Available online at www.sciencedirect.com
SCIENCE DIRECT®
Archives of Biochemistry and Biophysics 436 (2005) 333–345
ABB
www.elsevier.com/locate/yabbi

Ceramide increase cytoplasmic Ca^{2+} concentration in Jurkat T cells by liberation of calcium from intracellular stores and activation of a store-operated calcium channel

Claudia Colina [a,b], Adriana Flores [a], Héctor Rojas [c], Angie Acosta [b], Cecilia Castillo [a], María del Rosario Garrido [d], Anita Israel [d], Reinaldo DiPolo [c], Gustavo Benaim [a,b,*]

[a] Centro de Biociencias y Medicina Molecular, Instituto de Estudios Avanzados (IDEA), Caracas, Venezuela
[b] Instituto de Biología Experimental, Facultad de Ciencias, Universidad Central de Venezuela, Caracas, Venezuela
[c] Laboratorio de Permeabilidad Iónica, Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela
[d] Laboratorio de Neuropéptidos, Facultad de Farmacia, Universidad Central de Venezuela, Caracas, Venezuela

Received 27 December 2004, and in revised form 9 February 2005

Abstract

The effect of ceramide on the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) varies depending on the cell type. We have found that in Jurkat human T cells ceramide increases the $[Ca^{2+}]_i$ from a thapsigargin-sensitive calcium pool and the subsequent activation of a capacitative Ca^{2+} entry. This effect occurs both in the presence and in the absence of extracellular calcium. Addition of ceramine, a non-hydrolysable analogue of ceramide, reproduced its effect on the $[Ca^{2+}]_i$ ruling out that this is due to the conversion of ceramide to sphingosine. The effect of ceramide was additive to that obtained by sphingosine, but not to the Jurkat T cells specific antibody OKT3. However, different to the latter, ceramide do not induced an elevation of $InsP_3$. The opening of a store operated Ca^{2+} channel by ceramide was corroborated by experiments of Fura-2 quenching, using Mn^{2+} as a surrogate for Ca^{2+} and confirmed by whole-cell recording patch clamp techniques.

Keywords: Ceramide; Calcium; Ceramine; Capacitative calcium entry channel; $InsP_3$; Jurkat T cells

Sphingolipids are ubiquitous among eukaryotic organisms and have been implicated in cellular growth regulation, differentiation, and apoptosis [1–3]. The precursor of these second messengers is sphingomyelin, which generates ceramide via sphingomyelinase hydrolysis. Subsequently, sphingosine is derived from ceramide, by action of different ceramidases. Ceramide and sphingosine can be phosphorylated by specific kinases, generating ceramide-1-phosphate and sphingosine-1-phosphate, respectively [1,2]. Ceramide has been frequently implicated as an inductor of apoptosis in many cell types [1,3,4]. Even more, it has been recently reported that ceramide plays an important role in lymphocyte activation and generation of immune response, beside the induction of apoptosis [5], thus generating a pleiotropic response in these cells. However, its mechanism of action remains to be elucidated. On the other hand, an increase in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) contributes to essential triggering signals for T cell activation in the immune system [6,7]. Therefore, stimulation of the T cell receptor (TCR)[1] complex by

* Corresponding author. Fax: +58 212 903 5118.
E-mail address: gbenaim@reacciun.ve (G. Benaim).

[1] *Abbreviations used:* TCR, T cell receptor; KHBB, Krebs–Henseleit bicarbonate buffer; PLCγ, phospholipase C-γ; 2-APB, 2-aminoethoxydiphenylborate; MIC, Mg^{2+}-inhibited cation; SPP-1, sphingosine-1-P phosphohydrolase; PAK, p21-activated kinase (PAK).

0003-9861/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2005.02.014

the anti-CD3 specific antibody OKT3 generates inositol 1,4,5-trisphosphate (Ins(1,4,5)-P_3), which releases Ca^{2+} from the endoplasmic reticulum, with the subsequent opening of store-operated Ca^{2+} channels [6], also called capacitative Ca^{2+} entry, at the plasma membrane [8]. Thus, an increase in $[Ca^{2+}]_i$ in stimulated T cells is due to the release of Ca^{2+} from intracellular stores, sustained by a Ca^{2+} influx from the extracellular milieu [9].

While it have been reported that sphingosine produces an increase in $[Ca^{2+}]_i$ in Jurkat T cells and many other cell types [7,10], the effect of ceramide on $[Ca^{2+}]_i$ is more controversial since in some cells produces a decrease in the $[Ca^{2+}]_i$ [11], or an inhibition of extracellular calcium entry [12–14], whereas in others produced an increases in the $[Ca^{2+}]_i$ [15,16]. Concerning Jurkat T cells, the effect of ceramide on $[Ca^{2+}]_i$ is not documented at present.

In this work, we show that ceramide induce a rapid increase in the $[Ca^{2+}]_i$ in Jurkat T cells, with a biphasic form followed by a sustained Ca^{2+} influx in the presence of extracellular calcium. The effect of ceramide was additive to that obtained by the use of sphingosine, but not with OKT3. The opening of a store operated Ca^{2+} channel was corroborated by patch clamp experiments. Thus, our results show that ceramide constitutes per se a new route for the liberation of intracellular Ca^{2+} from intracellular organelles and for the subsequent opening of a plasma membrane Ca^{2+} channel in lymphocytes.

Materials and methods

Chemicals

Ceramide (C_2 and C_8), dihydro-C_2-ceramide, L-sphingosine, D-sphingosine, and sphingosine-1-phosphate were obtained from Avanti Polar Lipids (Alabaster, AL) or from Calbiochem (La Jolla, CA). Fura-2 acetoxymethyl ester (Fura-2/AM) was obtained from Molecular Probes (Eugene, OR). Myo-[2-^{3}H]inositol was obtained from Perkin-Elmer (Torrance, CA). Dowex anion exchange chromatography (AG 1-X8, 100-200 mesh formate form) was from Bio-Rad Laboratory (Hercules, CA). 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%. OKT3 was a gift from Dr. Martín Rodríguez (Universidad Central de Venezuela). Ceramine-C_8 and all other reagents were obtained from Sigma (St. Louis, MO).

