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Ceramide-1-P induces Ca^{2+} mobilization in Jurkat T-cells by elevation of Ins(1,4,5)-P_3 and activation of a store-operated calcium channel

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Abstract

Sphingolipids comprise a very important class of second messengers involved in cell growth, differentiation, and apoptosis, among other different functions. Recently, these lipids have been implicated in calcium mobilization in different cell lines, including Jurkat T-lymphocytes. However, the effect of each particular sphingolipid appears to be cell-line specific. Among them, the least studied is ceramide-1-P (Cer-1-P). Here, we show that Cer-1-P increased the intracellular Ca^{2+} concentration in Jurkat T-cells. Furthermore, laser-scanning confocal microscopy indicated that Ca^{2+} is released from the endoplasmic reticulum. An effect on store-operated Ca^{2+} channels was evidenced by whole-cell "patch clamp" measurements after Cer-1-P induced Ca^{2+} store depletion. The mechanism of action of Cer-1-P resembles that of the Jurkat anti-TCR antibody, but differs from that of ceramide, since Cer-1-P induced an increase in Ins(1,4,5)-P_3.

Keywords: Ceramide 1-P; Ins(1,4,5)-P_3; Capacitative calcium entry channel; Confocal; Jurkat T-cells

Sphingolipids have been implicated in growth regulation, differentiation, and apoptosis, among other important cellular functions [1]. This group of lipid biosignals has been involved in calcium mobilization in different cell lines, affecting the enzymes involved in calcium regulation [2] and directly modulating the levels of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in many cells [3–5]. Interestingly, the action of different sphingolipids on calcium mobilization varies on distinct cell lines. Thus, in some cells, some sphingolipids induce a rapid increase in the $[Ca^{2+}]_i$, while in others, addition of sphingolipids does not affect the $[Ca^{2+}]_i$ levels [6]. The effect of ceramide and sphingosine in Jurkat T-cells is well documented [7,8]. Both sphingolipids produce an increase in the $[Ca^{2+}]_i$ which is known to be essential for T-cell activation at the immune system [9]. It is well known that Jurkat T-cells can be activated by the anti-CD3 specific antibody OKT3 that binds to the T-cell receptor (TCR) complex. This activation is induced in part by the Ca^{2+} release from intracellular stores, sensitive to inositol 1,4,5-trisphosphate ($InsP_3$), and sustained by Ca^{2+} influx from the extracellular milieu [10]. On the other hand, the action of ceramide 1-P (Cer-1-P), which is derived from ceramide by the action of a ceramide kinase, although it has received some attention recently, is

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far less known, and even a lesser extent is known about the effect of Cer-1-P on calcium mobilization. It has been reported that in some cell lines this sphingolipid induces a rapid increase in the $[Ca^{2+}]_i$ [3–5], while in others, there is no effect on calcium mobilization [11–14]. Thus, the effect of ceramide 1-P on intracellular calcium movements appears to be cell-line specific. We have recently reported that ceramide increases the $[Ca^{2+}]_i$ in Jurkat T-cells [7]. Interestingly, albeit liberation of $[Ca^{2+}]_i$ occurs from endoplasmic reticulum, this sphingolipid is not able to generate inositol 1,4,5-trisphosphate ($InsP_3$). In the present work, we investigate the effect of Cer-1-P on Jurkat T-cells, demonstrating that this sphingolipid, at a concentration lower than that of ceramide, induces a rapid increase in the $[Ca^{2+}]_i$. Even more, unlike ceramide, Cer-1-P elevates the concentration of $InsP_3$, inducing the liberation of Ca^{2+} from the endoplasmic reticulum, which in turn provokes the opening of store-operated calcium channels (SOCC) at the plasma membrane.

Materials and methods

Chemicals. Ceramide 1-phosphate (C_8) and ceramide (C_8) were from Sigma (St. Louis, MO). All lipids were microdispersed by sonication at 4 °C under N_2 before use. Stocks of concentrated lipids were dissolved in DMSO and its final concentration in the cuvette was always below 0.1%. Myo-[2-^{3}H]inositol (25 C/mmol) was from Perkin-Elmer (Torrance, CA). Fura-2 acetoxymethyl ester (Fura-2/AM) was from Molecular Probes (Eugene, OR). OKT3 was kindly supplied by Dr. Martín Rodríguez (Universidad Central de Venezuela). All other reagents were obtained from Sigma (St. Louis, MO).

