inhibited by neither Xestospongine C, 2-aminoethoxydiphenylborate nor neomycin. C1P mobilized calcium from an IP_3 -sensitive calcium store, as C1P did not increase $[Ca^{2+}]_i$ in cells pretreated with thapsigargin. The effect of C1P on $[Ca^{2+}]_i$ was potentially attenuated by dihydrosphingosine and dimethylsphingosine, two inhibitors of sphingosine kinase, but not by the inactive SP-derivative *N*-acetyl sphingosine. Stimulating the cells with C1P evoked an increase in the production of intracellular sphingosine 1-phosphate. C1P did not modulate DNA synthesis or the forskolin-evoked production of cAMP. The results indicate that C1P may be an important SP participating in cellular signalling.

Key words: calcium entry, receptor, sphingolipid.

INTRODUCTION

Sphingomyelin derivatives, i.e. sphingosines and ceramides, potentially modulate a multitude of cellular events, including proliferation, differentiation and migration [1,2]. Some of these derivatives, especially sphingosine, sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC), also have diverse effects on the regulation of intracellular free calcium concentration ($[Ca^{2+}]_i$) in both non-excitabile and excitabile cells. In non-excitabile cells, S1P and SPC mobilize sequestered calcium and activate calcium entry [2,3]. Many of these effects are mediated by G-protein-coupled plasma membrane receptors for sphingolipids. Five S1P receptors have been identified so far, namely Edg1, Edg3, Edg5, Edg6 and Edg8 [2], along with the SPC-specific receptors OGR1 [4] and GPR4 [5].

Sphingosine is a potent inhibitor of store-operated calcium (SOC) entry (i.e. by inhibiting calcium release-activated calcium current, or I_{CRAC}) [6]. In addition to effects mediated by plasma membrane receptors, an intracellular receptor for sphingolipids, SCA_{MPER} ('sphingolipid calcium-release-mediating protein of the endoplasmic reticulum'), has been cloned [7]. The physiological importance of this receptor is still not clear [2], and evidence against a role for SCA_{MPER} as a receptor has been obtained [8]. Sphingosine and SPC also potentially attenuate voltage-operated calcium channels and ryanodine receptors in

excitabile cells, like cardiac and pituitary cells [3,9]. Ceramides themselves, which are produced prior to sphingosine when sphingomyelin is hydrolysed, have been shown in a few reports to invoke changes in $[Ca^{2+}]_i$ [10,11]. Furthermore, C_8 -ceramide attenuates SOC entry in non-excitabile cells [12,13]. C_8 -ceramide also inhibits the L-type voltage-operated calcium channels in pinealocytes [14] and attenuates K^+ channels [15,16].

In addition to ceramide, C_2 -ceramide 1-phosphate (C1P) is a putative modulator of cellular functions (see [1]), although little is known with regard to its physiological significance. Some reports have shown that ceramide 1-phosphates enhance DNA synthesis in fibroblasts, although the mechanism of action is still not known [17,18]. According to these reports, C1P itself does not modulate $[Ca^{2+}]_i$. On the other hand, recent results suggest that C1P may attenuate noradrenaline-evoked changes in $[Ca^{2+}]_i$ in rat aortic smooth muscle [19]. However, C1P may be considered a sphingoid form of phosphatidic acid, and both phosphatidic acid and lysophosphatidic acid (lysoPA) bind to Edg2, Edg4 and Edg7, receptors that are closely related structurally to the sphingolipid receptors [20,21]. Furthermore, at least lysoPA is a weak agonist for the Edg1 receptor [22]. LysoPA stimulates proliferation and mobilizes sequestered calcium in thyroid FRTL-5 cells [23,24]. The FRTL-5 cells also express the sphingolipid receptor Edg5 [25,26]. Thus we considered it important to investigate the effects of C1P on both the regulation of $[Ca^{2+}]_i$

Abbreviations used: 2-APB, 2-aminoethoxydiphenylborate; $[Ca^{2+}]_i$, intracellular free calcium concentration; C1P, C_2 -ceramide 1-phosphate; C_8 -C1P, C_8 -ceramide 1-phosphate; DHS, dihydrosphingosine; DMS, dimethylsphingosine; IP_3 , inositol 1,4,5-trisphosphate; lysoPA, lysophosphatidic acid; PLC, phospholipase C; SOC, store-operated calcium; SPC, sphingosylphosphorylcholine; S1P, sphingosine 1-phosphate; TSH, thyrotropin.