Cell culture

Jurkat human leukaemia T cells line was 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 mg/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 were substituted for $CaCl_2$. A Jurkat T cells suspension (1.0×10^6 cells/ml) was loaded with 5 μM Fura-2/AM for 45 min at 37 °C in culture medium. After the loading period, the Jurkat T cells were washed twice with extracellular Ringer solution. The fluorescence of the Fura-2-loaded cell suspension was monitored in a stirred cuvette with a ratiometric fluorescence spectrophotometer HITACHI F-2000. The excitation wavelengths were 340 and 380 nm and emission was measured at 510 nm. All the experiments were performed at 37 °C. Intracellular Ca^{2+} concentration was estimated as described by Grynkiewicz et al. [17] from the relationship:

$$[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)]F_{min}(380)/F_{max}(380),$$

where R is F_{340}/F_{380} ratio, R_{min} and R_{max} are the ratios at 0 Ca^{2+} (6 mM digitonin plus 4 mM EGTA) and saturating Ca^{2+} (2 mM $CaCl_2$ plus 6 mM digitonin), respectively. K_d represents the apparent dissociation constant of Fura-2 (224 nM) and $F_{min}(380)/F_{max}(380)$ are the fluorescence values of digitonized cells (6 mM digitonin) plus 4 mM EGTA and 2 mM $CaCl_2$, respectively. Values of R_{min} and R_{max} were measured in situ in Jurkat T cells.

Detection of subcellular Ca^{2+} signals with confocal microscopy

Subcellular Ca^{2+} was monitored in individual cells by using time-scan confocal microscopy. Jurkat T cells were incubated with the cell permeant form of Rhod-2 (10 μM) for 50 min at 37 °C in culture medium and washed with extracellular Ringer solution. Cells were plated onto a 22×40 mm glass coverslip (0.15 mm thickness, Warner Instruments, Hamden, CT). Rhod-2 is a positively charged molecule that is largely retained within intracellular compartments, like endoplasmic reticulum and mitochondria. Moreover, the low-affinity of this dye allows it to be utilized as a Ca^{2+} concentration indicator in the endoplasmic reticulum. The incubation for relative large time and at 37 °C favours the compartmentalization of the dye. The cells were then incubated for additional 10 min at 37 °C with BODI-

PY-FL-Ryanodine (500 nM). This indicator interacts with the ryanodine sensitive Ca^{2+}-channels on the endoplasmic reticulum membranes, thus allowing locating this compartment. The coverslip with the loaded cells was attached to an open experimental chamber (RC-27, Warner Instruments, Hamden, CT) and mounted in a Diaphot-TMD Nikon inverted microscope (Tokyo, Japan) with Nikon 100/1.30 oil Ph4L oil-immersion objective (Tokyo, Japan) connected to a confocal Nikon C1, coupled to a C1-LU2 lasers unit with Neon (543 nm) and Argon cooled air (488 nm). This lasers unit was controlled by a D-eclipse C1 interface.

Experiments were performed using a DAD-12 perfusion system with a PTR-2000 perfusion temperature regulator (Adam & List Associates, Westbury, NY) stage to deliver the test solutions to the Jurkat T cells via pressure ejection from a 200 μm ID carbonated pipette. This device allows the computer to control the changes of different experimental solutions as fast as 20 ms (measured by changing the resistance of a microelectrode placed near the tip of the perfusion cannula after perfusion with a diluted Ringer solution) [18,19].

Phosphoinositide hydrolysis

The $InsP_3$ hydrolysis was assessed as the accumulation of $InsP_1$ in the presence of 10 mM LiCl, to block the inositol-1-phosphate phosphatase [20], as described [21]. Briefly, Jurkat T cells were labeled for 24 h at 37 °C under a humidified atmosphere of O_2/CO_2 (95:5) in DMEM (with low inositol) culture medium with 2 mCi/ml of myo-[2-^{3}H]inositol (specific activity 25.0 Ci/mmol). After labeling, the cells were washed twice and kept in Krebs–Henseleit bicarbonate buffer (KHBB) containing (mM): 111 NaCl, 26.2 $NaHCO_3$, 1.2 NaH_2PO_4, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 LiCl, 5.5 glucose, and 10 Hepes–NaOH (pH 7.4) and resuspended at 4.0×10^7 cells/ml. Aliquots (0.4 ml) of the cell suspension were transferred into individual 1.5 ml Eppendorf tubes and incubated for 5 min at 37 °C with or without the correspondent effector. The incubation was terminated by addition of 6% of ice-cold trichloroacetic acid and the sample was transferred to ice. The mixture was centrifuged at 10,000*g* for 15 min at 4 °C. The whole supernatant was used for analysis of inositol-1-phosphate by Dowex anion exchange chromatography (AG 1-X8, 100–200 mesh formate form). Fractions (5 ml) containing $InsP_1$ were collected and counted for radioactivity. To estimate the incorporation of radioactivity into phospholipids, membrane-associated radioactivity was extracted with chloroform–methanol and counted for radioactivity. For Jurkat T cells, the amount of $InsP_1$ was calculated as percentage of the radioactivity originally present in the membrane ($InsP_1/InsP_1$ + Lipids). This relationship is directly proportional to the $InsP_3$ production [21].

