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Ceramide 1-(2-cyanoethyl) phosphate enhances store-operated Ca^{2+} entry in thyroid FRTL-5 cells

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Abstract

Sphingolipid derivatives cause diverse effects towards the regulation of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in a multitude of nonexcitable cells. In the present investigation, the effect of C-8 ceramide-1-(2-cyanoethyl) phosphate (C1CP) on store-operated Ca^{2+} (SOC) entry was investigated. C1CP evoked a modest increase in $[Ca^{2+}]_i$. The increase was inhibited by the SOC channel antagonist 1-(β-[3-(4methoxyphenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole (SKF96365) but not by overnight pretreatment of the cells with pertussis toxin. C1CP did not invoke the production of inositol phosphates. When cells were stimulated with both C1CP and thapsigargin, the thapsigargin-invoked increase in $[Ca^{2+}]_i$ was enhanced in comparison to control cells. When Ca^{2+} was added to cells treated with both C1CP and thapsigargin in a Ca^{2+}-free buffer, the increase in $[Ca^{2+}]_i$ was enhanced in comparison to control cells. In patch-clamp experiments, C1CP hyperpolarized the membrane potential (E_m) of the cells and attenuated the thapsigargin-invoked depolarization of the E_m. The effects of C1CP came, in part, as a result of a decreased conductance of the cell membrane towards Cl^- ions, as C1CP in a Cl^--free solution also enhanced Ca^{2+} entry. Barium 2-cyanoethylphosphate (Ba2Cy), which also contains the 2-cyanoethyl group, did not modulate thapsigargin-invoked changes in $[Ca^{2+}]_i$ nor did it modulate the E_m. In conclusion, C1CP enhances SOC entry, in part, via hyperpolarization of the E_m and attenuation of the thapsigargin-invoked membrane depolarization, thus increasing the electrochemical gradient for Ca^{2+} ions. Hence, C1CP may be a useful reagent for investigating the cellular effects of ceramide derivatives.

Keywords: Ca^{2+}, intracellular; Sphingolipid; Thyroid; Current-clamp; Cl^- channel

1. Introduction

Sphingomyelin derivatives, i.e. sphingosines and ceramides, modulate a multitude of cellular events including proliferation, apoptosis, angiogenesis, and cellular migration (Mathias et al., 1998; Spiegel, 1999; Hla et al., 1999). Sphingosine derivatives, e.g. sphingosine, sphingosine 1-phosphate (S1P), and sphingosylphosphorylcholine (SPC), potently modulate the regulation of the intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ in several cell types. In non-excitable cells, S1P and SPC mobilize sequestered Ca^{2+} and activate Ca^{2+} entry (Meyer zu Heringdorf et al., 1997; Spiegel, 1999; Hla et al., 1999)). Most of these effects are mediated by G-protein-coupled plasma membrane receptors for S1P, and presently, five S1P-specifc receptors have been identified, namely Edg1, Edg3, Edg5, Edg6, and Edg8 (Hla et al., 1999; Im et al., 2000). In addition, two SPC-specific receptors, OGR1 (Xu et al., 2000) and GPR4 (Zhu et al., 2001), have been identified.

Sphingosine is a potent inhibitor of store-operated Ca^{2+} (SOC) entry (i.e. by inhibiting the Ca^{2+}-release activated Ca^{2+} current) (Mathes et al., 1998). In addition, sphingosine and SPC potently block voltage-operated Ca^{2+} channels and ryanodine receptors in several excitable cell types (Meyer zu Heringdorf et al., 1997; Titievsky et al., 1998). Ceramides per se, which are produced prior to sphingosine when sphingomyelin is hydrolyzed, have not been reported to

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invoke any changes in $[Ca^{2+}]_i$. In contrast to these observations, C8-ceramide has been shown to attenuate store-operated Ca^{2+} entry in nonexcitable cells (Breittmeyer et al., 1994; Törnquist et al., 1999) and to inhibit the L-type voltage-operated Ca^{2+} channels in pinealocytes (Chik et al., 1999).

In addition to ceramide, ceramide 1-phosphate (C1P) may be an important modulator of cellular functions (see Mathias et al., 1998), but comparatively little is known in regard to its physiological significance. In fibroblasts, C1P enhances DNA synthesis by an unknown mechanism (Gomez-Munoz et al., 1995, 1997). According to these reports, C1P per se does not modulate $[Ca^{2+}]_i$. C1P may, however, be considered a sphingoid form of phosphatidic acid, and phosphatidic acid and lyso-phosphatidic acid bind to Edg2, Edg4, and Edg7, receptors closely related structurally to the sphingolipid receptors (Goetzl and An, 1998; Bandoh et al., 1999). In addition, at least lyso-phosphatidic acid is a weak agonist for the Edg1 receptor (Lee et al., 1998). Several reports have shown that lyso-phosphatidic acid stimulates proliferation and mobilizes sequestered Ca^{2+} in Fisher rat thyroid cell line-5 (FRTL-5) cells (Falasca et al., 1995; Okajima et al., 1997), and the FRTL-5 cells also express the sphingolipid receptor Edg5 (Nikmo et al., 1999). Furthermore, we have shown that $C_2$1P increase $[Ca^{2+}]_i$ in FRTL-5 cells.[1] In the present study, we have investigated the effect of ceramide 1-(2-cyanoethyl) phosphate (C1CP), a compound produced in the final steps of C1P synthesis. The results showed that C1CP invoked a small increase in the $[Ca^{2+}]_i$ in FRTL-5 cells. A most remarkable finding was that C1CP potently enhanced store-operated Ca^{2+} entry in these cells. This effect was invoked, in part, as a result of hyperpolarizing the membrane potential (E_m) and attenuating the thapsigargin-invoked depolarization of the E_m, thus maintaining an electrochemical gradient for Ca^{2+}.

