Ceramide 1-phosphate enhances calcium entry through voltage-operated calcium channels by a protein kinase C-dependent mechanism in GH₄C₁ rat pituitary cells

Kid TÖRNQUIST*^{†1}, Tomas BLOM*, Ramin SHARIATMADARI‡ and Michael PASTERNAK§²

*Department of Biology, Åbo Akademi University, BioCity, Artillerigatan 6, 20520 Turku, Finland, †The Minerva Foundation Institute for Medical Research, Biomedicum Helsinki, 00290 Helsinki, Finland, ‡Department of Physiology, Institute of Biomedicine, University of Turku, 20520 Turku, Finland, and §The Institute of Biotechnology, 00014 University of Helsinki, Helsinki, Finland

Sphingomyelin derivatives modulate a multitude of cellular processes, including the regulation of $[Ca^{2+}]_i$ (the intracellular free calcium concentration). Previous studies have shown that these metabolites often inhibit calcium entry through VOCCs (voltage-operated calcium channels). In the present study, we show that, in pituitary GH₄C₁ cells, C1P (C₂-ceramide 1-phosphate) enhances calcium entry in a dose-dependent manner. The phospholipase C inhibitor U73122 attenuated the response. C1P invoked a small, but significant, increase in the formation of inositol phosphates. Pre-treatment of the cells with pertussis toxin was without an effect on the C1P-evoked increase in $[Ca^{2+}]_i$. The effect of C1P was critically dependent on extracellular calcium, since no increase in $[Ca^{2+}]_i$ was observed when cells in a calcium-free buffer were stimulated with C1P. Furthermore, if the cells were retreated with 300 nM of the VOCC inhibitor nimodipine, the effect of C1P was almost totally abolished. In addition, ceramide C₈-1-phosphate evoked an increase in $[Ca^{2+}]_i$, but the onset of

the response was slow compared with that of C1P. In cells treated with 1 μ M thapsigargin for 15 min, C1P still evoked an increase in $[Ca^{2+}]_i$. In patch-clamp experiments in the whole-cell mode, C1P enhanced calcium entry through the VOCCs compared with vehicle-treated cells. Dialysis of the cells with C1P did not enhance the calcium current. On-cell patch-clamp experiments showed an enhanced probability of the VOCCs being open (P_{open}) in the presence of C1P. Inhibition of PKC (protein kinase C) with GF109203X and down-regulation of PKC with PMA attenuated the C1P-evoked increase in $[Ca^{2+}]_i$. Furthermore, down-regulation of PKC abolished the effect of C1P on P_{open} . This is the first report showing that a sphingomyelin derivative enhances calcium entry through VOCCs.

Key words: calcium entry, calcium homeostasis, ceramide 1-phosphate, nimodipine, sphingolipid, voltage-operated calcium channel.

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, S1P (sphingosine 1-phosphate) and SPC (sphingosylphosphorylcholine), also have diverse effects on the regulation of $[Ca^{2+}]_i$ (the intracellular free calcium concentration) in both non-excitable and excitable cells. In non-excitable 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 S1P_{1–5} [2], and in addition two SPC-specific receptors, OGR1 (ovarian cancer G-protein-coupled receptor 1) [4] and GPR4 (G-protein-coupled receptor 4) [5]. Sphingosine is a potent inhibitor of SOC entry (store-operated calcium entry) via inhibition of I_{CRAC} (calcium-release-activated calcium current) [6]. In addition to effects mediated by plasma membrane receptors, a putative 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]. Sphingosine and SPC potently attenuate VOCCs (voltage-operated calcium channels) and ryanodine receptors in excitable cells, such as cardiac and pituitary cells [3,8,9]. Ceramides, 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, C₈-ceramide attenuates SOC entry in non-excitable cells [12–14]. C₈-ceramide also inhibits the L-type VOCCs in pinealocytes [15] and attenuates K⁺ channels [16,17].