Electrophysiological measurements

Patch clamp experiments were performed according to standard techniques, in the whole-cell configuration [22] using an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA, USA), and an Axolab 1200 interface in an Axiovert 10 inverted microscope (Zeiss, Jena, Germany). Data were acquired with a PC-computer, using pClamp6 software (Axon Instruments). Patch-pipettes were pulled in two stages from borosilicate glass (Sutter Instruments, Novato, CA) and then fired-polished. Electrodes used had a resistance of 3–5 MΩ. Data were sampled at a rate of 5 kHz. Capacitative transients and series resistance were cancelled by the compensation circuitry of the amplifier. The membrane potential was clamped at 0 mV, and 100 ms voltage ramps from −120 to +20 mV after an 8 s prepulse to −120 mV. Leak currents before channel activation were averaged and subtracted from records with the activated channels. Only experiments were I_{leak} remained constant were used.

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). Ceramide 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 $CaCl_2$, 1 EGTA, 8 $MgCl_2$, 10 NaCl, and 10 Hepes (pH 7.2). Depending on the experiment (described in the text), the Ca^{2+} chelators EGTA (Sigma), BAPTA tetracesium salt (Molecular Probes) was added to this solution at the specified concentrations.

Statistical analysis

The values shown in results and table are expressed as means ± SD, for the number (n) of experiments expressed in parentheses. Statistical analysis was made using the Student's t test for unpaired observations. Probability values below 0.01 ($P \leqslant 0.01$) or 0.05 ($P \leqslant 0.05$) were considered significant. The data are from at least three different batches of cells.

Results

Effect of ceramide on $[Ca^{2+}]_i$ in Jurkat T cell suspensions

To investigate the effect of ceramide on $[Ca^{2+}]_i$ in Jurkat T cells, a cell suspension was incubated in the presence of extracellular calcium (Fig. 1A). Under this condition, addition of ceramide (Cer-C_2, 5 μM) elicited a biphasic increase in the intracellular Ca^{2+}

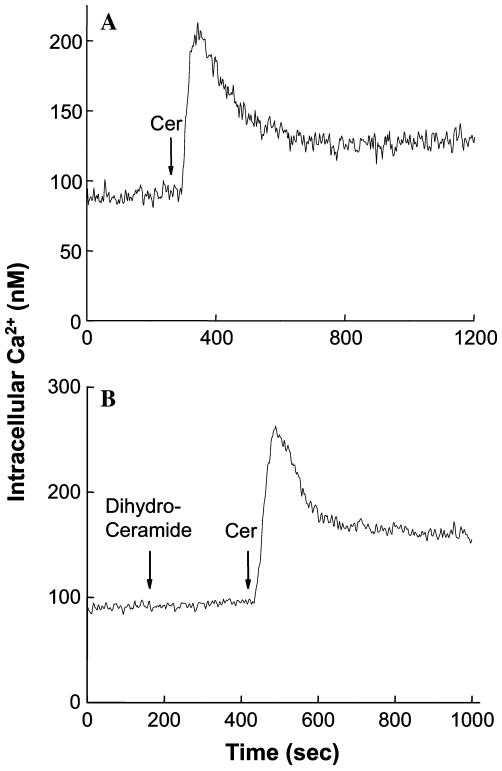

Fig. 1. Effect of ceramide on the $[Ca^{2+}]_i$ in Jurkat T cells. The $[Ca^{2+}]_i$ was measured in Fura-2 loaded cell suspensions as described under Materials and methods. All the experiments were done in the presence of 2 mM $CaCl_2$. (A) Arrow indicates the addition of ceramide (5 μM); (B) arrows indicate the addition of dihydroceramide (5 μM) and ceramide (5 μM). Traces are representative of at least three independent experiments.

concentration, reaching a Ca^{2+} peak (219.6 ± 14.9 nM) and then descending to a sustained a plateau (Fig. 1A). This result is typical of events occurring throughout an internal liberation of Ca^{2+} from cytoplasmic reservoirs and the subsequent onset of a capacitative calcium entry [8]. We also tested the effect of dihydroceramide, the precursor of ceramide [1], which is commonly used as an inactive analogue of the latter. Addition of dihydroceramide (5 mM) previous to ceramide neither has any effect (Fig. 1B) nor alters the action of the second messenger (Fig. 1B and Table 1). Ceramine (5 mM) (Fig. 2A), a non-hydrolysable analogue of ceramide [23], caused an identical effect on the $[Ca^{2+}]_i$ (Fig. 2B). Even more, the effect of ceramide was not elicited when added after its non-hydrolysable analogue (Fig. 2A). Accordingly, when ceramine is added after ceramide, there was no further increase in $[Ca^{2+}]_i$ (Fig. 2B), which indicates the same site of action of both compounds. These results taken together, ruled out that the effect of ceramide is due to its enzymatic conversion to sphingosine, pointing to a direct effect of this sphingolipid on calcium mobilization. We also tested the effect of ceramide-C_8 on the $[Ca^{2+}]_i$, obtaining identical results (Table 1).

We compared the effect of ceramide to the well-described effect of the Jurkat T cells anti-CD3 specific antibody, OKT3 on $[Ca^{2+}]_i$. The latter is known to activate phospholipase C-γ (PLCγ) via tyrosine phosphorylation producing in turn Ins(1,4,5)-P_3 from phosphatidylinositol (4,5) bis-phosphate, that liberates Ca^{2+} from the endoplasmic reticulum [6]. OKT3 produces an overall response similar to that observed with ceramide (203.8 ± 17.4 nM vs 219.6 ± 14.9 nM). It can be observed that when ceramide (5 mM) is added after OKT3 (Fig. 3A) or when OKT3 is added after ceramide (Fig. 3B), there was no additive effect, suggesting that both effectors elevate the $[Ca^{2+}]_i$ through the same mechanism. When EGTA was added to the extracellular milieu to abolish the extracellular calcium entry (Figs. 3C and D), it can be observed that OKT3 produced a