2. Materials and methods

2.1. Materials

Culture medium, serum, and hormones were purchased from Gibco (Grand Island, NY, USA), Biological Industries (Beth Haemek, Israel), and Sigma (St. Louis, MO, USA). Culture dishes were obtained from Falcon Plastics (Oxnard, CA, USA) or from Greiner (Germany). Ceramide 1-(2-cyanoethyl) phosphate, authentic $C_8$1P, and *N*-hexanoyl-sphingosine (C6-ceramide) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). $C_8$1P and $C_2$1P were also from Avanti Lipids (Alabaster, AL, USA). Staurosporin, phorbol 12-myristyl 13-acetate (PMA), okadaic acid, pertussis toxin, lyso-phosphatidic acid, sphingosine, sphingosylphosphorylcholin, arachidonyltrifluoromethyl ketone ($AACOCF_3$), and Wortmannin and 1-(6-[8[17*b*]-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-1*H*pyrrole-2,5-dione (U73122) were all purchased from Sigma. 4-Amino-1-*tert*-butyl-3-(1′-naphtyl)pyrazolo[3,4-*d*]pyrimidine (PP1) was from Calbiochem (Lutterworth, UK). Barium 2-cyanoethylphosphate (Ba2Cy) hydrate was from Aldrich Chemical (Milwaukee, WI, USA). Calphostin C was from Alexis (Laufelfingen, Switzerland). Amprep® mini-columns (SAX, 100 mg, RPN 1908), *myo*-[^{3}H]inositol with PT6-271 (1 mCi/ml), and [methyl-^{3}H]thymidine (70–90 Ci/mmol) were from Amersham International (Amersham, UK). Fura 2-acethoxymethylester (Fura 2-AM) and bisoxonol were purchased from Molecular Probes (Eugene, OR, USA). Thapsigargin was from LC Services (Woburn, MA, USA). All other chemicals used were of reagent grade. Bovine thyroid stimulating hormone was a generous gift from Dr. A.F. Parlow (the National Institute of Diabetes and Digestive and Kidney Diseases' National Hormone and Pituitary Program).

2.2. Cell culture

Rat thyroid FRTL-5 cells, originally obtained from the Interthyr Foundation (Bethesda, MD), was a generous gift of Dr. Egil Haug (Akers Hospital, Oslo, Norway). The cells were grown in Coon's modified Ham's F 12 medium supplemented with 5% calf serum and together with six hormones (Ambesi-Impiombato et al., 1980) (insulin, 10 μg/ml; transferrin, 5 μg/ml; hydrocortisone, 10 nM; tripeptide gly-L-his-L-lys, 10 ng/ml; thyroid stimulating hormone, 0.3 mU/ml; somatostatin, 10 ng/ml) 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 0.2% trypsin solution and plated onto plastic 100-mm culture dishes. The cells were grown for 7–8 days incorporating two to three changes of culture medium with the final medium change always occurring 24 h prior to an experiment.

2.3. Synthesis and identification of ceramide 1-(2-cyanoethyl) phosphate

The synthetic pathway and the structure of ceramide 1-(2-cyanoethyl) phosphate is shown in Fig. 1.

2.3.1. Synthesis of 1

Imidazole (3 eq.) followed by dimethyl-*t*-butylsilyl chloride (1.25 eq.) were added to C8-ceramide in dry dimethyl formamide (0.25 M) and the resulting solution left to stir overnight at room temperature. The reaction mixture was quenched with water and extracted with diethyl ether. The organic phase was washed with brine and dried over Na_2SO_4. Flash chromatography (silica gel; 50% ether/hexane) of the residue after removal of solvent produced (**1**) in 65% yield.

[1] Högback et al., manuscript in preparation.

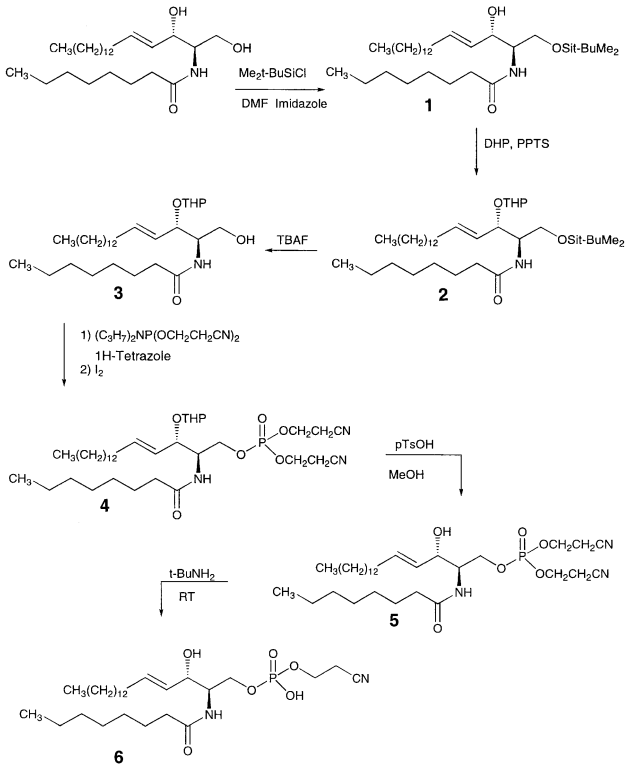

Fig. 1. Synthetic pathway and structure of C1CP. See Materials and methods for details.

2.3.2. Synthesis of 2

Dihydropyran (3 eq.) and pyridinium-*p*-toluenesulfonate (0.1 eq.) were added to 1-*O*-silyl-C8-ceramide (**1**) in methylene chloride (0.025 M), and the resulting solution was left to stir overnight at room temperature. The reaction mixture was diluted with ethyl acetate, washed with water followed by brine, and dried over Na_2SO_4. Flash chromatography (silica gel; 5–95% ethyl acetate/petroleum ether to 10%) produced the desired product (**2**) in 87% yield.

2.3.3. Synthesis of 3

Tetrabutylammonium fluoride (1.5 eq., 1 M in tetrahydrofuran) was added to 1-*O*-silyl-3-*O*-tetrahydropyranyl-C8-ceramide (**2**) in tetrahydrofuran (0.1 M) at 0 °C and the resulting solution was left to stir overnight at 0 °C. The reaction was diluted with ethyl acetate, washed with water followed by brine, and dried over Na_2SO_4. Flash chromatography (silica gel; 3% methanol/methylene chloride) produced the desired product (**3**) in 88% yield.

2.3.4. Synthesis of 4

Sublimed 1*H*-tetrazole (2 eq.) followed by bis-2-cyanoethyl diisopropyl phosphoramidite (1.6 eq.) was added to 3-*O*-tetrahydropyranyl-C8-ceramide (**3**) in dry acetonitrile/methylene chloride (50:50, 0.06 M). The reaction mixture was stirred at room temperature for 2 h; after which, 0.4 M I_2 solution (in 3:1:1 pyridine/water/methylene chloride) was added dropwise until the orange color persisted upon which the reaction was quenched with 10% $Na_2S_2O_3$. The organic phase was washed with water followed by brine and dried over Na_2SO_4. Flash chromatography (silica gel; 50–75% ethyl acetate/petroleum ether to 25%) produced the desired product (**4**) in 84% yield.

2.3.5. Synthesis of 5

p-Toluenesulfonic acid (0.1 eq.) was added to 3-*O*-tetrahydropyranyl-1-(bis(2-cyanoethyl)phosphoro)-C8-ceramide in methanol (0.03 M). After stirring overnight at room temperature, the reaction mixture was diluted with ethyl acetate washed with water and brine and dried over Na_2SO_4. The crude product was used directly in the next step.