In addition to ceramide, C1P (C₂-ceramide 1-phosphate) is a putative modulator of cellular functions (e.g. see [1]), although little is known with regard to its physiological significance. C1P enhances DNA synthesis in fibroblasts, although the mechanism of action is still not known [18,19]. According to these reports, C1P does not modulate $[Ca^{2+}]_i$. On the other hand, recent results suggest that C1P may attenuate phenylephrine-evoked changes in $[Ca^{2+}]_i$ in rat aortic smooth muscle [20]. In a recent study, we have shown that C1P evokes a potent increase in $[Ca^{2+}]_i$ in thyroid FRTL-5 cells, which seems to be due to an increased level of both inositol 1,4,5-trisphosphate and intracellular S1P [21]. However, C1P may be considered a sphingoid form of PA (phosphatidic acid). Furthermore, PA and lysoPA bind to Edg2, Edg4 and

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; C1P, C₂-ceramide 1-phosphate; C₈ 1P, C₈-ceramide 1-phosphate; $[Ca^{2+}]_i$, intracellular free calcium concentration; EDG, endothelial differentiation gene; fura 2/AM, fura 2 acetoxymethyl ester; (lyso)PA, (lyso)phosphatidic acid; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PKC, protein kinase C; PLC, phospholipase C; P_{open} , probability (of the VOCCs) being open; S1P, sphingosine 1-phosphate; SOC entry, store-operated calcium entry; SPC, sphingosylphosphorylcholine; TRH, thyrotropin-releasing hormone; VOCCs, voltage-operated calcium channels.

¹ To whom correspondence should be addressed, at the Department of Biology, Åbo Akademi University (e-mail kid.tornqvist@abo.fi).

² Present address: OrionPharma, Orionintie 1, 02200 Espoo, Finland.

Edg7, receptors that are closely related structurally to the sphingolipid receptors [22,23]. Furthermore, lysoPA, at least, is a weak agonist for the S1P₁ receptor [24]. The GH₄C₁ cells express at least the sphingolipid receptor S1P₂ [25]. Thus we considered it to be of importance to investigate the effects of C1P on the regulation of [Ca²⁺]_i. Our results have shown that C1P evoked an entry of extracellular calcium into GH₄C₁ cells by a PKC (protein kinase C)-mediated activation of VOCCs.

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.). C1P and C₈1P (C₈-ceramide 1-phosphate) were purchased from Avanti Lipids (Alabaster, AL, U.S.A.). Pertussis toxin, lysoPA, GF109203X, U73122 and U73343, OAG (1-oleoyl-2-acetyl-*sn*-glycerol) and PMA were all purchased from Sigma. Amprep[®] mini-columns (SAX, 100 mg; RPN 1908) and *myo*-[³H]inositol with PT6-271 (1 mCi/ml) were from Amersham International (Amersham, Little Chalfont, Bucks., U.K.). fura 2/AM (fura 2 acetoxymethyl ester) and BAPTA [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid] were purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Thapsigargin was from LC Services Corp (Woburn, MA, U.S.A.). All other chemicals used were of reagent grade.

Cell culture

Clonal rat pituitary cells were originally given by Dr Armen H. Tashjian, Jr (Harvard University, Boston, MA, U.S.A.). The cells were grown in monolayer culture in Ham's F10 nutrient mixture containing 15 % (v/v) horse serum and 2.5 % (v/v) fetal-calf serum in a water-saturated atmosphere of 5 % CO₂ and 95 % air at 37 °C, as described previously [26]. Before an experiment, cells from one culture dish were harvested with PBS containing 0.05 mM EDTA and plated on to 100 mm culture dishes. The cells were grown for 7–9 days, with two changes of the culture medium. Fresh medium was always added to the cells 24 h prior to an experiment.