Cell culture. Jurkat human leukemia T-cells were maintained in log-phase growth at 37 °C under a humidified atmosphere of O_2/CO_2 (95:5) in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Measurement of calcium concentration. Measurements of intracellular calcium concentration were performed by the use of the fluorescent calcium indicator Fura-2. The extracellular Ringer solution contains (mM): 155 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 Hepes–NaOH (pH 7.4). In the calcium-free solution, 2 $MgCl_2$ plus 1 mM EGTA was substituted for $CaCl_2$. A Jurkat T-cell suspension was loaded with 5 μM Fura-2/AM for 45 min at 37 °C in culture medium and then washed twice with extracellular Ringer solution. The fluorescence of the Fura-2-loaded cell suspension was monitored as reported [7,15] and $[Ca^{2+}]_i$ was estimated as described by Grynkiewicz et al. [16].

Detection of subcellular Ca^{2+} signal by laser-scanning confocal microscope. Subcellular Ca^{2+} was monitored in individual cells by using time-scan confocal microscopy. Jurkat T-cells were incubated with Rhod-2/AM (10 μM) for 50 min at 37 °C in culture medium and washed with extracellular Ringer solution for 5 min. Cells were plated onto a 22 × 40 mm glass coverslip as described [7] and then incubated for an additional 10 min at 37 °C with BODIPY-FL-Ryanodine (500 nM). The coverslip with the dual-loaded cells was placed in a superfusion open chamber on the laser scanning confocal microscope stage (LSCM, Nikon C1), mounted in a Eclipse TE300 Nikon inverted microscope with Nikon 100/1.30 oil Ph4L oil-immersion objective coupled to a C1-LU2 laser unit with neon-(543 nm) and argon-cooled air (488 nm). This laser unit was controlled by a D-eclipse C1 interface [7]. The cells were continuously superfused with physiologic buffer at 37 °C.

Hydrolysis of phosphoinositol(4,5)bis-phosphate. Hydrolysis of phosphoinositol(4,5)bis-phosphate was assessed as described before [7], by measuring the accumulation of inositol phosphate in the presence of 10 mM LiCl, to block the inositol-1-phosphate phosphatase [17]. The amount of inositol phosphate ($InsP_1$) was calculated as percentage of the radioactivity originally present in the cell membrane ($InsP_1/InsP_1$+lipids). This relationship is directly proportional to the $InsP_3$ levels [18].

Electrophysiological measurements. Patch-clamp experiments were performed according to standard techniques, in the whole-cell configuration [19] using an Axopatch 200A patch clamp amplifier, and an Axolab 1200 interface in an Axiovert 10 inverted microscope. Patch pipettes were pulled in two stages from borosilicate glass and then fire-polished. Electrodes used had a resistance of 3–5 MΩ. The membrane potential was clamped at 0 mV and currents were recorded during 100 ms voltage ramps from −120 to +40 mV, delivered after 8 s hyperpolarized pulse (−120 mV) [7].

Cells were plated on 0.01% poly-L-lysine-treated Thermanox coverslips 30 min before recording. All experiments were conducted at room temperature (~21 °C) in a standard external solution containing (mM): 155 NaCl, 4.5 KCl, 1 $MgCl_2$, 10 D-glucose, 2 or 20 $CaCl_2$, and 5 Hepes–NaOH (pH 7.4). Cer-1-P was added to the external solution and the solution was changed by a gravity-driven perfusion system. The internal solution contained (mM): 115 CsCl, 1 EGTA, 1 $CaCl_2$, 8 $MgCl_2$, 10 NaCl, and 10 Hepes (pH 7.2).

Statistical analysis. The values are expressed as means ± SD, for the number (n) of experiments expressed in parentheses. Statistical analysis was made using Student's t test for unpaired observations. Probability values below 0.05 ($p \leqslant 0.05$) were considered significant. Spatial profile of fluorescence intensity at region of interest was made with SigmaPlot software. The data are from at least four different batches of cells.