2.3.6. Synthesis of 6

t-Butylamine/methylene chloride (25%) was added to crude 1-*O*-(biscyanoethylphosphoro)-C8-ceramide and stirred at room temperature for 3 h. The reaction mixture was evaporated to dryness and ethyl acetate was added followed by acetic acid (1 ml). The organic phase was washed with water and brine, and then dried over Na_2SO_4. Flash chromatography (silica gel; 90:10:3 chloroform/methanol/acetic acid) produced the desired product (**6**) in 50% yield.

Nuclear magnetic resonance (NMR) spectra were acquired on a JEOL Alpha 500 NMR spectrometer equipped with either a 5-mm normal configuration tunable probe or a 5-mm inverse *z*-axis field-gradient probe operating at 500.16 MHz for ^1H, 125.78 MHz for ^{13}C, and 202.47 MHz for ^{31}P. The spectra were recorded at 30 °C in d_6-dimethylsulphoxide; ^1H spectra were referenced internally to tetramethylsilane (0 ppm), ^{13}C spectra were referenced internally to the middle peak of the solvent (39.5 ppm), whilst ^{31}P spectra were referenced externally to 90% H_3PO_4 in D_2O (0 ppm).

1D proton spectra were acquired with single-pulse excitation, 45° flip angle, pulse recycle time of 9 s, and with spectral widths of 7 kHz consisting of 32-k data points (digital resolution 0.22 Hz/pt) zero-filled to 128 k prior to Fourier transformation. The strong water signal was suppressed by presaturation. Field gradient double-quantum filtered correlation spectroscopy (FG DQF COSY) spectra were acquired in phase-sensitive mode with spectral widths appropriately optimised from the 1D spectra, and processed with zero filling ($\times 2$, $\times 4$) and exponential weighting (3–5 Hz) applied in both dimensions prior to Fourier transformation.

1D carbon spectra were acquired with single-pulse excitation, 45° flip angle, pulse recycle time of 4 s, and with spectral widths of 34 kHz consisting of 64 k data points (digital resolution 0.53 Hz/pt), zero-filled to 128 k and with 1 Hz exponential weighting applied prior to Fourier transformation. Distortionless enhancement by polarisation transfer (DEPT) 135° spectra were acquired with similar

spectral windows and with a pulse sequence delay time of 3 s. Field gradient heteronuclear single-quantum correlation (FG HSQC, phase-sensitive) and heteronuclear multiple-bond correlation (HMBC, magnitude mode) experiments were both acquired with spectral widths appropriately optimized from the 1D spectra and processed with zero filling ($\times 2$, $\times 4$) and exponential weighting applied in both dimensions (5 and 20 Hz) prior to Fourier transformation (an additional 2P/3-shifted sinebell function was applied in both dimensions of the HMBC spectra). Both experiments utilized a $^{1}J_{HC}$ coupling of 145 Hz, whilst the HMBC correlations were optimized for a long-range $^{n}J_{HC}$ coupling of 8 Hz.

1D phosphorous spectra were acquired with single-pulse excitation, 65° flip angle, pulse recycle time of 6 s, and with spectral widths of 21 kHz consisting of 32 k data points (digital resolution 0.66 Hz/pt), zero-filled to 64 k and with 1 Hz exponential weighting applied prior to Fourier transformation. Both proton decoupled and coupled spectra were acquired, the latter under much higher resolution conditions (digital resolution 0.05 Hz/pt, zero-filled $\times 4$). Low-resolution mass spectroscopy (MS) were acquired on a PE Sciex API 365 LC/MS/MS instrument using direct infusion and electrospray ionization (ESI) in -ve mode (source temperature 300 °C).

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

The medium was aspirated, and the cells were harvested with 1.0: *N*-(2-hydroxyethyl)piperazine-*N*′ -(2-ethanesulfonic acid) (HEPES) buffered saline solution (HBSS, in millimolar concentrations: NaCl, 118; KCl, 4.6; glucose, 10; $CaCl_2$, 1.0; HEPES, 20; pH 7.2) lacking Ca^{2+} but containing 0.02% ethylenediaminetetraacetic acid (EDTA) and 0.1% trypsin. After washing the cells three times by pelleting, the cells were incubated with 1 μM Fura 2-acethoxymethylester 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 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 ethylene glycol-bis(2aminoethyleter)-*N*,*N*,*N*′,*N*′-tetraacetic acid (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. (1985) using a computer program designed for the fluorimeter with a K_d value of 224 nM for Fura 2. For experiments in Cl^--free buffer, equimolar concentrations of the gluconate salts of Na^+, K^+, and Ca^{2+} were used in the buffer solution.

For the experiments, C1CP was applied as an ethanol solution. The concentration of the solute never exceeded 0.1% and in the control experiments ethanol alone was used.

2.5. Measurement of Ca^{2+} ATPase phosphorylation

The cells were grown on 100-mm dishes in 6H as described above. The method is a modification of Kuo et al. (1991). Confluent cells were washed three times in phosphate-free minimal essential medium (MEM) and then incubated with the same medium containing [^{32}P]orthophosphate (50 μCi/ml) for 4 h in a water-saturated atmosphere of 5% CO_2 and 95% air at 37 °C. The test compounds were then added for 15 min; at which time, the incubation was halted by removing the medium and washing the cells with cold phosphate-buffered saline solution (PBS, in millimolar concentrations: NaCl, 150; $NaHPO_4$, 10; pH 7.4). The cells were scraped and homogenized in 0.8-ml lysis buffer (in millimolar concentrations: NaCl, 150; phenylmethylsulfonyl fluoride, 1; EDTA, 5; EGTA, 5; sodium vanadate, 100; NaF, 50; dithiotreitol, 2; Tris/HCl, 10; pH 7.4, containing 1% Nonidet-P40, 1% bovine serum albumin, and 5 mg/ml leupeptin). The cells were extracted in the lysis buffer for 1 h. Cell debris was removed by centrifugation (20 000 × *g* for 30 min at 4 °C) and the lysates were cleared by incubating with 5 μg/ml rabbit anti-mouse immunoglobulin, 5% protein A-Sepharose, and 3 μl normal ascetic fluid for 40 min at 4 °C. The cleared lysates were then incubated for 12–16 h at 4 °C with 3 μl of the 5F10 monoclonal antibody under gentle shaking. Subsequently, 5 μg/ml of rabbit anti-mouse immunoglobulin was added and the incubation was continued for 30 min. Immunocomplexes were then adsorbed onto 5% protein A-Sepharose at 4 °C for 1 h under gentle shaking. The immunoprecipitate protein A-Sepharose was centrifuged (200 × *g* for 1 min at 4 °C) and then washed five times with lysis buffer (as above, but without bovine serum albumin). Laemmli sample buffer (50 μl) was then added to the pellet and the complex was dissociated by treatment at 92 °C for 5 min. The samples were then centrifuged (200 × *g* for 1 min at 4 °C) and the supernatants were stored at −20 °C. The proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel, whereafter the gel was dried and autoradiographied.