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and DNA synthesis in these cells. Our results showed that C1P mobilized sequestered calcium and evoked a substantial entry of extracellular calcium in FRTL-5 cells. The effect of C1P was mediated, at least in part, by a pertussis-toxin-sensitive G-protein. Interestingly, the increase in $[Ca^{2+}]_i$ evoked by C1P may be due to an increase in both inositol 1,4,5-trisphosphate (IP_3) and intracellular S1P.

MATERIALS AND METHODS

Materials

Culture medium, serum and hormones were purchased from Gibco (Grand Island, NY, U.S.A.), Biological Industries (Beth Haemek, Israel) and Sigma (St. Louis, MO, U.S.A.). Culture dishes were obtained from Falcon Plastics (Oxnard, CA, U.S.A.). C_8 -Ceramide 1-phosphate (C_8 -C1P), S1P, dimethylsphingosine (DMS), dihydrosphingosine (DHS) and *N*-acetyl sphingosine (C_2 -ceramide) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). C1P and C_8 -C1P were also purchased from Avanti Lipids (Alabaster, AL, U.S.A.). Xestospongine C was from Calbiochem (La Jolla, CA, U.S.A.), and U73122 and U73343 were from Biomol. Pertussis toxin, 2-aminoethoxydiphenylborate (2-APB), PMA, lysoPA, SPC, wortmannin, ATP and heparin were all purchased from Sigma. Calphostin C and 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*DL*]-pyrimidine (PP1) were from Alexis Corporation (Laufelfingen, Switzerland). Amprep® (SAX) minicolumns (100 mg), *myo*- $[^3H]$ inositol with PT6-271 (1 mCi/ml) and *[methyl-^3H]*thymidine (70–90 Ci/mmol) were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Fura 2/acetoxymethyl ester, BAPTA [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid] and bisoxonol were purchased from Molecular Probes (Eugene, OR, U.S.A.). Thapsigargin was from LC Services Corp. (Woburn, MA, U.S.A.). All other chemicals used were of reagent grade. Bovine thyrotropin (TSH) was a generous gift from Dr A. F. Parlow (NIDDK National Hormone and Pituitary Program, Torrance, CA, U.S.A.).

Cell culture

Rat thyroid FRTL-5 cells, originally obtained from the Interthyr Foundation (Bethesda, MD, U.S.A.) were a generous gift of Dr Egil Haug (Akers Hospital, Oslo, Norway). The cells were grown in Coon's modified Ham's F12 medium supplemented with 5% calf serum together with six hormones (6H medium; 10 μ g/ml insulin, 5 μ g/ml transferrin, 10 nM hydrocortisone, 10 ng/ml tripeptide Gly-L-His-L-Lys, 0.3 m-units/ml TSH and 10 ng/ml somatostatin) [27] in a water-saturated atmosphere of 5% CO_2 and 95% air at 37 °C. Prior to an experiment, cells from one donor culture dish were harvested with a 0.1% trypsin solution and plated on to plastic 100 mm culture dishes. The cells were grown for 7–8 days incorporating two or three changes of culture medium with the final medium change always occurring 24 h prior to an experiment.

Rat hepatoma RH7777 cells were a generous gift of Dr Sven-Olof Olofsson (Göteborg University, Göteborg, Sweden). The cells were grown in monolayers at 37 °C in a water-saturated atmosphere of 5% CO_2 and 95% air in growth medium consisting of Eagle's minimum essential medium containing 10% fetal calf serum, non-essential amino acids, 2 mM glutamine and 1 mM pyruvate. The cells were grown for 7–8 days incorporating two or three changes of culture medium with the final medium change always occurring 24 h prior to an experiment.

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

The medium was aspirated, and the cells were harvested with Hepes-buffered saline solution (HBSS: 118 mM NaCl, 4.6 mM KCl, 10 mM glucose, 1.0 mM $CaCl_2$ and 20 mM Hepes, pH 7.2) lacking Ca^{2+} but containing 0.02% EDTA and 0.1% trypsin. After washing the cells three times by pelleting, the cells were incubated with 1 μ M fura 2/acetoxymethyl ester for 30 min at 37 °C. Following the loading period, the cells were washed twice with HBSS buffer, incubated for at least 10 min at room temperature and washed once more. Fluorescence was measured at 37 °C with a Hitachi F2000 fluorimeter using excitation wavelengths of 340 and 380 nm and detecting emission at 510 nm. The signal was calibrated by the addition of 1 mM $CaCl_2$ and Triton X-100 to obtain maximum fluorescence. The chelation of extracellular Ca^{2+} with 5 mM EGTA and the addition of Tris base to elevate the pH above 8.3 were both used to obtain minimum fluorescence. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. [28], using a computer program designed for the fluorimeter with a K_d value of 224 nM for fura 2. We observed that the magnitude of the C1P-evoked increase in $[Ca^{2+}]_i$ changed during the time course of the study. Thus appropriate control experiments from the same batch of cells are always shown in the graphs. We suspect that the difference in the magnitude of the C1P-evoked increase in $[Ca^{2+}]_i$ obtained with the same dose of C1P was due to some factors in the serum we used.