Results

Effect of Cer-1-P on $[Ca^{2+}]_i$ in Jurkat T-cells

In order to investigate the effect of Cer-1-P on $[Ca^{2+}]_i$ in Jurkat T-cells, suspensions of cells were incubated in the presence of this phosphorylated sphingolipid and the $[Ca^{2+}]_i$ was quantified in real time. Addition of Cer-1-P (C_8) (1 μM) to the cell suspension, in the presence of extracellular calcium, elicited a rapid biphasic increase in the $[Ca^{2+}]_i$ (Fig. 1A). The biphasic form of this curve is compatible with the liberation of Ca^{2+} from intracellular reservoirs and the subsequent onset of a capacitative calcium entry [20]. This effect is mimicked by the Jurkat specific antibody OKT3 upon TCR/CD3 binding to these cells [9], which is known to induce the elevation of Ins(1,4,5)-P_3 with the concomitant depletion of the endoplasmic reticulum and the subsequent opening of a capacitative calcium entry (by SOCC) [9,10]. The dose–response curve shown in Figs. 1B and 1C demonstrates that Cer-1-P is more potent than ceramide, although the elevation of the $[Ca^{2+}]_i$ reaches the same magnitude.

We further investigated the origin of the intracellular calcium increase induced by Cer-1-P, by comparing its

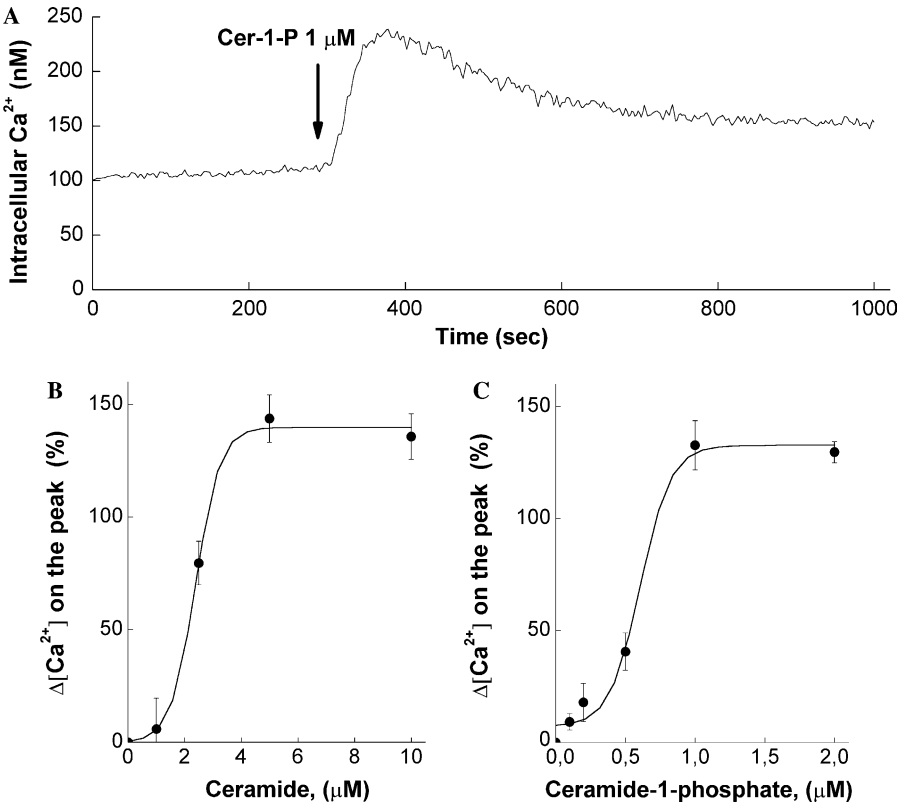

Fig. 1. Effect of Cer-1-P on $[Ca^{2+}]_i$ in Jurkat T-cells. The $[Ca^{2+}]_i$ was measured in Fura-2 loaded cell suspensions as described under Materials and methods. (A) Arrow indicates the addition of Cer-1-P (1 μM). (B) Dose–response curve in the presence of increasing concentrations of ceramide. (C) Dose–response curve in the presence of increasing concentrations of Cer-1-P. All experiments were done in the presence of 2 mM $CaCl_2$. The increase in $[Ca^{2+}]_i$ is expressed as a δ change in the peak of $[Ca^{2+}]_i$ from the resting level (90.1 ± 9.0 nM) of the $[Ca^{2+}]_i$. Each point in B and C are the means ± SD of five determinations. Trace in A are representative of at least four independent experiments.

effect with that of ceramide, which is known to release calcium from the endoplasmic reticulum [7]. As can be observed in Fig. 2A, when ceramide is added after Cer-1-P, there is no further increase in $[Ca^{2+}]_i$. Similarly, when Cer-1-P is added after ceramide, the former shows no effect. These data strongly suggest that Cer-1-P and ceramide induce the release of calcium from the same compartment.