Table 1
Effect of different sphingolipids on the $[Ca^{2+}]_i$ on Jurkat T cells

	Peak $[Ca^{2+}]_i$, (%) (2 mM $CaCl_2$)	Peak $[Ca^{2+}]_i$, (%) (0 mM $CaCl_2$)
Control	100	86.8 ± 13.0
OKT3 (5 μg/ml)	226.2 ± 19.3*	146.9 ± 6.3*
Ceramide-C_2 (0.5 μM)	105.8 ± 13.7	N.D
Ceramide-C_2 (2.5 μM)	179.5 ± 9.7*	124.6 ± 12.3*
Ceramide-C_2 (5 μM)	243.7 ± 16.5*	179.2 ± 2.8*
Ceramide-C_2 (10 μM)	225.7 ± 10.0*	150.4 ± 11.7*
Ceramide-C_8 (2.5 μM)	174.4 ± 10.7*	120.5 ± 10.1*
Ceramide-C_8 (5 μM)	253.6 ± 13.8*	168.8 ± 11.2*
Ceramine (2.5 μM)	176.6 ± 10.3*	116.9 ± 6.2*
Dihydroceramide (5 μM)	96.0 ± 4.6	N.D.
Sphingosine (5 μM)	164.9 ± 10.1*	129.1 ± 4.8*
Sphingosine-1-phosphate (5 μM)	95.6 ± 6.9	N.D.
L-Sphingosine (5 μM)	94.3 ± 2.0	N.D.

The $[Ca^{2+}]_i$ is expressed as the mean of the peak value, after addition of each sphingolipid. Control represent the value of the $[Ca^{2+}]_i$ in resting Jurkat T cells (90.1 ± 9.0 nM). Peak represents the maximal $[Ca^{2+}]_i$ reached after the indicated addition. Values are means ± SD of at least three different experiments, and represent the percentage with respect to the control. N.D., not determined. Asterisks indicate that differences were statistically significant ($P \leqslant 0.01$) when compared with the $[Ca^{2+}]_i$ at the resting level (control), as indicated under Materials and methods.

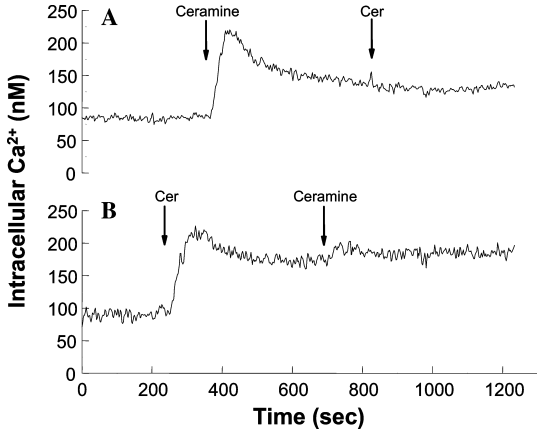

Fig. 2. Effect of ceramide and ceramine on the $[Ca^{2+}]_i$ in Jurkat T cells. All the experiments were done in the presence of 2 mM $CaCl_2$. (A) Arrows indicate the addition of ceramine (5 μM) and ceramide (5 μM); (B) arrows indicate the addition ceramide (5 μM) and ceramine (5 μM). Traces are representative of at least three independent experiments.

rapid increase in the $[Ca^{2+}]_i$, although of lower magnitude when compared to that obtained in the presence of extracellular calcium (Fig. 3A). Again, addition of ceramide after OKT3 (Fig. 3C) does not show any discernible effect on the $[Ca^{2+}]_i$. Accordingly, a similar result is obtained upon addition of ceramide in the absence of extracellular calcium (Fig. 3D), which supports that this sphingolipid is able to release Ca^{2+} from intracellular compartments. Again, the lack of effect of OKT3 after ceramide addition is observed (Fig. 3D), strongly suggesting a similar mechanism of action of these two effectors.

In the next series of experiments we present the effect of the combined action of ceramide and sphingosine. While neither sphingosine-1-P nor L-sphingosine has any effect on $[Ca^{2+}]_i$ (Table 1), the natural sphingolipid D-sphingosine, as reported by others [7], increases the $[Ca^{2+}]_i$, showing also a biphasic response (Fig. 4A). It can be also observed in the same figure that when ceramide was added to the cell suspension after sphingosine addition, the $[Ca^{2+}]_i$ is elevated to a higher level when compared with ceramide (Fig. 1A) or sphingosine alone, thus suggesting that this two sphingolipids liberate Ca^{2+} from different compartments, as reported for the action of sphingosine and OKT3 [7]. Since the optimal concentration of sphingosine is 20 μM [7], we used this concentration in our experiments, followed by 5 μM ceramide, which is the optimal concentration of this sphingolipid obtained during this work (Table 1). It can be observed in Fig. 4B that in the absence of extracellular calcium, again an additive effect of both messengers were observed supporting that they act through different calcium pools, but in both cases, after reaching a peak, the $[Ca^{2+}]_i$ returned to the basal level indicating that the plateau observed in the presence of extracellular Ca^{2+} is due to the activation of store-operated calcium channels.