For the experiments, C1P was applied as a DMSO solution, and C_8 -C1P as an ethanol solution. The concentration of the solute never exceeded 0.1% and in the control experiments DMSO or ethanol alone was used.

Measurement of membrane potential

A change in the membrane potential was measured using bisoxonol [29]. The cells were grown and harvested as for the calcium experiments. After the final wash, the cells were added to a quartz cuvette and bisoxonol (final concentration, 100 nM) was added. The cells were equilibrated with bisoxonol for at least 15 min prior to an experiment. The excitation wavelength was 540 nm, and the emission wavelength was 580 nm. Each experiment was calibrated by the addition of 50 mM K^+ , to make comparison between different experiments possible.

Measurement of cellular cAMP

Prior to an experiment, cells grown in 6H medium were washed with PBS and then grown for an additional 7 days in medium lacking TSH. The cells were harvested as described for the calcium experiments, and were preincubated in HBSS buffer for 20 min in the presence of 0.3 mM isobutylmethylxanthine (to inhibit phosphodiesterase activity). Then aliquots of cells (0.5×10^6 cells/tube) were stimulated with 1 μ M forskolin and C1P (0.3–3 μ M). After 20 min the reaction was stopped with 50 μ l perchloric acid to a final concentration of 0.5 M. The samples were neutralized by KOH and the cAMP concentrations were determined using a protein-binding method [30].

Measurement of inositol phosphates

The cells were preincubated with *myo*- $[^3H]$ inositol (6 μ Ci/100 mm dish) for 48 h. After harvesting, the cells were suspended in HBSS buffer. The cells were first incubated for

10 min at 37 °C, then with HBSS containing 10 mM LiCl for 10 min and finally the cells were stimulated with either vehicle or C1P (final concentration, 3 μM) for 30 s or 10 min at 37 °C. Inositol phosphates were extracted using 10% perchloric acid and then separated using Amprep® (SAX) mini-columns [31]. The radioactivity of the samples was measured by liquid-scintillation counting.

Measurement of sphingosine kinase activity

The method was essentially as described in [32]. Cells grown on 35 mm coverslips were serum-starved in Coon's medium containing 0.2% essentially fatty acid free BSA for 24 h. The cells were then equilibrated in 900 μl of medium for 10 min at 37 °C. Approx. 200 000 c.p.m. [^3H]sphingosine together with 3 μM C1P or vehicle in 100 μl medium was then added to the plates and the cells were incubated for the appropriate time at 37 °C. The reaction was stopped by aspirating the medium and by the addition of ice-cold methanol. The cells were scraped into polypropylene tubes and the plates were washed once with methanol. The suspension was then sonicated twice for 20 s each and centrifuged at 2000 g for 10 min to remove cell debris. The supernatant was transferred to a glass vial and authentic sphingosine and S1P was added to the samples for identification of [^3H]sphingosine and [^3H]S1P. The supernatants were then evaporated. After redissolving in methanol, samples were spotted on to HPTLC plates and the products were separated in butanol-1-ol/acetic acid/water (3:1:1, by vol.) as a solvent system. Sphingosine and S1P spots were visualized using ninhydrin and, after scraping off the spots, radioactivity was measured by liquid-scintillation counting. As we repeatedly observed that the uptake of [^3H]sphingosine was lower in the presence of C1P, the results were normalized to the [^3H]S1P/[^3H]sphingosine ratio of control cells at time zero. For plotting the graph, the normalized control values for the respective time points were subtracted from the corresponding normalized values obtained after stimulation with C1P.

Measurement of [^3H]thymidine incorporation in FRTL-5 cells

The cells were plated on to 35 mm dishes and grown in 6H medium for 3 days. Then the cells were washed twice with PBS and grown in Coon's medium without hormones or serum containing 0.2% BSA (0H/BSA) for 2 days. The medium was then changed to 0H/BSA containing the appropriate concentrations of the test compounds or vehicle and the cells were further incubated for 24–96 h. During the final 4 h of incubation, [^3H]thymidine (0.4 $\mu\text{Ci/ml}$) was added to the cells. After the final incubation the cells were washed twice with cold PBS solution, and once with cold 5% trichloroacetic acid. The trichloroacetic acid-insoluble precipitate was dissolved in 0.1 M NaOH and the radioactivity measured by liquid-scintillation counting.