When the cellular suspension was incubated in the absence of extracellular calcium (Fig. 2C) and then Cer-1-P was added to the medium, a smaller Ca^{2+} transient was observed, which rapidly returned to the basal $[Ca^{2+}]_i$ level, suggesting that part of the increase in $[Ca^{2+}]_i$ induced by Cer-1-P was due to a substantial entry of extracellular calcium. Again, it can be observed (Figs. 2C and D) that there is no additive effect of these sphingolipids, independently of the order of addition. These data further support that the liberation of Ca^{2+} by these two sphingolipids occurs from the same intracellular Ca^{2+} compartment. Addition of thapsigargin (1 μM) prior to Cer-1-P fully abolished the effect of the sphingolipid on the $[Ca^{2+}]_i$ (data not shown), further supporting that, similar to ceramide [7], Cer-1-P released calcium from the endoplasmic reticulum.

Identification of the Cer-1-P sensitive Ca^{2+} store by laser-scanning confocal microscope

We explored the location of the Cer-1-P sensitive Ca^{2+} pool by the use of confocal microscope, taking advantage of the fact that the endoplasmic reticulum can be marked by BODIPY-FL-Ryanodine (Fig. 3). Cells were loaded with Rhod-2 under conditions that favored the compartmentalization of the dye [7]. The red color in Fig. 3A, which coincides with the green color of the BODIPY-FL-Ryanodine dye in Fig. 3D, indicates that the endoplasmic reticulum indeed accumulates a large Ca^{2+} concentration. The yellow-orange color in the merge (Fig. 3E) indicates the portion of the endoplasmic reticulum which is highly loaded with calcium. When Cer-1-P is added, an evident bleaching of the orange-yellow zones occurred, after 5.5 min (Fig. 3C), consistent with the notion that this sphingolipid liberates Ca^{2+} from the endoplasmic reticulum.

When the picture was taken after 1 min after Cer-1-P addition (Fig. 3B), the cytoplasm of the whole cell appeared strongly red-colored, indicating a large increase in the $[Ca^{2+}]_i$ which is now high enough to be detected by the low-affinity calcium indicator Rhod 2. These