2.6. Measurement of inositol phosphates

The cells were preincubated with *myo*-[^{3}H]inositol (6 μCi per 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 then finally, the cells were stimulated with either vehicle or C1CP (final concentration 10 μM) for 10 min at 37 °C. Inositol phosphates were extracted using 10% perchloric acid and then separated using Amprep® (SAX) mini-columns (Oldham, 1990). The radioactivity of the samples was measured by liquid scintillation counting. In control experiments where the cells were stimulated with ATP, this protocol gave the most reliable results.

2.7. Measurement of [^{3}H]thymidine incorporation in FRTL-5 cells

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

2.8. Measurement of membrane potential using bisoxonol

The cells were grown and harvested as for the Ca^{2+} experiments. After the final wash, the cells were added to a quartz cuvette and bisoxonol was added (final concentration 100 nM). The cells were equilibrated with bisoxonol for at least 15 min prior to an experiment. The cells were stimulated with either the appropriate agonist or vehicle. The excitation wavelength was 540 nm and emission was measured at 580 nm (Rink et al., 1980). Each experiment was calibrated by the addition of 50 mM K^+ to depolarize the cells to enable comparison between different experiments.

2.9. Electrophysiological measurements of the membrane potential

The studies were performed using the patch-clamp whole-cell technique in voltage- and current-clamp mode (Hamill et al., 1981). Prior to the experiments, cells were harvested with 0.02% EDTA-trypsin solution and subcultured on coverslips (10^5 cells/well) on 24-well plates (Falcon, Beckton & Dickinson, NJ, USA) for 2–5 days. The coverslips were placed in a perfusion chamber with a volume of approximately 500 μl. During recordings, the cells were continuously perfused with a standard solution containing (in mM): NaCl, 150; KCl, 5.4; $MgCl_2$, 1; $CaCl_2$, 1.8; HEPES, 5 (pH adjusted to 7.4 with NaOH) at 0.5 ml/min or a low-Cl^- (5.6 mM Cl^-) solution with Cl^- substituted for gluconate. C1CP (10 μM) and thapsigargin (1 μM) were added to the superfusate from stock solutions of 10 mM C1CP in ethanol and 1 mM thapsigargin in dimethylsulphoxide. No significant effects on the membrane potential (E_m) recordings were observed with respect to the agonist solute concentrations (<0.2%).

Patch pipettes were made from GC150TF glass micropipettes (Harvard Apparatus, Kent, UK) and had a resistance of 2–5 MΩ (when filled with an internal solution containing (in mM): KCl, 150; $MgCl_2$, 2; 1,2-bis(aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraaceric acid (BAPTA), 5; HEPES, 10; pH was adjusted to 7.2 with KOH). Cells were also dialyzed with C1CP. All recordings were made at room temperature. Currents were recorded with an EPC-9 amplifier (HEKA, Lambrecht, Germany). Analyses were made using Pulse and Pulse Fit software (HEKA). A liquid junction potential of 3 mV was added to all data.

2.10. Statistics

The results are expressed as the mean ± S.E. Statistical analysis was made using Student's *t*-test for paired observations. When three or more means were tested, analysis of variance was used.

3. Results

3.1. Structure of C1CP

The synthetic route and the structure of C1CP are shown in Fig. 1. The structural elucidation of CICP followed accordingly from MS and NMR studies. LC/MS analysis (ESI, -ve ion mode) of an authentic sample of C1P showed the correct M^--H ion at 504 amu whilst C1CP displayed an M^--H ion at 557 amu, implying substitution of a phosphate H in C1P with a 2-cyanoethyl group ($-CH_2CH_2CN$) to yield C1CP.

The ^{1}H and ^{13}C NMR spectra of an authentic sample of C1P were consistent with the given structure of C1P; in comparison, the ^{1}H and ^{13}C spectra of C1CP were essentially identical to those of C1P, respectively, except for three additional signals in the carbon spectra—two methylenes, both with phosphorous coupling at 59 and 19 ppm, and a quaternary at 119 ppm all pertaining to, and consistent with, the 2-cyanoethyl group—and two additional multiplets of 2Hs, each in the proton spectra—one triplet at 2.75 ppm with apparent phosphorous coupling, and the other of higher order at 3.8 ppm, again pertaining to, and consistent with, the extra pair of methylene sets in the 2-cyanoethyl group. The assignments, where possible, were made by the standard application of DEPT and 2-D COSY, HSQC, and HMBC experiments. The high degree of overlap in the alkyl chains precluded full assignment, and indeed, resolution of these inconsequential signals.

The ^{31}P chemical shifts of the two samples were essentially the same (ca. 4 ppm). The proton-coupled ^{31}P spectrum of C1CP displayed an approximate quintet in the ratio of 1:4:6:4:1, i.e. equal coupling to four protons ($J \sim 8.3$ Hz).

The sample used in the cell experiments was determined to be pure C1CP as indicated by the aforementioned 1D NMR spectra. As many of the intermediate compounds in the synthesis may have cellular effects, a rigorous purity determination of the sample was made.

3.2. Action of C1CP on $[Ca^{2+}]_i$

Stimulation of FRTL-5 cells with C1CP invoked a modest, concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 2). At the highest concentration tested (10 μM), a transient increase in $[Ca^{2+}]_i$ was first observed, followed by an elevated plateau phase in $[Ca^{2+}]_i$, which was inhibited by 1-(β-[3-(4methoxyphenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride (SKF96365), an inhibitor of store-operated Ca^{2+} entry (Fig. 2). In a Ca^{2+}-free buffer, C1CP had a negligible effect per se on $[Ca^{2+}]_i$ (10 μM C1CP invoked a 5 ± 2-nM increase in $[Ca^{2+}]_i$). However, after stimulation of the cells with 10 μM C1CP in a Ca^{2+}-free buffer, an enhanced increase in $[Ca^{2+}]_i$ was obtained upon addition of Ca^{2+} to the cells compared with control cells (Fig. 2). This effect of C1CP was inhibited by SKF96365 (Fig. 2). In control experiments with 10 μM $C_8$1P, the increase in $[Ca^{2+}]_i$ was 129 ± 27 and 67 ± 19 nM in a Ca^{2+}-containing and Ca^{2+}-free buffer, respectively. These results obtained with $C_8$1P must be taken with caution, however, due to the poor solubility of $C_8$1P in our hands.