Statistics

The results are expressed as means $\pm$ S.E.M. Statistical analysis was made using Student's t test for paired observations. When three or more means were tested, ANOVA was performed using the Prism program. Curve fitting was also performed using the Prism program. The data are from at least two different batches of cells and were obtained on at least 2 separate days. Each experiment was repeated at least three times.

RESULTS

Effects of C1P on $[\text{Ca}^{2+}]_i$ in FRTL-5 cells

Previous studies in fibroblasts have suggested that C1P does not affect $[\text{Ca}^{2+}]_i$ [17,18]. However, in thyroid FRTL-5 cells we obtained a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ in response to C1P (Figure 1). The increase was more marked in a calcium-containing buffer compared with a calcium-free buffer, suggesting that part of the increase in $[\text{Ca}^{2+}]_i$ was due to a substantial entry of extracellular calcium. Furthermore, the increase in $[\text{Ca}^{2+}]_i$ was, at least in part, mediated by a G-protein-dependent mechanism, as the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ was attenuated in cells pretreated with pertussis toxin (50 ng/ml for 24 h; Figure 1). Statistical analysis (ANOVA) showed that the response curves obtained with cells in a calcium-free buffer, and in cells pretreated with pertussis toxin, were significantly decreased compared with cells in a calcium-containing buffer ($P < 0.05$). The inhibitory effect of pertussis toxin was more pronounced in experiments conducted in a calcium-containing buffer compared with experiments in a calcium-free buffer. In pertussis toxin-treated cells stimulated with 3 μM C1P in calcium-containing buffer, the increase in $[\text{Ca}^{2+}]_i$ was only 56% of that of control cells. The increase was 72% of that in control cells when the experiments were conducted in a calcium-free buffer (results not shown). Furthermore, in cells pretreated with 1 μM thapsigargin for 15 min to deplete intracellular calcium stores, C1P was without an effect on $[\text{Ca}^{2+}]_i$ (Figure 1). Qualitatively similar results were obtained with C_8 -C1P, but the poor solubility of the compound hampered detailed investigations. In calcium-containing and calcium-free buffers, 10 μM C_8 -C1P (the highest dose tested) increased $[\text{Ca}^{2+}]_i$ by 128 ± 33 and 67 ± 10 nM, respectively.

We next compared the EC_{50} values for the increase in $[\text{Ca}^{2+}]_i$ evoked by C1P, S1P, SPC and lysoPA. The EC_{50} values for C1P, S1P and SPC were 0.24, 0.56 and 2.4 μM , respectively, and that for lysoPA was 0.45 μM . With C_8 -C1P we obtained an EC_{50} value of 1.2 μM , but the result is uncertain due to the poor solubility of the compound. Thus all tested sphingolipid agonists evoked an increase in $[\text{Ca}^{2+}]_i$ with comparable potencies. It is worth noting that the maximal increase in $[\text{Ca}^{2+}]_i$ evoked with C1P was two to three times that of the S1P-evoked response (results not shown).

We tested whether C1P evoked an increase in $[\text{Ca}^{2+}]_i$ in rat hepatoma RH7777 cells. This cell line expresses very low levels of sphingolipid receptors in the plasma membrane [33], although the existence of intracellular S1P-receptors has not been evaluated. We were unable to obtain any change in $[\text{Ca}^{2+}]_i$ when the cells were stimulated with 3 μM S1P (results not shown). Cells stimulated with 3 μM C1P evoked a modest, but distinct, change in $[\text{Ca}^{2+}]_i$ (33 ± 5 nM). Stimulating the cells with 3 μM of either lysoPA or SPC increased $[\text{Ca}^{2+}]_i$ by 67 ± 6 and 94 ± 21 nM, respectively.

The above results suggested that C1P mobilized sequestered calcium and evoked calcium entry by a G-protein-dependent mechanism. When cells were pretreated with 5 μM of the phospholipase C (PLC) inhibitor U73122, the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ in a calcium-free buffer was clearly attenuated ($P < 0.05$; Figure 2A). The inactive analogue U73343 did not have an effect. The experiments were performed in a calcium-free buffer as C1P evoked a substantial entry of calcium, and we observed that U73122 attenuated the thapsigargin-evoked entry of calcium in control experiments (results not shown; see also [34]).