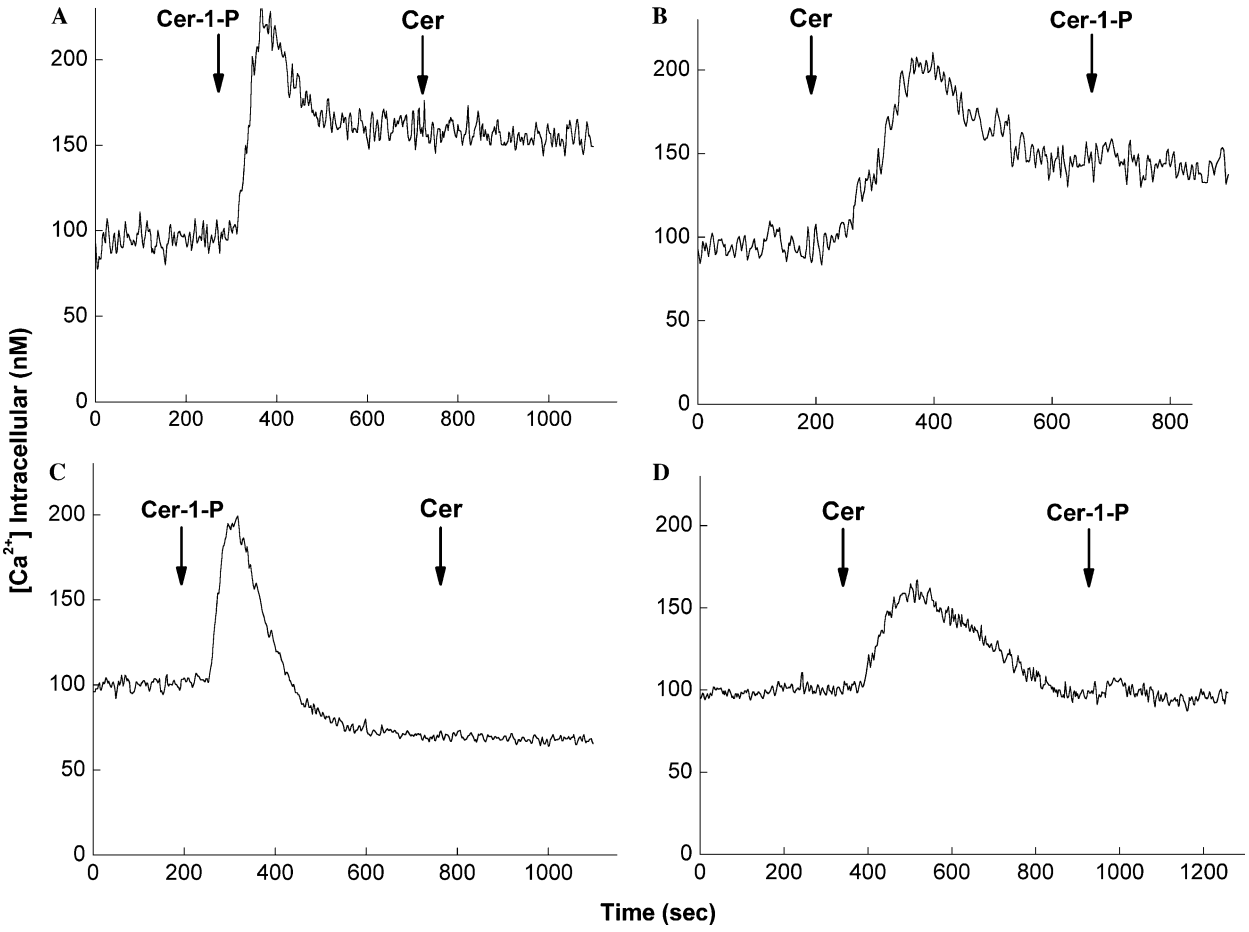

Fig. 2. Effect of Cer and Cer-1-P on the $[Ca^{2+}]_i$ in Jurkat T-cells. The $[Ca^{2+}]_i$ was measured in Fura-2 loaded cell suspensions as described in Fig. 1. (A) Arrows indicate the addition of Cer-1-P (1 μM) and ceramide (5 μM) in the presence of 2 mM $CaCl_2$. (B) Arrows indicate the addition of Cer (5 μM) and Cer-1-P (1 μM) in the presence of 2 mM $CaCl_2$. (C) Arrows indicates the addition of Cer-1-P (1 μM) and ceramide (5 μM) in the absence of extracellular $CaCl_2$ (1 mM EGTA). (D) Arrows indicates the addition of Cer (5 μM) and Cer-1-P (1 μM) in the absence of extracellular $CaCl_2$. Traces are representative of at least four independent experiments.

results are fully compatible with the time-course observed in Fig. 1A. Accordingly, upon Cer-1-P addition the $[Ca^{2+}]_i$ reached a peak after 1 min and, then after a short time, the cytoplasmic Ca^{2+} is rapidly regulated. When comparing Figs. 3A and C, other small compartments, which were not evident at the beginning of the experiment, now appeared red-colored in the cytoplasm of the cell, probably associated with Ca^{2+} accumulation by mitochondria or even Ca^{2+} recapture by other zones of the endoplasmic reticulum.

The three-dimensional fluorescence quantification made in the spatial profile (Figs. 3F, G, and H), at the region of interest indicated by the arrow in Figs. 3A and C, also reflected the appearance of a calcium peak after incubation of cells with Cer-1-P, which decreased after the indicated time (Fig. 3H).

Effect of ceramide-1-P on phosphoinositide hydrolysis

To determine if the effect of Cer-1-P occurred through the elevation of $InsP_3$ as is the case for the action of the anti-TCR, OKT3 on Jurkat T-cells, a cell suspension was incubated with myo-[2-^{3}H]inositol for 24 h prior to the stimulation with Cer-1-P (1 μM) for 5 min. The results demonstrated that, unlike ceramide, Cer-1-P, similar to OKT3, produces an accumulation of $InsP_3$ (Fig. 4).