Measurement of [Ca²⁺]_i in GH₄C₁ cells: cell-suspension experiments

Cells were harvested with Hepes-buffered salt solution (HBSS, containing in mM: NaCl, 118; KCl, 4.6; CaCl₂, 1; glucose, 10; and Hepes, 20 mM, pH 7.4) lacking CaCl₂, but containing 0.05 mM EDTA. After washing the cells three times with HBSS buffer containing 1 mM CaCl₂, the cells were incubated with fura 2/AM (final concentration 2 μM) for 30 min at 37 °C. Following the loading period, the cells were incubated for at least 20 min to ensure complete cleavage of the AM group from fura 2. After washing the cells once more, 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 (Hitachi, Tokyo, Japan). The excitation wavelengths were 340 nm and 380 nm, and emission was measured at 510 nm. The signal was calibrated by the addition of 1 mM CaCl₂ and 10 % (v/v) Triton X-100 to obtain maximal fluorescence (*R*_{max}). Then, extracellular Ca²⁺ was chelated with 5 mM EGTA and the pH was elevated above 8.3 with Tris base to obtain minimum fluorescence (*R*_{min}). [Ca²⁺]_i was calculated as described by Grynkiewicz et al.

[27] using a computer program designed for the fluorimeter, with a *K*_d value of 224 nM for fura 2.

Calcium-imaging experiments

Cells from one 100 mm cell culture dish were harvested using 0.02 % EDTA solution, and plated on to polylysine-coated round coverslips (diameter 2.5 cm). After 2–4 days of culture, the cells were incubated with fura 2/AM (final concentration 4 μM) for 30 min at 37 °C. Following the loading period, the cells were incubated for at least 20 min to ensure complete cleavage of the AM-group from fura 2. After washing the cells once, the coverslip was mounted on a holder that was mounted on an Axiovert 35-inverted microscope with a Fluor 40× objective, and perfused with HBSS. The excitation filters were set at 340 nm and 380 nm, and the excitation light was obtained using an XBO 75W/2 xenon lamp. Emission was measured at 510 nm. The shutter was controlled by a Lambda 10-2 control device (Sutter Instruments, Novato, CA, U.S.A.). Fluorescence images were collected with a SensiCam 12BIT charge-coupled-device (CCD) camera (PCO/CD Imaging, Kelheim, Germany). Images were taken every 2.6 s to avoid bleaching, and the images were processed using Axon Imaging Workbench 2.1 software (Axon Instruments, Foster City, CA, U.S.A.). The results are given as the 340 nm/380 nm ratio. The experiments were performed at 37 °C.

Measurement of inositol phosphates

The cells were pre-incubated with *myo*-[³H]inositol (10 μCi/100 mm dish) for 48 h. After harvesting, the cells were suspended in HBSS. 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 10 μM) for 30 s at 37 °C. Inositol phosphates were extracted using 10 % (v/v) HClO₄, and then separated using Amprep[®] (SAX) mini-columns [28]. The radioactivity of the samples was measured by liquid scintillation counting.

Measurement of cellular cAMP

The cells were harvested as described for the calcium experiments, and were pre-incubated in HBSS for 20 min in the presence of 0.3 mM isobutylmethylxanthine (to inhibit phosphodiesterase activity). Then, aliquots of cells (0.5 × 10⁶ cells/tube) were stimulated with 10 μM C1P. After 15 min, the reaction was stopped with 50 μl of HClO₄ added 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 [29].

Patch-clamp experiments

The studies were performed using the patch-clamp technique in the whole-cell mode and in the cell-attached mode [30]. The electrodes were made from GC150TF glass capillaries (Harvard Apparatus, Edenbridge, Kent, U.K.). In the whole-cell experiments, the electrodes had a resistance of 3–5 MΩ when filled with Hepes-buffered salt solution I (HBSS-I buffer, containing in mM: CsCl, 90; MgCl₂, 5; CaCl₂, 2.5; MgATP²⁻, 4; GTP, 0.1; BAPTA, 10; glucose, 10; tetraethylammonium chloride, 20; leupeptin, 0.1; creatine phosphate, 6; creatine phosphokinase, 20 units/ml; Hepes, 20; pH 7.2, adjusted with CsOH). Leak and capacitive currents in the whole-cell recordings were compensated for by the P/4 routine from a holding potential of –80 mV. The access resistance was 7–15 MΩ. No series resistance compensation was made. Before an experiment, the coverslips were attached to a perfusion chamber, and the cells were perfused with HBSS-Ba (containing