Next we investigated whether the effect of C1P could be modulated by Xestospongins C or 2-APB, two agents that block

Figure 1 Effect of C1P on $[Ca^{2+}]_i$ in FRTL-5 cells

The cells were loaded with fura 2 as described in the Materials and methods section. (A) Cells incubated in calcium-containing (a, a') or calcium-free (b, b') buffer were stimulated with 3 μ M C1P. In some experiments (a', b') the cells had been pretreated with pertussis toxin (50 ng/ml) for 24 h. (B) Concentration-dependent effect of C1P on $[Ca^{2+}]_i$ in cells incubated in calcium buffer (■), calcium-free buffer (▲) and in calcium buffer after pretreatment with pertussis toxin (Ptx; 50 ng/ml for 24 h; □) for 24 h. Each point is the mean $\pm$ S.E.M. of 4–8 separate experiments. (C) Lack of an effect of C1P (final concentration, 3 μ M) on $[Ca^{2+}]_i$ in cells pretreated with thapsigargin (final concentration, 1 μ M) for 15 min. Each trace is representative of at least four separate experiments.

the IP_3 receptor, thus blocking the IP_3 -evoked mobilization of sequestered calcium [35]. Pretreatment of the cells with 20 μ M Xestospongine C for 30 min had no effect on the change in $[Ca^{2+}]_i$ evoked by 3 μ M C1P (690 ± 89 nM compared with 613 ± 28 nM in control cells). In control experiments, Xestospongine C also did not affect the increase in $[Ca^{2+}]_i$ evoked by 1 μ M ATP (results not shown). A similar lack of an effect for Xestospongine C has also been shown in other cell types (see [36]). Treating the cells for 3 min with 30 μ M 2-APB in a calcium-free buffer did not attenuate the C1P-evoked increase in $[Ca^{2+}]_i$ (69 ± 11 nM compared with 107 ± 12 nM in control cells and in 2-APB-treated cells, respectively). As 2-APB effectively attenuated the thapsigargin-evoked entry of calcium (results not shown), the experiments were conducted in a calcium-free buffer. In control experiments, 2-APB attenuated the ATP-evoked increase in $[Ca^{2+}]_i$ (227 ± 12 nM compared with 342 ± 25 nM in control cells; $P < 0.05$). Furthermore, in cells pretreated for 30 min with 5 mM neomycin, which binds to phosphatidylinositol 4,5-bisphosphate and inhibits the production of IP_3 [37], C1P still evoked a substantial increase in $[Ca^{2+}]_i$ (789 ± 124 nM compared with 713 ± 62 nM in control cells).

Effect of C1P on inositol phosphate production

Several reports suggest that sphingolipids may or may not stimulate the production of IP_3 (see [3]). We obtained a small but

significant ($P < 0.05$) increase in the formation of IP_3 after stimulating the cells with 3 μ M C1P for 30 s (280 ± 18 and 456 ± 9 c.p.m./tube in control and C1P-stimulated cells, respectively; Figure 2B). After 10 min of stimulation, no significant effect was detected (372 ± 41 and 398 ± 44 c.p.m./tube in control and C1P-stimulated cells, respectively).

Effects of C1P on SOC entry

Of the sphingomyelin derivatives previously tested, both sphingosine and ceramide attenuate SOC entry [6,13]. When FRTL-5 cells were first stimulated with 3 μ M C1P, and then with 1 μ M thapsigargin, the thapsigargin-evoked increase in $[Ca^{2+}]_i$ was neither attenuated nor enhanced (results not shown). This is a surprising finding, as 3 μ M C1P depolarized the membrane potential by $48 \pm 8\%$ of that obtained with 50 mM K^+ (as measured with the fluorescent membrane potential indicator bisoxonol), thus decreasing substantially the electrochemical driving force for calcium [38]. In control cells stimulated with 1 μ M ATP, which transiently increased $[Ca^{2+}]_i$ and depolarized the membrane potential, the thapsigargin-evoked increase in $[Ca^{2+}]_i$ was attenuated (119 ± 23 and 278 ± 9 nM in ATP-stimulated and control cells, respectively; $P < 0.05$). Furthermore, in cells stimulated with 3 μ M S1P, the thapsigargin-evoked increase in $[Ca^{2+}]_i$ (180 ± 10 nM) was attenuated compared with that observed in control cells (258 ± 9 nM; $P < 0.05$).

Figure 2 C1P-evoked increase in $[\text{Ca}^{2+}]_i$ is mediated by a PLC-dependent pathway

(A) Fura 2-loaded cells were pretreated with 5 μM U73122 or U73343 for 1.5 min. The cells were then stimulated with 3 μM C1P in a calcium-free buffer. Each bar gives the mean $\pm$ S.E.M. of 4–8 separate experiments. (B) Effect of 3 μM C1P or vehicle (Control) on the production of IP_3 in FRTL-5 cells. Each bar gives the mean $\pm$ S.E.M. of three separate experiments. * $P < 0.05$ compared with control cells.