Induction of a capacitative calcium entry in Jurkat T-cells by ceramide-1-P

As mentioned above, when the cellular suspension was incubated in the presence (see above, Figs. 1A and 2A) and in the absence (see above, Fig. 2C) of extracellular calcium a different kinetics was observed, suggesting that part of the increase in $[Ca^{2+}]_i$ was due to an entry of extracellular calcium. We confirmed the effect of Cer-1-P on calcium release activated channels (CRAC) measuring calcium currents by the use of patch-clamp in whole-cell configuration. Internal solutions included 8 mM $MgCl_2$ to hinder Mg^{2+}-inhibited cation (MIC) channels [7,20]. As shown in Fig. 5, an

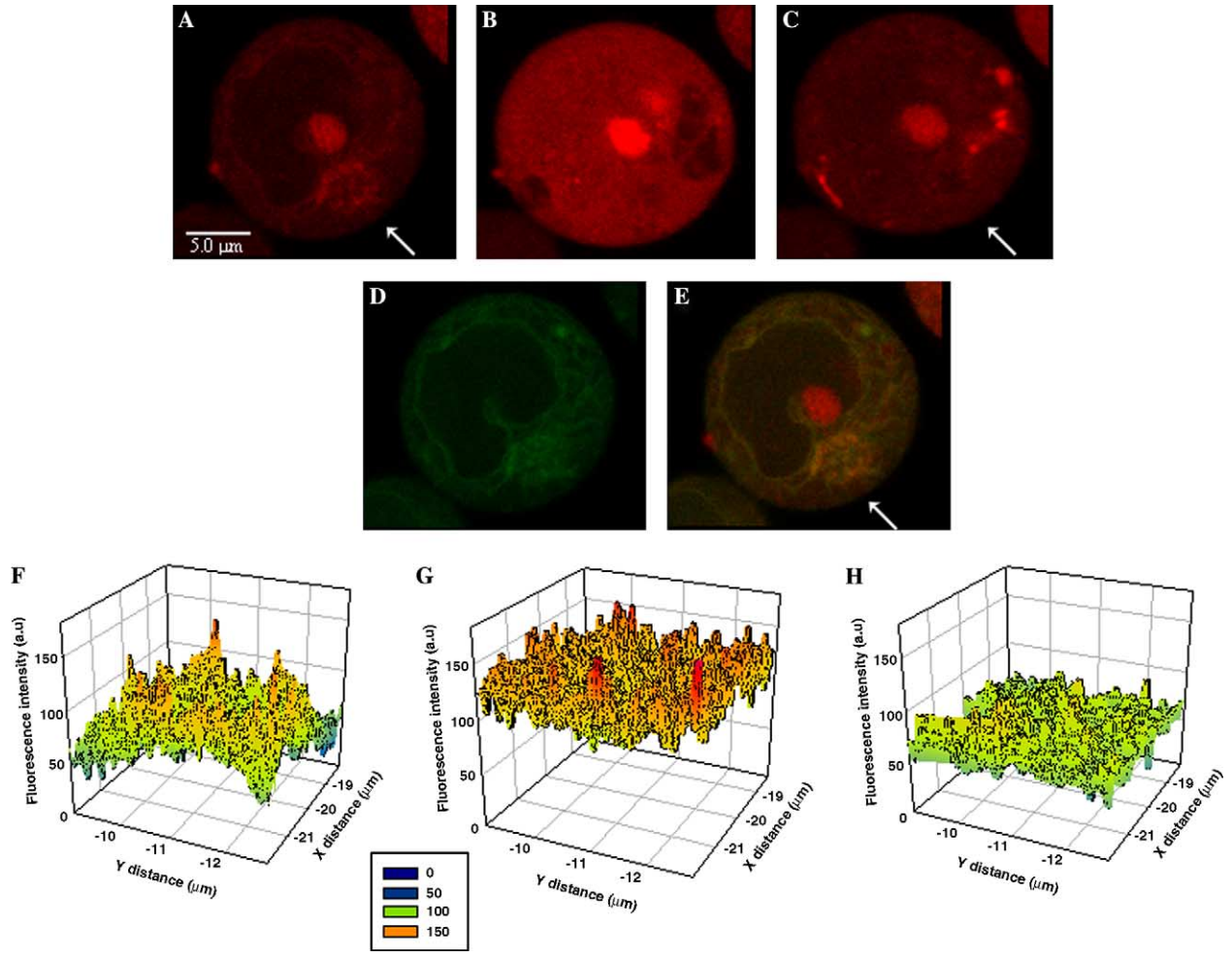

Fig. 3. Endoplasmic reticulum calcium release resulting from Cer-1-P action in Jurkat T-cell visualized by confocal microscopy. Representative time series of *XY* confocal scans taken every 30 s for 7 min from a cell. (A) Frame 0 shows a control cell loaded with Rhod-2. (B) Frame shows the Ca^{2+} peak after 1 min of Cer-1-P (1 μM) treatment. (C) Frame shows a Ca^{2+} release from endoplasmic reticulum, after 5.5 min of Cer-1-P treatment. (D) BODIPY-FL-Ryanodine labelling. (E) Merge images of A and D. (F) Representation of the three-dimensional mesh plot of the pixel-by-pixel fluorescence intensity for the region of interest of the image A. (G) Representation of the three-dimensional mesh plot of the pixel-by-pixel fluorescence intensity for the region of interest of the image B. (H) Representation of the three-dimensional mesh plot of the pixel-by-pixel fluorescence intensity for the region of interest of the image C. Fluorescence intensity is encoded by both, height on the *z*-axis and colors and depicts the *X*, *Y* localization and magnitude of fluorescence changes. Bar, 5 μm. The arrows indicate the region of interest with an area of 15.5 μm^2, which show the bleaching of the Cer-1-P sensitive endoplasmic reticulum calcium pool before and after the addition of Cer-1-P. The image is representative of at least four independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