in mM: BaCl_2 , 60; tetraethylammonium chloride, 75; glucose, 10; Hepes, 10; pH 7.4, adjusted with tetraethylammonium hydroxide) with or without the appropriate concentrations of C1P using a gravity perfusion system. The recordings were made with an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) at room temperature (22 °C). The cells were held at -80 mV. A stock solution of C1P (10 mM) was made in DMSO. The final concentration of the solvent did not exceed 0.2 %. This concentration of the solvent did not have any effects on VOCCs in GH_4C_1 cells. Data analysis of all experiments was made using the Pulsefit program (HEKA Elektronik, Lambrecht, Germany).

In the cell-attached single-channel recordings, the cells were bathed in a high-potassium HBSS buffer (HBSS-K, containing in mM: KCl, 140; MgCl_2 , 1.13; glucose, 10; Hepes, 10; pH 7.2, adjusted with KOH) to abolish the membrane potential. In these experiments, the pipettes were filled with HBSS-BaII buffer (containing in mM: BaCl_2 , 110; tetraethylammonium chloride, 10; Hepes, 10; pH 7.2, adjusted with tetraethylammonium hydroxide), and had a resistance of 10–20 M Ω . Leak and capacitive currents in the single-channel experiments were subtracted and compensated for in all recordings. Data analysis of all experiments was made using the TAC and TACFit programs (Heka Elektronik).

Statistics

The results are expressed as the means $\pm$ S.E.M. Each experiment was repeated at least three times. Statistical analysis was made by using Student's *t* test for paired observations. When three or more means were tested, one way analysis of variance was used, followed by Bonferroni's multiple comparison test.

RESULTS

Effects of C1P on $[\text{Ca}^{2+}]_i$ in GH_4C_1 cells

Previous studies have shown that sphingosine, S1P or SPC do not invoke an increase in $[\text{Ca}^{2+}]_i$ in GH_4C_1 cells [31]. However, when the cells were stimulated with C1P, a dose-dependent increase (EC_{50} 0.2 μM) in $[\text{Ca}^{2+}]_i$ was observed (Figure 1). The increase in $[\text{Ca}^{2+}]_i$ was critically dependent on extracellular calcium, as no C1P-evoked increase in $[\text{Ca}^{2+}]_i$ was obtained with cells incubated in a calcium-free buffer (Figure 1). The increase appeared to be mediated by calcium entry through VOCCs, as the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ was substantially attenuated in cells incubated with 300 nM nimodipine (Figure 1).

We tested whether the effect of C1P on $[\text{Ca}^{2+}]_i$ was sensitive to pertussis toxin, but observed that the increase in $[\text{Ca}^{2+}]_i$ evoked with 10 μM C1P in cells pre-treated with pertussis toxin (50 ng/ml for 24 h) was indistinguishable from that observed in control cells (Figure 1). Thus the effect of C1P was not mediated by a G_i/G_o protein. However, when the cells were pre-treated for 2 min with 5 μM of the PLC (phospholipase C) inhibitor U73122, the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ was attenuated (Figure 1). The inactive analogue U73343 was without an effect. In addition, stimulating the cells with 10 μM C1P for 30 s evoked a small, but significant ($P < 0.05$) increase in the formation of inositol phosphates (Figure 1). In cells pre-treated with 1 μM thapsigargin for 15 min to deplete intracellular calcium stores, C1P evoked an increase in $[\text{Ca}^{2+}]_i$ that was of a similar magnitude to that seen in control cells (Figure 1). Furthermore, stimulating the cells with 10 μM C1P for 15 min did not increase the intracellular concentration of cAMP (49 ± 3 fmol/ 0.5×10^6 cells and 42 ± 3 fmol/

0.5×10^6 cells; $n = 3$) in control cells and cells stimulated with C1P respectively.