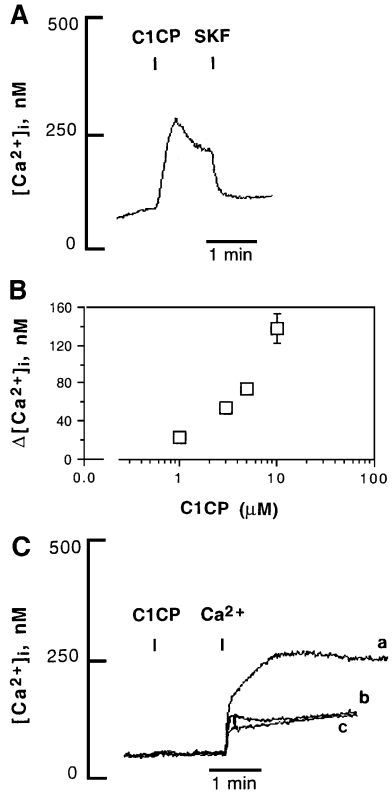

Fig. 2. C1CP modulates $[Ca^{2+}]_i$ in thyroid FRTL-5 cells. The cells were harvested and loaded with Fura 2 as described in Materials and methods. (A) The cells were stimulated first with ceramide 1-(2-cyanoethyl) phosphate (C1CP, final concentration 10 μM), and then with SKF96365 (final concentration 30 μM), and the changes in $[Ca^{2+}]_i$ were measured. (B) Concentration-dependent effect of C1CP on $[Ca^{2+}]_i$ in FRTL-5 cells. Each point gives the mean ± S.E. of four to eight separate experiments. (C) The cells were stimulated with C1CP (final concentration 10 μM) in a Ca^{2+}-free buffer, and then Ca^{2+} (final concentration 1 mM) was added to the cells (trace a). In trace b, the cells were first treated with SKF96365 (final concentration 30 μM), then with C1CP, and finally, Ca^{2+} was re-added to the cells. Trace c shows control cells treated with vehicle and Ca^{2+}. Each trace shows a representative recording of four to six separate experiments.

We next investigated the mechanism of action of C1CP on $[Ca^{2+}]_i$ and measured whether C1CP could induce the production of inositol phosphates. In these experiments, 10 μM C1CP did not stimulate hydrolysis of phosphatidyl-4,5-bisphosphate to inositol phosphates (372 ± 66 cpm/tube, compared with 327 ± 7 cpm/tube in control cells, $N=3$). In control experiments, we were unable to obtain an increase in inositol phosphates after stimulation of the cells with 10 μM $C_8$1P (results not shown).

Furthermore, in cells pretreated with pertussis toxin (50 ng/ml for 24 h) or with the phospholipase C-inhibitor U73122 (5 μM for 10 min), 10 μM C1CP still invoked an increase in $[Ca^{2+}]_i$ (214 + 33 and 105 + 13 nM, respectively, $N=4$). The results thus suggest that C1CP increases $[Ca^{2+}]_i$ in FRTL-5 cells apparently by a pertussis toxin- and U73122-insensitive mechanism. In control experiments, pretreatment with pertussis toxin resulted in a small but significant decrease in the $C_8$1P-evoked $[Ca^{2+}]_i$ response (38 ± 3 nM compared with 55 ± 8 nM in control cells).

3.3. Lack of an effect of C1CP on DNA synthesis

It was not possible to obtain a stimulatory effect of 10 μM C1CP on the incorporation of [^{3}H]thymidine into DNA after either a 24-h incubation period (78 ± 14 and 73 ± 9 cpm/plate in C1CP-treated cells and vehicle-treated cells, respectively, $N=3$) or a 48-h incubation period (215 ± 27 and 197 ± 19 cpm/plate in C1CP-treated cells and vehicle-treated cells, respectively, $N=3$). In control experiments, $C_8$1P was also without an effect on the incorporation of [^{3}H]thymidine (results not shown).

3.4. Effects of C1CP on store-operated Ca^{2+} entry

Of the sphingomyelin derivatives, both sphingosine and ceramide attenuate SOC entry (Mathes et al., 1998; Törnquist et al., 1999). In our experiments, C1CP potently enhanced the thapsigargin-invoked Ca^{2+} entry. The increase in $[Ca^{2+}]_i$ was 386 ± 53 nM in control cells and 786 ± 35 nM ($P<0.05$) in cells treated with 10 μM C1CP. We also tested the action of barium 2-cyanoethylphosphate (Ba2Cy) on $[Ca^{2+}]_i$. In these experiments, Ba2Cy was ineffective on the $[Ca^{2+}]_i$ per se or on the thapsigargin-invoked entry of Ca^{2+} (374 ± 38 and 329 ± 28 nM in control cells and cells treated with 10 μM Ba2Cy, respectively). Interestingly, 10 μM $C_8$1P did neither enhance nor inhibit the thapsigargin-evoked increase in $[Ca^{2+}]_i$ (265 ± 14 and 246 ± 30 nM in control- and $C_8$1P-stimulated cells, respectively). In a Ca^{2+}-free buffer, C1CP did not affect the thapsigargin-invoked release of sequestered Ca^{2+}. The thapsigargin-invoked increase in $[Ca^{2+}]_i$ was 148 ± 12 and 143 ± 16 nM in control cells and in cells treated with 10 μM C1CP,

respectively. However, if Ca^{2+} (final concentration 1 mM) was re-added to these cells, C1CP enhanced the Ca^{2+}-invoked increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 3). Also in these experiments, Ba2Cy was again found to be ineffective (Fig. 3). In cells treated with SKF96365, the effect of C1CP on Ca^{2+} entry was abolished (Fig. 3). In control experiments (i.e. cells not treated with either SKF96365 or C1CP), the Ca^{2+}-evoked transient increase and the new plateau level of $[Ca^{2+}]_i$ was 870 ± 20 and 449 ± 27 nM. In a Ca^{2+}-free buffer, C_81P was without an effect on the thapsigargin-evoked release of sequestered Ca^{2+}, and was without an effect on the thapsigargin-evoked entry of Ca^{2+} in the Ca^{2+} re-addition experiments (results not shown).

We next investigated the mechanism of action of C1CP on SOC entry. We first tested whether C1CP enhanced the entry of Ba^{2+} in our cells. As Ba^{2+} is a poor substrate for Ca^{2+}ATPase, it cannot be sequestered in the cells or transported out of the cell. Our experiments showed that C1CP also enhanced the entry of Ba^{2+} (Fig. 4). Furthermore, C1CP did not modulate the thapsigargin-invoked phosphorylation of the plasma membrane Ca^{2+}ATPase (results not shown). Taken together, our results suggest that C1CP increased $[Ca^{2+}]_i$ by enhancing Ca^{2+} entry, and not by blocking plasma membrane Ca^{2+}ATPases.