inwardly rectifying current developed after the application of Cer-1-P. Judged by its current–voltage relationship and positive reversal potential, this current resembles that of CRAC channels [20] and is similar to that elicited by the addition of ceramide or OKT3 under the same conditions [7].

Discussion

At present, very little is known about the cellular events that are regulated by Cer-1-P. This sphingolipid is a potent mitogen in some cell lines as fibroblasts [12,13] and osteoblastic cells [21], while in others, like FRLT-5 cells, it is inert [4,5]. These results suggest that the effect of Cer-1-P on cell proliferation is cell specific. Concerning its effect on the $[Ca^{2+}]_i$, Cer-1-P induces Ca^{2+} mobilization in calf pulmonary artery endothelial cells [3] as well as in thyroid FRTL-5 cells [4] and increase $[Ca^{2+}]_i$ (but indirectly, through voltage-operated Ca^{2+} channels) in GH_4C_1 rat pituitary cells [5]. Nonetheless, Cer-1-P did not induce Ca^{2+} mobilization in neutrophils [11], fibroblasts [12,13] or A549 lung adenocarcinoma cells [14]. In this work, we are reporting that in Jurkat T-cells, Cer-1-P induces a rapid increase in the $[Ca^{2+}]_i$. Therefore, from the above results it can be concluded, as suggested previously [6], that the effect of Cer-1-P on calcium mobilization is celltype specific.

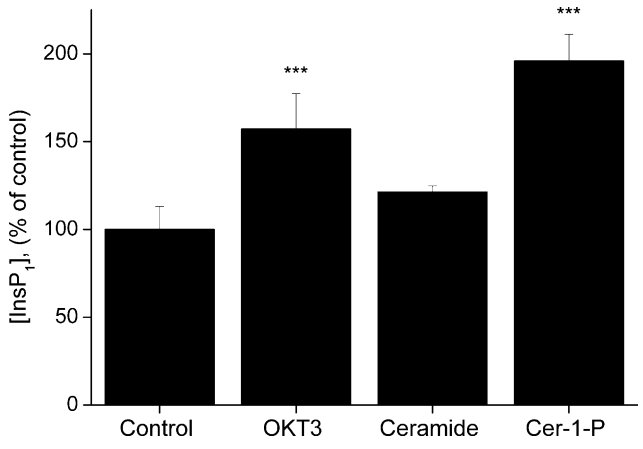

Fig. 4. Effect of Cer-1-P on the accumulation of $InsP_3$ on Jurkat T-cells. The $InsP_3$ synthesis was assessed as accumulation of $InsP_1$ incubated in KHBB containing 10 mM LiCl after labeling the cells with inositol (myo-[2-^{3}H]inositol). Control, DMSO 1.6 μl, OKT3 5 μg/ml, Cer 5 μM, and Cer-1-P 1 μM. The asterisks (***) indicate that results are statistically significant when compared to the control ($p \leqslant 0.05$). Values shown are averages of four determinations ± SE.