In control experiments using 10 μM C_8IP , we also observed an increase in $[\text{Ca}^{2+}]_i$ (162 ± 23 nM; $n = 4$), but the response was much slower in onset compared with that for C_2IP (Figure 2). As with C1P, the increase evoked by C_8IP was attenuated by nimodipine.

To characterize further the effects of C1P on $[\text{Ca}^{2+}]_i$ in GH_4C_1 cells, we performed calcium-imaging studies on single cells. Two main types of responses were obtained: a sustained increase in $[\text{Ca}^{2+}]_i$ and a more transient response (Figure 3). The different response of individual cells to exogenous stimulation is typical for GH_4C_1 cells [32]. In the presence of 300 nM nimodipine, we never observed an increase in $[\text{Ca}^{2+}]_i$ in response to C1P (Figure 3).

Next, we wanted to compare the effects of C1P with that of other sphingolipid derivatives. Our previous study showed that ceramide, S1P, sphingosine and SPC did not increase $[\text{Ca}^{2+}]_i$ in GH_4C_1 cells [31]. However, as lysoPA binds to the same family of Edg receptors as S1P, we wanted to investigate the effects of lysoPA on $[\text{Ca}^{2+}]_i$ in GH_4C_1 cells. LysoPA evoked an increase in $[\text{Ca}^{2+}]_i$ that was of similar magnitude to that of C1P (Figure 4). However, in contrast with C1P, lysoPA evoked a small increase in $[\text{Ca}^{2+}]_i$ in cells incubated in a calcium-free buffer (Figure 4). Furthermore, in cells incubated with 300 nM nimodipine, the plateau phase of the lysoPA-evoked increase in $[\text{Ca}^{2+}]_i$ was attenuated, but not abolished (Figure 4). Thus the mechanisms of action of C1P and lysoPA on $[\text{Ca}^{2+}]_i$ were distinctly different. In cells pre-treated with 1 μM thapsigargin, 10 μM lysoPA evoked an increase in $[\text{Ca}^{2+}]_i$ (168 ± 8 nM) that was blocked by 300 nM nimodipine. In control experiments, we observed that stimulating thapsigargin-treated cells with 100 nM TRH (thyrotropin-releasing hormone) also evoked a nimodipine-sensitive entry of calcium (216 ± 32 nM). We thus conclude that depletion of intracellular calcium stores does not abolish receptor-invoked activation of the VOCCs (see [33]).

Action of C1P on VOCCs in GH_4C_1 cells

To characterize the mechanism of action of C1P on $[\text{Ca}^{2+}]_i$, we investigated the effect of C1P on VOCCs using patch-clamp. When patch-clamp was used in the whole-cell mode, we obtained a rapid rundown of the VOCCs (results not shown), similar to that in a previous study [8]. In cells perfused with 10 μM C1P, the rundown was consistently slower compared with control cells (Figure 5). A significant effect was observed at 10 μM only, due to the fact that the experiments were performed at room temperature. When control calcium experiments were performed at room temperature, the cells also had to be stimulated with 10 μM C1P to obtain an increase in $[\text{Ca}^{2+}]_i$ (results not shown).

As several transport mechanisms for lipids may exist in cells, we wanted to investigate whether the effect of C1P was mediated by cellular uptake of C1P. However, in experiments where the pipette solution contained 10 μM C1P, we were unable to observe any difference in the I/I_{max} relationship compared with control cells (results not shown). Thus the effect of C1P was not due to an intracellular effect of C1P.

We have recently shown that C1P increases the production of S1P in thyroid cells [21]. Thus it is possible that the effect of C1P was mediated by the formation of intracellular S1P. To test this possibility, we dialysed the cells with 30 μM S1P in the pipette solution. In these experiments we could not observe an enhanced calcium current in cells dialysed with S1P, compared with control cells (results not shown). Thus the effect of C1P was not mediated by the formation of intracellular S1P.