In cells stimulated with 3 μM C1P in a calcium-free buffer, readdition of calcium (final concentration, 1 mM) evoked a rapid increase in $[\text{Ca}^{2+}]_i$ (428 ± 30 nM, compared with 175 ± 19 nM in control cells; $P < 0.05$) which then decreased to control levels (143 ± 16 and 136 ± 9 nM in C1P-treated and control cells, respectively; Figure 3A). Furthermore, when barium (final concentration, 1 mM) was added instead of calcium, we obtained a more pronounced increase in intracellular barium in cells stimulated with C1P compared with control cells (Figure 3B). In addition, when cells were stimulated with 3 μM C1P in a calcium-containing buffer containing 1 μM gadolinium, the C1P-evoked calcium entry was substantially hampered (Figure 3C). Thus C1P invokes SOC entry in our cells. Interestingly, in cells stimulated with both C1P and thapsigargin, the entry of calcium was not enhanced compared with cells stimulated with thapsigargin only (349 ± 14 and 413 ± 28 nM in control and C1P-stimulated cells, respectively).

In cells treated with wortmannin (an inhibitor of phosphoinositide 3-kinase; 1 μM for 10 min) or PP1 (an inhibitor of Src kinase; 1 μM for 30 min), the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ was of similar magnitude to the increase obtained in control cells (results not shown). In addition, calphostin C (an inhibitor of protein kinase C; 100 nM for 15 min) did not affect the C1P-

Figure 3 C1P-evoked capacitative entry of calcium and barium

(A) Fura 2-loaded cells were stimulated with 3 μM C1P in a calcium-free buffer and then calcium (final concentration, 1 mM) was added (trace b). Trace a, control cells. (B) The cells were stimulated with 3 μM C1P in a calcium-free buffer and then barium (final concentration, 1 mM) was added (trace b). Trace a, control cells. (C) To the cells was first added 1 μM gadolinium and then the cells were stimulated with 3 μM C1P (trace b) in calcium buffer. Trace a, control cells. Each trace is representative of 4–6 separate experiments.

evoked response. Furthermore, pretreatment of the cells with 100 nM PMA for 2.5 min attenuated the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ (145 ± 17 compared with 262 ± 31 nM in control cells; $P < 0.05$). We also observed that activation of G-protein-coupled receptors attenuated the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ in a calphostin C-sensitive manner (results not shown). Thus, the increase in $[\text{Ca}^{2+}]_i$ was probably not the result of an activation of protein kinase C.

Effect of C1P on intracellular S1P production

G-protein-coupled receptors may evoke an increase in $[\text{Ca}^{2+}]_i$ by activating a sphingosine kinase and the production of intracellular S1P [32]. In cells pretreated for 10 min with 15 μM of

Figure 4 C1P and intracellular S1P

(A) Fura 2-loaded cells were pretreated with 15 μ M DHS (trace b) or 15 μ M DMS (trace c) for 10 min and then stimulated with 3 μ M C1P. Trace a, control cells. (B) Summary of the effects of a 10 min pretreatment of the cells with 15 μ M either DHS, DMS or *N*-acetyl sphingosine (N-AcS) on the change in $[Ca^{2+}]_i$ evoked by 3 μ M C1P. The bars give the means $\pm$ S.E.M. of 4–6 separate experiments. (C) The cells were stimulated with 3 μ M C1P at 37 $^{\circ}$ C (trace a) and 22 $^{\circ}$ C (trace b). (D) Effect of 3 μ M C1P on the relative amount of intracellular S1P in FRTL-5 cells. Each point gives the mean $\pm$ S.E.M. of four separate determinations.

either DMS or DHS, two inhibitors of sphingosine kinase, the C1P-evoked increase in $[Ca^{2+}]_i$ was clearly attenuated (Figure 4). Incubating the cells with 15 μ M *N*-acetyl sphingosine, an inactive SP derivative, for 10 min did not modulate the increase in $[Ca^{2+}]_i$ evoked by 3 μ M C1P (474 ± 19 and 454 ± 16 nM in *N*-acetyl sphingosine-treated and control cells, respectively). Furthermore, in control experiments, neither DHS nor DMS attenuated the ATP-evoked increase in $[Ca^{2+}]_i$ or the thapsigargin-evoked calcium entry (results not shown). In cells incubated at 22 $^{\circ}$ C, stimulation with 3 μ M C1P evoked only a marginal increase in $[Ca^{2+}]_i$ compared with cells incubated at 37 $^{\circ}$ C (Figure 4). In control experiments, the increase in $[Ca^{2+}]_i$ evoked by 1 μ M ATP was also decreased ($P < 0.05$) in cells at 22 $^{\circ}$ C (236 ± 16 nM) compared with cells at 37 $^{\circ}$ C (371 ± 29 nM), but the shape of the calcium transient was indistinguishable in the two groups (results not shown). The result thus suggests that the effect of C1P on $[Ca^{2+}]_i$ apparently requires a metabolic process, possibly via activation of a sphingosine kinase.