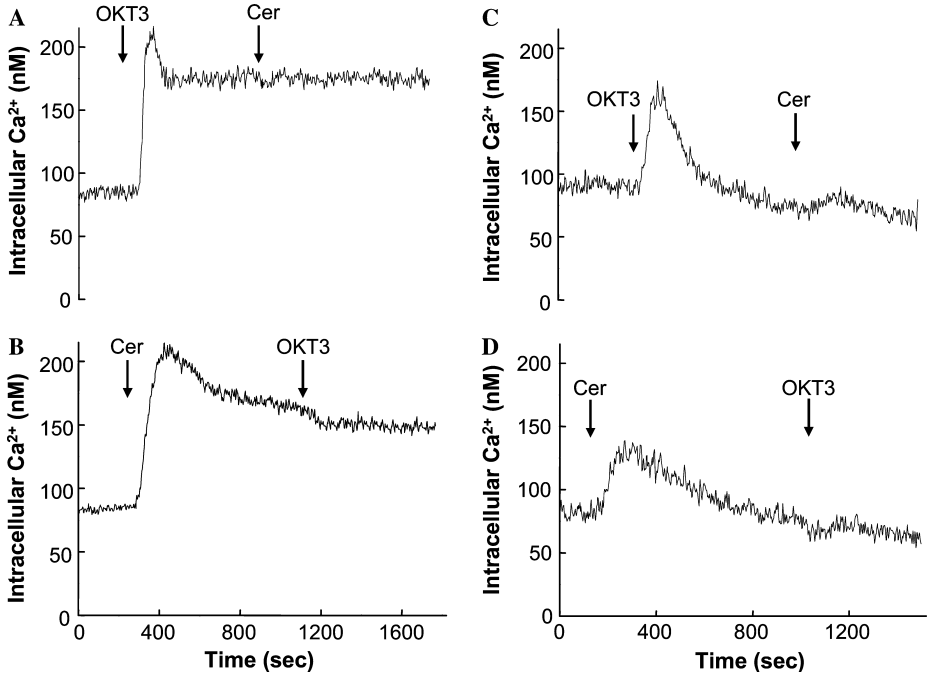

Fig. 3. Effect of OKT3 and ceramide on the $[Ca^{2+}]_i$ in Jurkat T cells. (A) Arrows indicate the addition of OKT3 (5 μg/ml) and ceramide (5 μM) in the presence of 2 mM $CaCl_2$; (B) arrows indicate the addition of ceramide (5 μM) and OKT3 (5 μg/ml) in the presence of 2 mM $CaCl_2$; (C) arrows indicate the addition of OKT3 (5 μg/ml) and ceramide (5 μM) in the absence of extracellular $CaCl_2$; (D) arrows indicate the addition of ceramide (5 μM) and OKT3 (5 μg/ml) in the absence of extracellular $CaCl_2$. Traces are representative of at least three independent experiments.

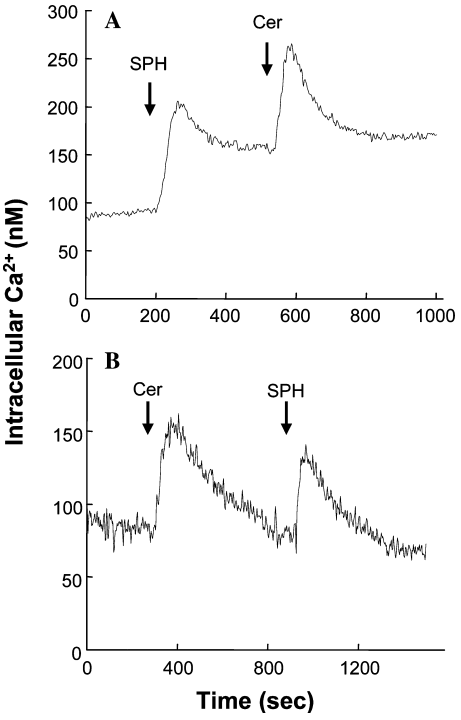

Fig. 4. Effect of ceramide and sphingosine on the $[Ca^{2+}]_i$ in Jurkat T cells. (A) Arrows indicate the addition of sphingosine (20 μM) and ceramide (5 μM) in the presence of 2 mM $CaCl_2$; (B) arrows indicate the addition of ceramide (5 μM) and sphingosine (20 μM) in the absence of extracellular $CaCl_2$. Traces are representative of at least three independent experiments.

Effect of SERCA inhibitors on ceramide signaling in cell populations

To investigate the origin of the intracellular calcium increase induced by ceramide, we used thapsigargin, a known Ca^{2+}-ATPase (SERCA) inhibitor that releases calcium from the endoplasmic reticulum [24], thereby depleting calcium stores which in turn results in the activation of store-operated calcium entry through plasma membrane (I_{CRAC}) channels [25–27]. As can be observed in Fig. 5A, thapsigargin indeed induces an elevation in the $[Ca^{2+}]_i$, which is followed by a calcium decrease to a plateau level higher than the basal calcium concentration. Ceramide added after the plateau reached by the action of thapsigargin elicited no effect. On the contrary, when thapsigargin is added after the onset of the ceramide effect (Fig. 5B), a clear increase in the $[Ca^{2+}]_i$ can be observed. This additional effect of thapsigargin after ceramide action can also be observed in the absence of extracellular calcium (Fig. 5C). This results indicates that albeit ceramide liberates Ca^{2+} from a thapsigargin sensitive calcium pool, there is another calcium pool also sensitive to the SERCA blocker but insensitive to ceramide. Fig. 5D shows the effect of ceramide in the absence of extracellular calcium. Under this condition, ceramide induces an increase in the $[Ca^{2+}]_i$ which is lower than that obtained in the presence of extracellular calcium (Fig. 5B). Interestingly, in these experiments in the absence of extracellular calcium, it can be observed that after getting a peak upon addition of ceramide, the $[Ca^{2+}]_i$ reached a level well below to that of the initial basal calcium concentration (Fig. 5D), which was not revealed in the presence of calcium.