Figure 1 Effect of C1P on $[Ca^{2+}]_i$ in GH₄C₁ cells

The cell suspension was loaded with fura 2, as described in the Materials and methods section. **(A)** The cells were stimulated with 10 μ M C1P in a calcium-containing buffer (panel a), in a calcium-containing buffer in the presence of 300 nM nimodipine (panel b) and in a calcium-free buffer (panel c). Each trace is representative of four to seven separate experiments. **(B)** Dose-dependent effect of C1P on $[Ca^{2+}]_i$. **(C)** Cells in suspension were stimulated with 10 μ M C1P: in a calcium buffer (Control); in a calcium-free buffer (Ca-free); in a calcium buffer containing 300 nM nimodipine (Nimo); after treatment of the cells with 50 ng/ml pertussis toxin for 24 h (Ptx); and after treating the cells with 1 μ M thapsigargin for 15 min (Tg-pre). **(D)** Effect of 5 μ M of either U73122 or U73443 on the C1P-evoked increase in $[Ca^{2+}]_i$. **(E)** C1P-evoked increase in inositol phosphates. The cells were stimulated with 10 μ M C1P for 30 s. Each bar represents the mean $\pm$ S.E.M. of four to seven separate experiments, except in **(E)**, where $n = 3$. Asterisks show $P < 0.05$.

Figure 2 Effect of C₈1P on $[Ca^{2+}]_i$ in GH₄C₁ cells in suspension

Fura 2-loaded cells were stimulated with 10 μ M C₈1P, and the increase in $[Ca^{2+}]_i$ was measured. Nimodipine (Nimo; 300 nM) was added to the cells, as shown by the arrow on the right. The trace is representative of four separate recordings.

To characterize further the effects of C1P, we performed on-cell patch-clamp experiments. In our hands, the patches were very silent, and showed very few openings of the VOCCs, probably due to the fact that the experiments were performed at room temperature. Thus the probability of the VOCCs being open (P_{open}) was very low. However, during the course of the experiments we were able to obtain an increase in P_{open} ($P < 0.05$) of the VOCCs in cells in which the pipette contained 10 μ M C1P ($P_{open} 0.06 \pm 0.01$), compared with control cells ($P_{open} 0.01 \pm 0.003$; Figure 6). These results thus suggest that the C1P-evoked increase in $[Ca^{2+}]_i$ is due to an enhanced calcium entry through VOCCs.

Previous studies on the regulation of $[Ca^{2+}]_i$ in GH₄C₁ cells have shown that agonists activating the PLC-signalling pathway activate calcium entry through VOCCs by a mechanism dependent on the activation of PKC, and that stimulation of the cells with OAG or PMA also increased $[Ca^{2+}]_i$ [34]. When we stimulated our cells with 10 μ M OAG, $[Ca^{2+}]_i$ increased by 44 ± 5 nM. The increase was attenuated by nimodipine. Similar results were obtained with 30 nM PMA. We thus pre-treated the cells with

Figure 3 Effect of C1P on $[\text{Ca}^{2+}]_i$ in single GH_4C_1 cells

The cells were loaded with fura 2, and the experiments were performed as described in the Materials and methods section. The effect of $10 \mu\text{M}$ C1P on $[\text{Ca}^{2+}]_i$ in three representative cells is shown (a–c). For comparison, the cells were stimulated with 100 mM K^+ (KCl). A total of 16 cells were investigated. In (c), the cell was incubated in the presence of 300 nM nimodipine. A total of 11 cells were investigated.