Sphingomyelin derivatives may modulate protein kinase C (PKC) in several cell types (Hannun and Bell, 1989) and PKC potently modulates SOC entry in FRTL-5 cells (Törnquist, 1993). In FRTL-5 cells, the α, βI, βII, γ, δ, ε, ζ, and η isoforms of PKC are expressed (Wang et al., 1996). Down-regulation of PKC by pre-incubating the cells with 2 μM PMA for 24 h, or by pretreatment of the cells with PKC-inhibitor calphostin C (100 nM for 15 min) did not attenuate the effect of C1CP on thapsigargin-evoked SOC entry (results not shown).

We also tested whether the effect of C1CP on SOC could be inhibited by okadaic acid (an inhibitor of type 1 and type 2A serine/threonine protein phosphatases), Wortmannin (an inhibitor of phosphoinositide 3-kinase), PP1 (an inhibitor of Src kinase), $AACOCF_3$ (an inhibitor of phospholipase A_2) or by pertussis toxin. None of these compounds attenuated the effect of C1CP on thapsigargin-invoked SOC entry (results not shown). Furthermore, an effect similar to that of C1CP on thapsigargin-invoked SOC entry could not be obtained with SPC, S1P, lyso-phosphatidic acid, or C6-ceramide (results not shown).

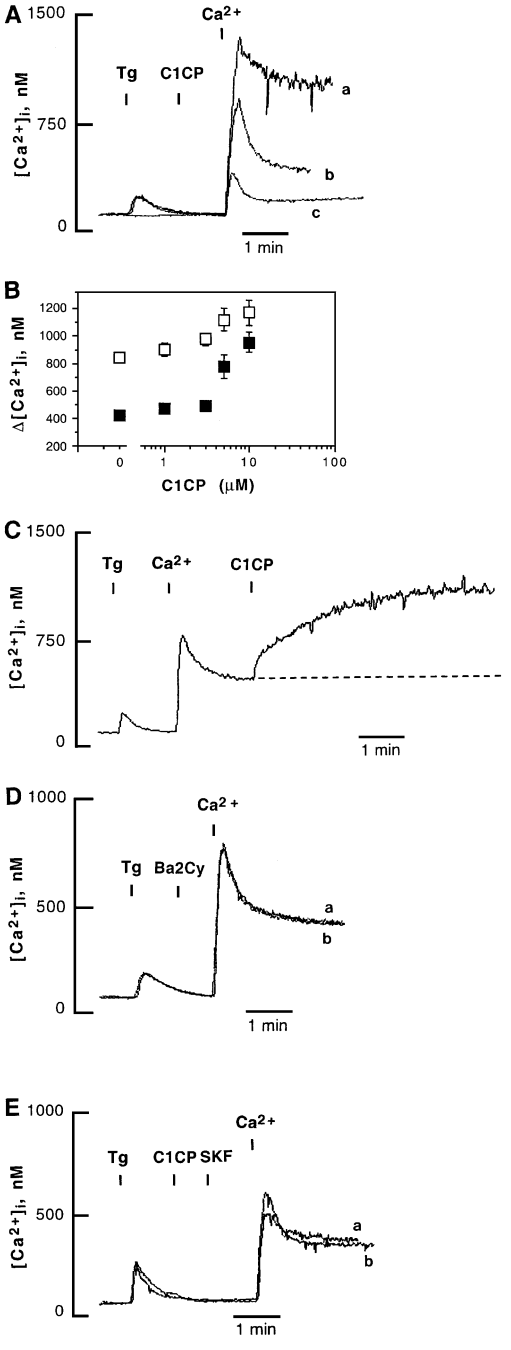

Fig. 3. C1CP enhances store-operated Ca^{2+} entry in thyroid FRTL-5 cells. The cells were harvested and loaded with Fura 2 as described in Materials and methods. (A) The cells were stimulated first with thapsigargin (final concentration1 μM), then with ceramide 1-(2-cyanoethyl) phosphate (C1CP, final concentration 10 μM) in a Ca^{2+}-free buffer, and then Ca^{2+} (final concentration 1 mM) was added to the cells (trace a). In trace b, the cells were first treated with thapsigargin and vehicle, and then Ca^{2+} was re-added to the cells. Trace c shows control cells stimulated with Ca^{2+} only. (B) Concentration-dependent effect of C1CP on the Ca^{2+}-invoked peak in $[Ca^{2+}]_i$ (□), and the new plateau level of $[Ca^{2+}]_i$ (■) in cells stimulated with thapsigargin. Each point gives the mean ± S.E. of four to eight separate experiments. (C) Cells in a Ca^{2+}-free buffer were first stimulated with thapsigargin (Tg) and then Ca^{2+} was re-added to the cells, and finally, the cells were stimulated with C1CP (final concentration 10 μM). (D) The cells were stimulated with thapsigargin, and then barium 2-cyanoethyl phosphate (Ba2Cy, final concentration 10 μM) in a Ca^{2+}-free buffer, and then Ca^{2+} was added to the cells (trace a). In trace b, the cells were first treated with thapsigargin and vehicle and then Ca^{2+} was re-added to the cells. (E) The cells were stimulated first with thapsigargin (Tg), then C1CP (final concentration 10 μM), and finally with SKF96365 (final concentration 10 μM) in a Ca^{2+}-free buffer, and then Ca^{2+} was added to the cells (trace a). Trace b shows control cells stimulated first with thapsigargin, then vehicle was added, followed by SKF96365 and Ca^{2+}. Each trace is a representative recording of five to eight separate experiments.

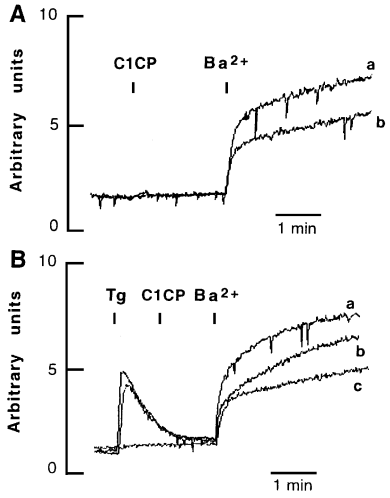

Fig. 4. C1CP enhances store-operated Ba^{2+} entry in thyroid FRTL-5 cells. The cells were harvested and loaded with Fura 2 as described in Materials and methods. (A) The cells were stimulated with ceramide 1-(2-cyanoethyl) phosphate (C1CP, final concentration 10 μM) in a Ca^{2+}-free buffer, and then Ba^{2+} (final concentration 1 mM) was added to the cells (trace a). In trace b, the cells were stimulated with vehicle, and then Ba^{2+} was re-added to the cells (trace b). (B) The cells were stimulated with thapsigargin (Tg, final concentration1 μM), and then with ceramide 1-phosphate ester (C1CP, final concentration 10 μM) in a Ca^{2+}-free buffer, and then Ba^{2+} was added to the cells (trace a). The cells were stimulated with thapsigargin and vehicle, and then Ba^{2+} was re-added to the cells (trace b). Trace c shows control cells to which Ba^{2+} only was added. Each trace is a representative recording of four separate experiments.