To further quantify the magnitude of the calcium release by the different effectors, we use the Ca^{2+} ionophore ionomycin on the cell suspension and measured the ionomycin-releasable pool [28], which can be taken as the 100% (if acidic pools are neglected, since this ionophore interchange Ca^{2+} by H^+ electroneutrally). In Fig. 6, it can be observed the integrated area under the curve obtained after addition of ionomycin (1 μM) in a medium without calcium. This register represents the calcium flux from the intracellular stores to the extracellular space, which encounters the fluorescence probe present in the cytoplasm, before leaving the cell. It can be noticed that this area is larger in magnitude to that obtained upon addition of thapsigargin under the same conditions. If a higher concentration of the ionophore (10 μM) is used, the area under the curve obtained is lower, suggesting that calcium ions passed the cytoplasm too fast to be detected by the fluorophore. With this approach it is also possible to quantify if the ceramide and sphingosine releasable calcium pools add up to the thapsigargin releasable calcium store. Our results show that indeed, it seems to be the case (Fig. 6), since the sum of the area obtained after ceramide and sphingosine added separately is equal to that obtained in the presence of thapsigargin. This appears to be the case in both, when the experiments were done in the presence or in the absence of extracellular calcium. Albeit it would be expected that in the absence of the thapsigargin some redistribution of the calcium could occur, under the experimental condition used this was not seen. The lack of calcium redistribution was also observed in the experiments when ceramide and ceramine were added in sequence (Fig. 2) and also when OKT3 was added after ceramide addition and vice versa (Fig. 3). In all of these cases, the addition of the second effector did not show any additional significant calcium release, which would be expected in the case of some re-accumulation of calcium after its release.

Identification of the ceramide-sensitive Ca^{2+} pool by confocal microscopy

We then explored by the use of confocal microscopy the location of the ceramide-sensitive Ca^{2+} pool, taking advantage of the fact that endoplasmic reticulum can be easily marked by BODIPY-FL-Ryanodine (Fig. 7). This organelle appears in Jurkat T cells surrounding the cytoplasm just below the plasma membrane. On the other hand, cells were loaded with Rhod 2 under conditions

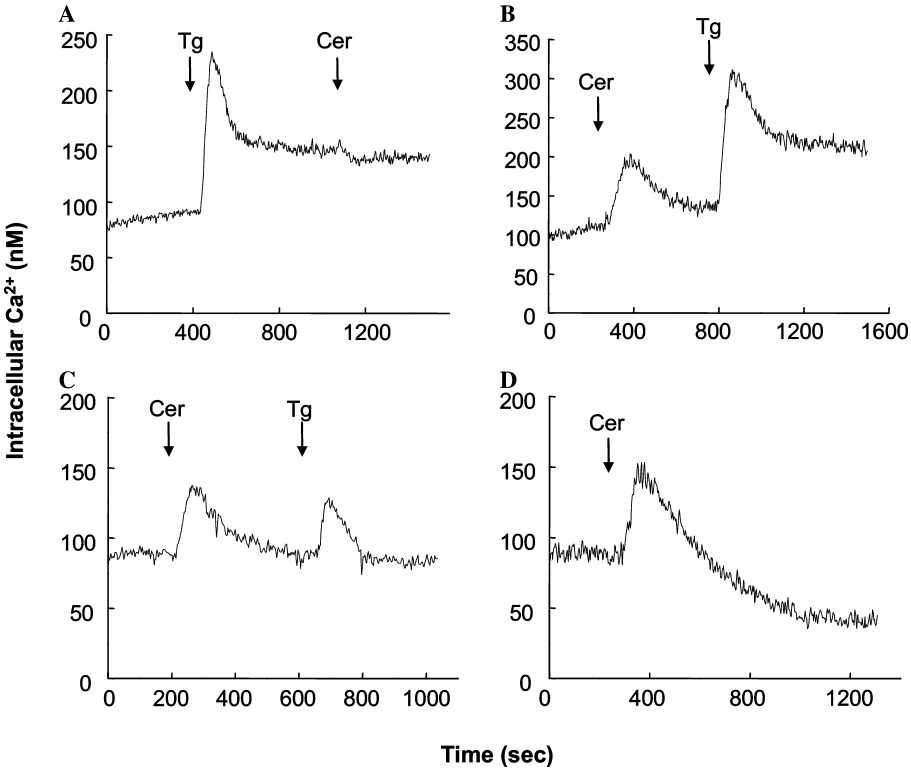

Fig. 5. Effect of thapsigargin and ceramide on the $[Ca^{2+}]_i$ in Jurkat T cells. (A) Arrows indicate the addition of thapsigargin (1 μM) and ceramide (5 μM) in the presence of 2 mM $CaCl_2$; (B) arrows indicate the addition of ceramide (5 μM) and thapsigargin (1 μM) in the presence of 2 mM $CaCl_2$; (C) arrow indicates the addition of ceramide (5 μM) and thapsigargin (1 μM) in the absence of extracellular $CaCl_2$. (D) Arrow indicates the addition of ceramide (5 μM) in the absence of extracellular $CaCl_2$. Traces are representative of at least three independent experiments.

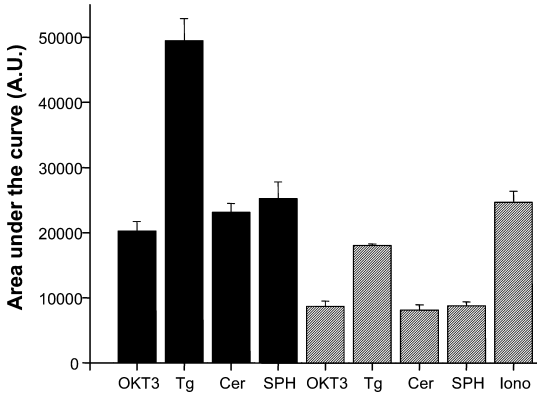

Fig. 6. Calculated area under the curve of the integrated calcium responses after different effectors. Thapsigargin (Tg), 1 μM; ceramide (Cer), 5 μM; sphingosine (SPH), 20 μM; ionomycin (Iono), 1 μM. Filled blocks were done in the presence of 2 mM $CaCl_2$. Dashed blocks, in the absence of $CaCl_2$ (EGTA 0.1 mM). Bars are representative of at least three independent experiments.

which favoured the compartmentalization of the dye (see Materials and methods). The red color (Fig. 7) indicates that the endoplasmic reticulum indeed possesses a large Ca^{2+} concentration. Some other calcium pools are also apparent, but these clearly do not correspond to endoplasmic reticulum as can be seen in the merge of the two indicators. Thus, a yellow-orange color indicates the position of this organelle (Fig. 7). When ceramide is added, after 7 min an evident bleaching of the orange-yellow zones appeared, consistent with the notion that this sphingolipid liberates Ca^{2+} from the endoplasmic reticulum. It is also apparent from this figure that ceramide its not able to release Ca^{2+} from the whole endoplasmic reticulum, thus indicating that another Ca^{2+} pool which is part of the endoplasmic reticulum is not sensitive to the action of ceramide.