200 nM PMA for 24 h to down-regulate PKC. In these experiments, the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ was attenuated (Figure 7). Furthermore, in cells treated for 2 min with $10 \mu\text{M}$ of the PKC inhibitor GF109203X, the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ was attenuated. We next performed patch-clamp experiments in the on-cell mode with cells pre-treated with 200 nM PMA for 24 h to down-regulate PKC. In these experiments, P_{open} was extremely low, and $10 \mu\text{M}$ C1P was unable to enhance P_{open} (Figure 7). Our results thus suggest that, in GH_4C_1 cells, C1P enhances calcium

Figure 4 Effects of lysoPA on $[\text{Ca}^{2+}]_i$ in GH_4C_1 cells

Cells in suspension were stimulated with $10 \mu\text{M}$ lysoPA in a calcium-containing buffer (a), in the presence of 300 nM nimodipine (b), or in a calcium-free buffer (c). Each trace is representative of four to seven separate experiments.

entry through VOCCs by a mechanism dependent on activation of PKC.

DISCUSSION

In the present paper, we have described the effects of C1P on calcium fluxes in pituitary GH_4C_1 cells, and show that C1P enhances calcium entry through VOCCs. This is a remarkable observation, because most sphingolipids previously tested on excitable cells inhibit VOCCs. In cardiac cells, brain cells and pituitary GH_4C_1 cells, both sphingosine and SPC inhibit VOCCs [8,9,35]. In the sarcoplasmic reticulum, sphingosine inhibits the ryanodine receptor [36]. Furthermore, in pinealocytes ceramide inhibits VOCCs [15]. Our previous study showed that ceramide slightly attenuated high potassium-evoked calcium entry in GH_4C_1 cells [31]. Thus C1P provides a novel mechanism of action of sphingolipids on VOCCs.

At present, there are only a few reports on the cellular effects of C1P. In fibroblasts, C1P is a potent mitogen, although the mechanism of action is currently not understood [18,19]. C1P also activates phospholipase A_2 , and enhances the release of arachidonic acid [37]. In osteoblastic cells, C1P stimulates proliferation by a mitogen-activated-protein-kinase-dependent mechanism [38]. Furthermore, C1P has been shown to inhibit the phenylephrine-invoked changes in $[\text{Ca}^{2+}]_i$ in rat aortic smooth muscle, and to relax these arteries after a phenylephrine-invoked contraction [20]. 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 [39,40]. A role for C1P during neutrophil phagocytosis and liposome fusion has also been indicated [41]. However, in all these reports, the mechanism of action of C1P was not determined.

Our results suggest that C1P enhances calcium entry through VOCCs by enhancing the probability of the channels being open. Furthermore, this effect seems to involve activation of the PLC-signalling pathway, since the effect of C1P on the VOCCs was attenuated by U73122 and inhibition of PKC. We also showed that C1P enhanced the formation of inositol phosphates.

The GH_4C_1 cells express the S1P_2 receptor [25]. Thus we cannot exclude the possibility that C1P could modulate $[\text{Ca}^{2+}]_i$ and the VOCCs by interacting with this receptor. However, it is interesting to note that S1P, which binds to the S1P_2 receptor with high affinity, is without an effect on either $[\text{Ca}^{2+}]_i$ or VOCCs in GH_4C_1 cells [31]. Binding experiments in cells overexpressing the receptors for S1P have also shown that C1P competes poorly with S1P for binding to the receptors [42,43]. Thus C1P is probably not acting through the S1P-sensitive receptors known

Figure 5 Effect of C1P on calcium channel currents in GH₄C₁ cells

Representative traces of Ba^{2+} currents obtained by depolarizing the cells from -80 mV to 0 mV. (A) Currents obtained from a cell prior to (trace a) and after (trace b) application of vehicle (0.1% DMSO). (B) Currents obtained from a cell prior to (trace a) and after (trace b) application of $10\ \mu\text{M}$ C1P. (C) Summary of the effect of C1P on the normalized peak Ba^{2+} current. The panel shows vehicle currents (■) and currents obtained after application of $10\ \mu\text{M}$ C1P (▲). (D) Concentration-dependent effect of C1P on the normalized peak Ba^{2+} current. All recordings were made at room temperature ($\approx 22^\circ\text{C}$). Each bar or data point shows the mean $\pm$ S.E.M. of five to seven separate recordings (* $P < 0.05$).