We next investigated whether C1P stimulated the activation of a sphingosine kinase and the concomitant production of S1P in the cells. We were unable to measure a C1P-evoked increase in $[^3H]S1P$ production after stimulation of cells grown in normal serum-containing medium (results not shown). However, in cells that had been serum starved for 24 h, 3 μ M C1P evoked a significant ($P < 0.05$) increase in the intracellular S1P concentration (Figure 4). In serum-starved cells, C1P evoked an increase in $[Ca^{2+}]_i$ (558 ± 40 nM), comparable with that seen in control cells (514 ± 39 nM).

Lack of an effect of C1P on cellular cAMP levels

Several sphingolipid derivatives decrease cellular cAMP levels, and S1P decreases the cAMP level in FRTL-5 cells in a pertussis toxin-sensitive manner [29,39]. However, we were unable to show any effects of C1P on the forskolin-induced cellular cAMP content (the highest tested dose was 3 μ M C1P; results not shown).

Lack of an effect of C1P on DNA synthesis

It was not possible to obtain a stimulatory effect of 3 μ M C1P itself on the incorporation of $[^3H]$ thymidine into DNA after a 24–96 h incubation period (results not shown). Furthermore, 3 μ M C1P did not enhance or inhibit the incorporation of $[^3H]$ thymidine into DNA in cells stimulated with either 1 m-unit/ml TSH, 5 μ g/ml insulin or 100 nM PMA (results not shown). Furthermore, C_8 -C1P itself was also without an effect on the incorporation of $[^3H]$ thymidine into DNA (results not shown).

DISCUSSION

In the present report we show, for the first time, that C1P is capable of evoking an increase in $[Ca^{2+}]_i$, and that this effect is probably mediated via the production of both IP_3 and intracellular S1P. The response in $[Ca^{2+}]_i$ is composed of both the

mobilization of sequestered calcium and the influx of extracellular calcium. Presently, very little is known about the effects of C1P itself on cellular events, or of the interactions of C1P with signalling cascades. In fibroblasts, C1P is a potent mitogen, although the mechanism of action is not understood [17,18]. In osteoblastic cells, C1P stimulates proliferation by a mitogen-activated protein kinase-dependent mechanism [40]. C1P has also been shown to inhibit the adrenaline-evoked changes in $[\text{Ca}^{2+}]_i$ in rat aortic smooth muscle and to relax these arteries after an adrenaline-evoked contraction [19]. In the brain, a role for C1P is implicated by the findings that a ceramide kinase and a C1P phosphatase have been identified in membranes of synaptic vesicles [41,42]. A role for C1P during neutrophil phagocytosis and liposome fusion has also been indicated [43].

We show that C1P evokes an increase in $[\text{Ca}^{2+}]_i$, which is, in part, sensitive to pertussis toxin, suggesting the involvement of a G_i/G_o protein and the activation of the PLC/ IP_3 signalling cascade. This was also shown to be the case, as the PLC inhibitor U73122 attenuated the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ and C1P evoked an increase in the production of IP_3 . Interestingly, we were unable to abolish the effects of C1P on $[\text{Ca}^{2+}]_i$ using several compounds known to modulate the production of inositol phosphates (neomycin) and the binding of IP_3 to its receptor (Xestospongine C, 2-APB). Xestospongine C has been proven to be ineffective also in other cells [36]. We cannot exclude the possibility that part of the effect of U73122 is the result of a depletion of intracellular calcium stores. In addition, we cannot exclude the possibility that the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ was mediated by a protein kinase C isoform insensitive to PMA and calphostin C, but sensitive to DHS and DMS.

The present results also suggest that the effect of C1P on $[\text{Ca}^{2+}]_i$ is mediated by an increase in intracellular S1P. We base our suggestion on the following observations. First, C1P evoked an increase in cellular S1P. Second, the effect of C1P on $[\text{Ca}^{2+}]_i$ was clearly decreased in cells pretreated with either DMS or DHS, two inhibitors of sphingosine kinase [44,45]. Although both compounds may have effects other than inhibition of sphingosine kinase [46], they are presently the most specific inhibitors available. The marked effect of both inhibitors also suggests that the effect of C1P is not due to a deacylation of C1P to S1P. Thus in a manner similar to that observed with several other calcium-mobilizing agonists activating G-protein-coupled receptors (see [47]), C1P evokes an increase in intracellular production of S1P. This increase in S1P then mobilizes sequestered calcium.