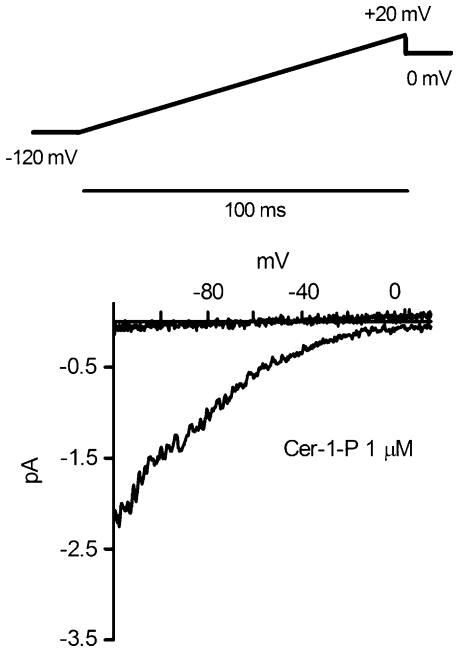

Fig. 5. Effect of Cer-1-P on CRAC channels in Jurkat T-cells. Upper panel represents a voltage ramp used for the experiment. Lower panel represents current traces before and after the addition of Cer-1-P (1 μM). Traces are representative of at least three independent experiments. Records were digitally filtered at 1 kHz.

The effect of Cer-1-P is partially mimicked by ceramide since they appear to liberate calcium from the same compartment, the endoplasmic reticulum, and both induced the opening of store-operated calcium channels at the plasma membrane [7]. Remarkably, and different to ceramide, our results show conclusively that Cer-1-P evoked the production of $InsP_3$. In this respect, activation of T-lymphocytes by antigen-presenting cells involves tyrosine phosphorylation of PLCγ (simulated by the use of the anti-CD3 OKT3) which provokes the production of $InsP_3$. In this sense, the effect of Cer-1-P greatly resembles this well-known route of T-cell activation.

Another important difference between Cer-1-P and ceramide obtained in this work is that the former is more potent than its precursor by one order of magnitude. In any case, it is not obvious why this two sphingolipids induce a similar increase in the $[Ca^{2+}]_i$ by two different mechanisms, and with distinct effectiveness. Cer-1-P, ceramide kinase [4], and ceramide 1-P phosphatase [22], as reported so far, are known to be present at the plasma membrane [4], where incidentally the PLC system is located. In T-lymphocytes, the activation of the pathway which increases PLCγ and induces $[Ca^{2+}]_i$ increase is necessary but not sufficient to commit the cell to produce IL-2. The co-stimulation of the CD28 signalling pathway should also be activated, which in turn triggers the activation of an acidic sphingomyelinase which results in the generation of ceramide [23]. This sphingolipid eventually triggers the proteolytic degradation of I-κB required for the subsequent nuclear translocation of NF-κB, which elicits the IL-2 and IL-2 receptor-α-chain that is essential for T-cell proliferation [23]. Thus, the effect of ceramide in these cells, as in many others, is pleiotropic. Nevertheless, the effect of Cer-1-P in T-lymphocytes has not been studied to any extent. Thus, at present we do not know about the physiological meaning of Cer-1-P-induced elevation in the $[Ca^{2+}]_i$ in Jurkat T-cells. In fact, this is the first report, to our knowledge, about the effect of Cer-1-P in T-lymphocytes, where calcium is a well-known and important second messenger involved in T-cell activation [9].

It has been proposed that in FRTL-5 cells that the effect of Cer-1-P on $[Ca^{2+}]_i$ is mediated by elevation of $InsP_3$ and also by elevation of sphingosine-1-P [4]. Interestingly, we have demonstrated previously that sphingosine 1-P is not able to induce an increase in $[Ca^{2+}]_i$ in Jurkat T-cells [7]. Thus, in these cells the elevation of $[Ca^{2+}]_i$ appears to be essentially due only to the production of $InsP_3$.

Recently, it has been proposed that ceramide and Cer-1-P are antagonistic in their cellular function [6,24], which is an extension of the proposed "rheostat" model proposed for ceramide and sphingosine 1-P [25]. In the case of Jurkat T-cells, however, ceramide and Cer-1-P, albeit through different mechanisms and with distinct potency, both increase the $[Ca^{2+}]_i$. Thus, this model, although very attractive and based in substantial evidences, cannot be extended to all effects in different cells.

In conclusion, we show for the first time that Cer-1-P, a very little understood bioactive sphingolipid, causes a direct effect on calcium regulation in Jurkat T-cells,

which is known to be essential for T-lymphocyte activation.

Acknowledgments

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