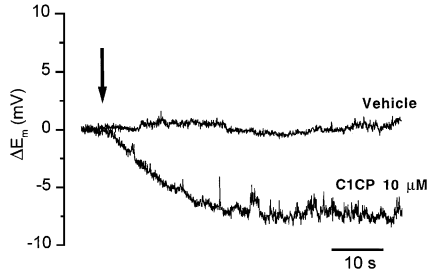

Fig. 5. A whole-cell current-clamp recording of the effect of C1CP on membrane potential in thyroid FRTL-5 cells. Ceramide 1-(2-cyanoethyl) phosphate (C1CP final concentration 10 μM) hyperpolarizes the E_m by 14 mV while a vehicle-treated cell shows no change in the E_m. The resting membrane potential in the vehicle-treated cell was −66 mV and in the C1CP-treated cell, −70 mV. Each trace is the representative recording of seven separate recordings.

3.5. Effect of C1CP on the membrane potential

In cells loaded with bisoxonol, a small but significant hyperpolarization of the E_m in response to 10 μM C1CP was observed, indicating that the effect of C1CP was not due to a nonspecific permeabilization of the cell membrane and a concomitant influx of Ca^{2+} ions. C1CP also potently attenuated the thapsigargin-invoked depolarization of the membrane potential when compared with vehicle-treated cells. In control cells, the relative thapsigargin-invoked depolarization was 50 ± 4% of that invoked by 50 mM KCl; whereas in C1CP-treated cells, the depolarization was only 24 ± 3% ($P < 0.05$). Ba2Cy had no effect on either the membrane potential per se (results not shown) or on the thapsigargin-invoked depolarization (64 ± 4% of the K^+-invoked depolarization, compared with 59 ± 5% in control cells). In control experiments, $C_8$1P depolarized the membrane potential 53 ± 5% of that obtained with 50 mM KCl.

To investigate more closely the effect of C1CP, we used the whole-cell configuration of the patch-clamp method to measure C1CP-induced membrane potential and conductance changes. In current-clamp experiments, addition of 10 μM C1CP resulted in a small but significant hyperpolarization in seven out of the seven cells examined ($P < 0.05$; Fig. 5 and Table 1). Under the experimental conditions used, a hyperpolarization is likely to be due to either an increase in membrane potassium conductance, or a decrease in Cl^- conductance due to the electrochemical gradient of these ions. Alternatively, the membrane hyperpolarization could result from the activation or inactivation of an active electrogenic transport mechanism. We measured the C1CP-induced changes in membrane conductance using sequential 5 mV hyper- and depolarizing voltage steps at a frequency of 1 Hz under voltage-clamp. In three out of three cells, addition of 10 μM C1CP induced a 90% decrease in the basal holding current coupled to a decrease in membrane conductance (Fig. 6A,B). Thus, the C1CP-evoked hyperpolarization is associated with a decrease in conductance. This supports the hypothesis that the C1CP-induced hyperpolarization is a consequence of a decrease in membrane Cl^- conductance. To confirm this, we repeated the experiments in a low-Cl^- extracellular solution (5.6 mM Cl^-). In low-Cl^- solution, C1CP did not change the membrane potential or conductance significantly (see Table 1), strengthening the conclusion that the effect of C1CP is

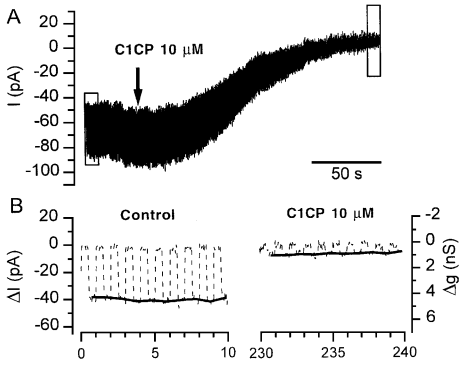

Fig. 6. A whole-cell voltage-clamp recording of the effect of C1CP on membrane conductance in thyroid FRTL-5 cells. The changes in membrane conductance were recorded under voltage-clamp using sequential 5-mV hyper- and depolarizing voltage steps at a frequency of 1 Hz. Addition of ceramide 1-(2-cyanoethyl) phosphate (C1CP, final concentration 10 μM) decreased the basal holding current, which is shown in (A). In (B), the first [Control (i)] and last [C1CP (ii)] 10 s of the trace in (A) are shown on a larger time scale. The holding current is shown with dashed line (left y-axis) and conductance with solid line (right y-axis). In this cell, the C1CP-invoked decrease in conductance was about 3 ns. The holding potential was −70 mV.

Table 1
Changes in the membrane potential of thyroid FRTL-5 cells for normal and low Cl^- concentrations under various conditions

Conditions	E_m (mV)	N
Normal Cl^-	-59 ± 2	19
Low Cl^-	-75 ± 3	9
Normal Cl^- plus	**ΔE_m (mV)**	**N**
Vehicle	-0.7 ± 0.5	7
C1CP (10 μM)	-14 ± 3*	7
Thapsigargin (1 μM)	$+22 \pm 2$	6
C1CP + thapsigargin	$+11 \pm 2$**	4
Low Cl^- plus	**ΔE_m (mV)**	**N**
C1CP (10 μM)	0 ± 2	4

The results are given as the mean ± S.E.
* $P < 0.05$ compared with vehicle.
** $P < 0.05$ compared with thapsigargin.

mediated via the closure of Cl^- channels. In addition to the above effects, 10 μM C1CP also potently attenuated ($P < 0.05$) the thapsigargin-invoked depolarization of the membrane potential (Fig. 7 and Table 1). Interestingly, when cells were dialyzed with 10 μM C1CP in the patch pipette solution, no effects on the membrane potential were observed (results not shown).

When the effect of C1CP on the thapsigargin-evoked Ca^{2+} entry was investigated in a Cl^--free buffer, we observed that the response in $[Ca^{2+}]_i$ was markedly altered but still significantly ($P < 0.05$) enhanced compared with control cells (Fig. 8). In these experiments, the Ca^{2+} evoked transient increase and plateau level of $[Ca^{2+}]_i$ was 668 ± 98

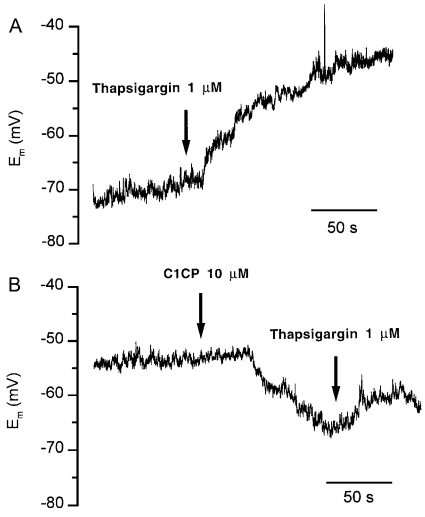

Fig. 7. A whole-cell current-clamp recording showing the attenuation of the thapsigargin-evoked depolarization by C1CP in thyroid in FRTL-5 cells. (A) Thapsigargin (final concentration 1 μM) depolarizes the E_m. (B) In the presence of ceramide 1-(2-cyanoethyl) phosphate (C1CP, final concentration 10 μM), the thapsigargin-invoked depolarization is attenuated by 50%. The data in (A) and (B) are from different cells and each trace is the representative recording of four to six separate recordings.