An interesting suggestion is that the PLC/ IP_3 signalling pathway may in some way interact with the activation of sphingosine kinase [48]: a small, local IP_3 -evoked increase in $[\text{Ca}^{2+}]_i$ could trigger the activation of sphingosine kinase. The possibility thus exists that inhibitors of the IP_3 receptor (e.g. Xestospongine C, 2-APB) would be less effective in attenuating the overall calcium response, as even small IP_3 -evoked changes in $[\text{Ca}^{2+}]_i$ may activate sphingosine kinase and the production of S1P. On the other hand, inhibitors of PLC (e.g. U73122), by hampering the production of IP_3 and the concomitant local change in $[\text{Ca}^{2+}]_i$, may interfere more effectively with the activation of sphingosine kinase, resulting in an attenuated overall change in $[\text{Ca}^{2+}]_i$.

The FRTL-5 cells express the sphingolipid receptor Edg5 [25,26] and a presently uncharacterized lysoPA receptor [24]. It is also possible that our cells express some of the other known sphingolipid receptors. Thus it is possible that C1P could modulate $[\text{Ca}^{2+}]_i$ by interacting with either one of these receptors, and naturally we cannot exclude this possibility. However, the following observations suggest that this is less likely. First, SPC,

S1P and lysoPA all are mitogens in FRTL-5 cells [23,24,39,49], whereas we were unable to show any mitogenic effects for C1P. Second, S1P decreased TSH-evoked cAMP levels in FRTL-5 cells [39], whereas C1P was without an effect. Third, in RH7777 cells, S1P was without an effect on $[\text{Ca}^{2+}]_i$, whereas C1P evoked a small, but distinct, increase in $[\text{Ca}^{2+}]_i$ (as the IP_3 signalling pathway apparently exists in RH7777 cells, the modest increase in $[\text{Ca}^{2+}]_i$ we observed can possibly be due to a lack of intracellular S1P receptors). Finally, both S1P and C1P depolarized the plasma membrane potential, but only S1P attenuated the thapsigargin-evoked entry of $[\text{Ca}^{2+}]_i$. Furthermore, previous binding studies have shown that C1P binds poorly, if at all, to the Edg family of receptors [50]. It is thus possible that C1P may activate a novel sphingolipid receptor. It is likely that several unknown SP receptors exist, as shown by recent findings [51]. Our result can also indicate an effect of intracellular C1P. In this case intracellular C1P could interact directly with a G-protein, resulting in activation of the PLC/ IP_3 pathway. Another possibility is that a G-protein is acting in a permissive manner on an intracellular pathway activated by C1P, enhancing the effect of C1P. Thus the effect of C1P need not be mediated by a G-protein-coupled receptor. Additional investigations are needed to clarify this possibility.

The physiological importance of C1P is presently obscure. Sphingomyelin is present predominantly in the outer leaflet of the plasma membrane [52] and a ceramide kinase is associated with the plasma membrane [41,43]. It is thus conceivable that high concentrations of C1P can be formed in the plasma membrane, where it could efficiently interact with several proteins, e.g. receptors or channels. Earlier studies have also shown that the membrane-associated ceramide kinase in synapses is activated in a calcium-dependent manner [41]. Furthermore, some cell types also express a ceramide phosphate phosphatase, which is regulated by a calcium-dependent mechanism [42]. In addition, a lipid phosphate phosphohydrolase is present on the outside of the plasma membrane, at least in Rat2 fibroblasts [53]. Thus several enzymes regulating the production and degradation of C1P are present in cells.

Previous studies have shown that agonists that invoke changes in $[\text{Ca}^{2+}]_i$ in FRTL-5 cells rapidly depolarize plasma membrane potential by activating calcium-dependent Cl^- channels [54]. Depolarization of the plasma membrane potential attenuates calcium entry in these cells by decreasing the electrochemical driving force for calcium [38]. It is thus surprising that C1P, although it depolarizes the plasma membrane potential, does not attenuate thapsigargin-evoked SOC entry in our cells. Presently we have no explanation for this effect. One possibility is that C1P (or intracellular S1P) can activate an additional calcium-entry pathway, thus enhancing calcium entry.

In conclusion, the effects of C1P on calcium regulation in rat thyroid cells have been characterized. The results showed that C1P potently modulates $[\text{Ca}^{2+}]_i$, apparently by the production of both IP_3 and intracellular S1P. As enzymes regulating the production and degradation of C1P exist in many cell types, C1P may be a sphingolipid of an importance not hitherto fully recognized.

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