Effect of ceramide on the phosphoinositide hydrolysis

To determine if the effect of ceramide occur through the elevation of $InsP_3$, as is the case for the action of OKT3, Jurkat T cells were labeled with myo-[2-^{3}H]inositol for 24 h prior to the stimulation with ceramide (5 mM) for 5 min. Unexpectedly, Jurkat T cells stimulated with ceramide did not produce an accumulation of $InsP_3$, when compared to the control (Fig. 8). This is in contrast to the stimulation by OKT3, which caused a significant increase on $InsP_3$ accumulation (Fig. 8). The production of $InsP_3$ by OKT3 stimulation indicates that cells are perfectly sensitive to the appropriate stimuli. Additionally, stimulation with sphingosine (20 mM) did not stimulate $InsP_1$ accumulation (Fig. 8), as reported by others [7] for the action of this sphingolipid.

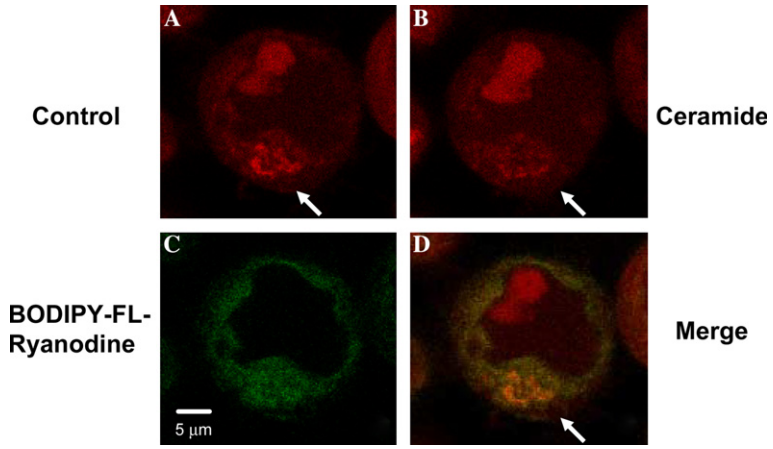

Fig. 7. Identification of the subcellular localization of the calcium pool sensitive to ceramide in Jurkat T cells by confocal fluorescence. Cells were double-labeled with BODIPY-FL-Ryanodine (green) and with Rhod-2 (red) to visualize stored Ca^{2+}. (A) Cells labeled with Rhod-2. (B) Cells labeled with Rhod-2 after ceramide (5 μM) treatment for 7 min. (C) BODIPY-FL-Ryanodine labeling. (D) Merge image demonstrates the coincidence of the labeling of the calcium pool at the endoplasmic reticulum and the ryanodine receptors as markers for this organelle. The arrow indicates the bleaching of the ceramide-sensitive endoplasmic reticulum calcium pool after the addition of ceramide.

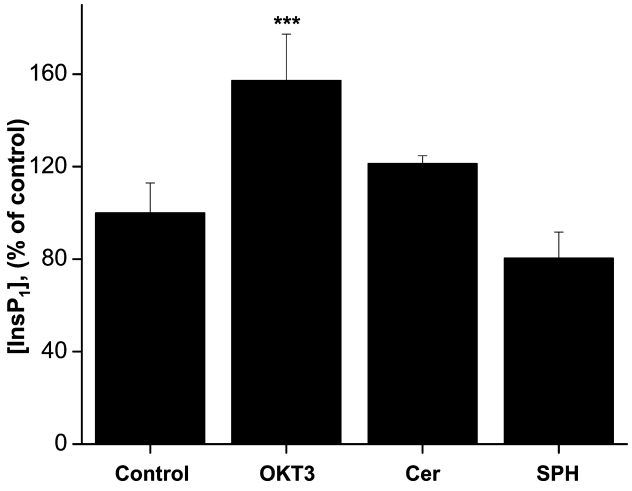

Fig. 8. Effect of ceramide, OKT3, and sphingosine on the accumulation of $InsP_3$ in Jurkat T cells. The $InsP_3$ formation was assessed as accumulation of radiolabeled $InsP_1$, incubated in KHBB medium containing 10 mM LiCl. Control, DMSO 1.6 μl, OKT3 5 μg/ml, ceramide 5 μM, and sphingosine 20 μM (see text for details). The asterisks indicate that results are statistically significant when compared to the control ($p \leqslant 0{,}05$). Values represent averages of four determinations ±SE.

Effect of ceramide on the capacitative calcium entry

Fig. 9A shows a typical effect of ceramide on $[Ca^{2+}]_i$ in the presence of extracellular calcium. When the I_{CRAC} inhibitor 2-aminoethoxydiphenylborate (2-APB) was added after the $[Ca^{2+}]_i$ plateau reached by the action of ceramide, a clear decrease in the $[Ca^{2+}]_i$ was observed, reaching values similar to that prior to the addition of ceramide. Fig. 9B shows that when 2-APB was added previous to ceramide under the same condition, i.e., in the presence of extracellular calcium, the capacitative calcium entry is fully abolished, thus resembling the effect of ceramide in the absence of calcium (Fig. 5D).