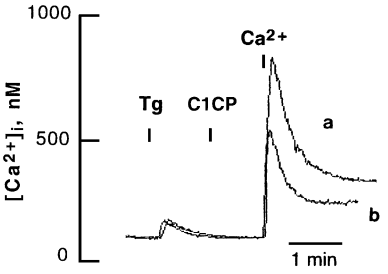

Fig. 8. C1CP enhances store-operated Ca^{2+} entry in a Cl^--free buffer solution. The cells were harvested and loaded with Fura 2 as described in Materials and methods. The cells were stimulated first with thapsigargin (final concentration1 μM), then with ceramide 1-(2-cyanoethyl) phosphate (C1CP, final concentration 10 μM) in a Cl^- and Ca^{2+}-free buffer, and then Ca^{2+} (final concentration 1 mM) was added to the cells (trace a). In trace b, the cells were first treated with thapsigargin and vehicle, and then Ca^{2+} was re-added to the cells. Each trace is the representative recording of four separate recordings.

and 379 ± 56 nM, respectively, compared with 322 ± 46 and 157 ± 26 nM in control cells.

4. Discussion

In the present report, we have described the effects of C1CP on Ca^{2+} fluxes in thyroid FRTL-5 cells. We show that C1CP modulates cells, in part, by rapidly inhibiting the conductance of Cl^- ions. This observation is supported by the fact that C1CP induced a hyperpolarization coupled to a decrease in membrane conductance, which were both absent when extracellular Cl^- concentration was decreased. Previous studies have shown that agonists that invoke changes in $[Ca^{2+}]_i$ in thyroid cells rapidly depolarizes E_m by activating Ca^{2+}-dependent Cl^- channels (Maruyama et al., 1985; Martin, 1992). In accordance with this, thapsigargin depolarizes FRTL-5 cells in a Ca^{2+}-dependent manner.[2] We suggest that thapsigargin induces a depolarization via the opening of Ca^{2+}-sensitive Cl^- channels and that C1CP modulates these channels. We base our conclusion on the above observation and the fact that C1CP decreased the thapsigargin-invoked depolarization by about 50%. This is a very interesting observation per se, as very few compounds are known to modulate Cl^- channels. Presently, we do not know whether C1CP modulates the channel protein directly or accomplishes the observed result by modulation of some signaling pathways. However, the effects of C1CP could not be abolished by a multitude of inhibitors, and C1CP hyperpolarized the cells only when present exterior to the cell. This observation implies that C1CP may have a direct effect on the channels. Furthermore, the effect of C1CP appeared to be very specific, as neither SPC, S1P, C8-ceramide nor lyso-phosphatidic acid invoked a similar response. Furthermore, authentic $C_8$1P and $C_2$1P[1] depolarized the membrane potential.

[2] Törnquist, unpublished observations.

In addition to the above described effect, C1CP also appears to enhance SOC entry by some presently unknown mechanism, as C1CP enhanced Ca^{2+} entry in a Cl^--free buffer. We also observed that $C_8$1P did not attenuate thapsigargin-evoked SOC entry, although $C_8$1P depolarized the membrane potential. Similar results have been obtained with $C_2$1P.[1] This is an interesting finding, as depolarization of the membrane potential always decreases Ca^{2+} entry in our cells (Törnquist, 1993). Furthermore, both C1CP and $C_8$1P per se evoked SOC entry in Ca^{2+} re-addition experiments, although both compounds evoked a negligible or a very small release of sequestered Ca^{2+}. We have also shown that $C_2$1P evokes SOC entry.[1] Taken together, our results suggest that C1CP (and C1P) have profound effects on Ca^{2+} entry in our cells. Sphingomyelin is present predominantly in the outer leaflet of the plasma membrane (Kolesnick and Golde, 1994) and both a ceramide kinase and a ceramide phosphate phosphatase are associated with the plasma membrane (Bajjalieh et al., 1989; Shinghal et al., 1993). Earlier studies have also shown that a membrane-associated ceramide kinase in synapses is activated in a Ca^{2+}-dependent manner (Bajjalieh et al., 1989). 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. The mechanism of action of C1Ps on SOC entry is presently not known. Electrophysiological studies are now in progress to further clarify these observations.

Ceramide 1-phosphate is a sphingoid form of phosphatidic acid, and phosphatidic acid and lyso-phosphatidic acid bind to the receptors Edg2, Edg4, and Edg7, which are structurally closely related to the sphingolipid Edg receptors (Goetzl and An, 1998; Bandoh et al., 1999). The FRTL-5 cells also express the Edg5 receptor (Nikmo et al., 1999). Thus, we cannot exclude the possibility that C1CP could modulate $[Ca^{2+}]_i$ and E_m by interacting with these receptors.

Presently, very little is known on the cellular effects of C1P. In fibroblasts and in osteoblastic cells, C1P is a potent mitogen (Gomez-Munoz et al., 1995, 1997; Carpio et al., 1999). In our experiments, we were, however, unable to show an effect of C1CP and $C_8$1P on proliferation. These results suggest that the proliferative effects of C1P are cell specific. Furthermore, C1P has been shown to inhibit the adrenaline-invoked changes in $[Ca^{2+}]_i$ in rat aortic smooth muscle and to relax these arteries after an adrenaline-invoked contraction (Zheng et al., 2000). A role for C1P during neutrophil phagocytosis and liposome fusion has also been indicated (Hinkovska-Galcheva et al., 1998).

In conclusion, the effects of C1CP, a ceramide derivative structurally very similar to that of C1P, have been characterized. The results showed that C1CP evoked an increase in $[Ca^{2+}]_i$ in a manner comparable to other C1Ps. In addition, C1CP decreased the Cl^- conductance, resulting in a hyperpolarization of the E_m and an attenuated Ca^{2+}-dependent depolarization of the plasma membrane. These effects resulted in an enhanced store-operated Ca^{2+} entry in the cells. Furthermore, C1CP also modulated Ca^{2+} fluxes in a manner similar to authentic C1Ps. Thus, C1CP may potentially be a useful tool for investigating the effects of ceramide derivatives on Ca^{2+} fluxes.

Acknowledgements

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