at present. Theoretically, C1P could interact with the lysoPA receptors Edg2, Edg4 and Edg7, which are structurally closely related to the sphingolipid receptors S1P₁, S1P₃ and S1P₂ [22,23]. In our experiments, however, the effects of lysoPA on $[\text{Ca}^{2+}]_i$ were strikingly different from that of C1P. First, lysoPA evoked a small, but distinct increase in $[\text{Ca}^{2+}]_i$ in cells incubated in a calcium-free buffer. Furthermore, in the presence of nimodipine, lysoPA evoked a substantial increase in $[\text{Ca}^{2+}]_i$, very similar to that of other agonists acting through a G-protein-coupled mechanism (e.g. TRH; see [33]). The results obviously suggest that lysoPA and C1P modulate $[\text{Ca}^{2+}]_i$ by different mechanisms. Interestingly, C1P, lysoPA and TRH all evoked an increase in $[\text{Ca}^{2+}]_i$ in cells treated with thapsigargin to deplete intracellular calcium

Figure 6 Effect of C1P as seen in single-channel recordings in GH₄C₁ cells

The experiments were performed using the cell-attached patch-clamp configuration. (A) Representative sweeps from recordings in a control cell. (B) Representative sweeps from a recording with $10\ \mu\text{M}$ C1P in the pipette solution. Both silent sweeps and sweeps with openings are shown for comparison. (C) The calculated P_{open} from a control recording (Control) and a recording with $10\ \mu\text{M}$ C1P (C1P) in the pipette solution. The results are the means $\pm$ S.E.M. of six to eight separate recordings (* $P < 0.05$). For the calculations, the sweeps obtained 2 min after formation of the gigaseal were used.

stores. This increase in $[\text{Ca}^{2+}]_i$ was sensitive to nimodipine. One possibility is thus that C1P acts through some as-yet-unknown receptor. It is clear that C1P does not evoke a non-specific permeabilization of the plasma membrane, because the C1P-evoked increase in $[\text{Ca}^{2+}]_i$ obtained with all doses of C1P tested always was abrogated by nimodipine.

Figure 7 Inhibition of PKC attenuates the effect of C1P on $[\text{Ca}^{2+}]_i$ in GH_4C_1 cells

(A) Cells in suspension were treated with 10 μM GF109203X (GF) for 2 min, or were treated with 200 nM PMA for 24 h to down-regulate PKC (PKC d-r), and then stimulated with 10 μM C1P. Each bar shows the mean $\pm$ S.E.M for three to seven separate recordings (* $P < 0.05$). (B) The calculated P_{open} from single-channel recordings from cells treated with 200 nM PMA for 24 h to down-regulate PKC. The bars show the mean $\pm$ S.E.M of recordings from four control cells (Vehicle) and five cells with 10 μM C1P (C1P) in the pipette solution. For the calculations, the sweeps obtained 2 min after formation of the gigaohm were used.

Sphingomyelin is present predominantly in the outer leaflet of the plasma membrane [44], and ceramide kinase is associated with the plasma membrane [40,41]. 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. channel proteins. Thus our observation that C1P modulates VOCCs may have important consequences for the cells. First, the enhanced entry of calcium can have a beneficial effect on the proliferation of the cells. An enhanced proliferation was also observed in fibroblasts stimulated with C1P [18,19]. Secondly, an increased influx of calcium enhances secretion from secretory cells, including GH_4C_1 cells [33], and may potentially enhance hormone gene expression [45]. Furthermore, earlier studies have shown that the membrane-associated ceramide kinase in synapses is activated in a calcium-dependent manner [40]. A possibility is that C1P forms a part of the mechanisms regulating calcium entry, and thus secretion, in cells.

In conclusion, the effects of C1P on $[\text{Ca}^{2+}]_i$ have been characterized in GH_4C_1 cells. The results showed that C1P enhanced calcium entry through VOCCs. Thus our results reveal a novel mechanism of action of sphingolipid derivatives on cellular calcium homeostasis.

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