The opening of CRAC channels by ceramide was supported by experiments carried out with Mn^{2+} as a surrogate permeable ion for calcium, and measuring the rate of the Mn^{2+}-induced quench of cytosolic Fura-2 fluorescence [26]. As can be seen in Fig. 10, when the excitation wavelength is set at the isosbestic point for Fura-2 (360 nm) and the emission is recorded at 510 nm, it can be followed the quenching of the fluorescent probe by addition of Mn^{2+}. Under this condition, there is a large decay in the fluorescence after the addition of ceramide (lower line in Fig. 10), when compared to the control without the sphingolipid (upper line in Fig. 10), which can be readily explained by the entrance of Mn^{2+} through the CRAC channels and the subsequent fluorescence quenching of the intracellular calcium indicator. When digitonin is added to the cell suspension to make the plasma membrane leaky to the divalent cation, a rapid decline in the fluorescence is obtained under both conditions, which is accordingly larger in the absence of ceramide.

To corroborate that ceramide indeed induces the opening of a capacitative calcium entry, we studied the plasma membrane 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 [29], with the exception of Fig. 11B, where intracellular $MgCl_2$ was omitted. In Fig. 11A, inwardly rectifying currents that developed after the application of ceramide is shown. Judged by its current–voltage relationship and positive reversal potential, this current resembles that of the I_{CRAC}. Several experiments were conducted to verify I_{CRAC} currents induced by passive dialysis using EGTA or BAPTA and by OKT3 (data not shown). Different $[Ca^{2+}]_i$ were assayed

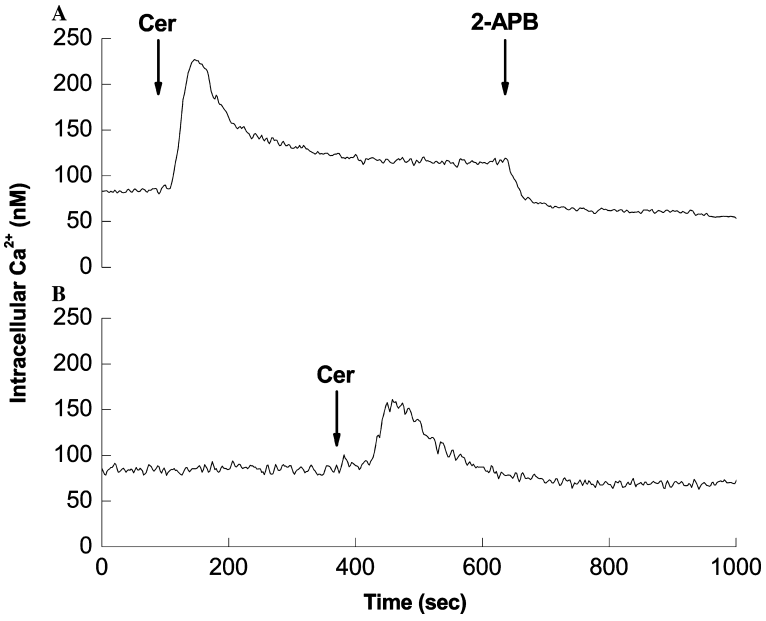

Fig. 9. Effect of ceramide and 2-APB on the $[Ca^{2+}]_i$ in Jurkat T cells. All the experiments were done in the presence of 2 mM $CaCl_2$. (A) Arrows indicate the addition of ceramide (5 μM) and 2-APB (20 μM); (B) 2-APB (20 μM) was added 5 min before the addition of ceramide. Arrow indicates the addition of ceramide (5 μM). Traces are representative of at least three independent experiments.

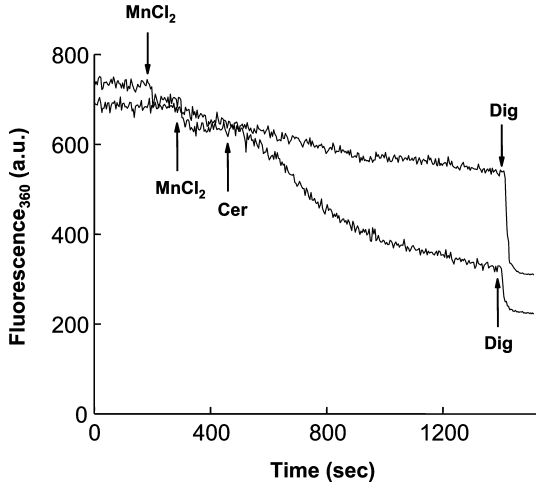

Fig. 10. Effect of ceramide on Mn^{2+} influx in Jurkat T cells. In the upper trace (control), arrows indicate the addition of $MnCl_2$ (100 μM) and digitonin (6 μM) at the end of the experiment. In the lower trace, arrows indicate the addition of $MnCl_2$ (100 μM), ceramide (5 μM), and of digitonin (6 μM). Entry of extracellular $MnCl_2$ was detected by its quenching effect on the Ca^{2+}-insensitive Fura-2 fluorescence signal, excited at 360 nm (isosbestic point), and the emission (F_{360}) recorded at 510 nm. Traces are representative of at least three independent experiments.

to verify if I_{CRAC} opened spontaneously. In the range of 10–200 nM Ca^{2+}, only 10 out of 69 patches have spontaneous opening of I_{CRAC}; BAPTA 10 mM induced always I_{CRAC} currents (3 out of 3). Ceramide was only added when no spontaneous activation of I_{CRAC} currents was observed. This sphingolipid induced a Ca^{2+} current in approximately half of the cells (combined data 20 out of 39). The peak current amplitude evaluated at −120 mV was -2.3 ± 0.65 pA ($n = 5$), and −7.4 pA ($n = 2$) at 2 and 20 mM external calcium, respectively. Currents induced by OKT3 had an amplitude, in 2 mM Ca^{2+}, of -6.1 ± 1.5 pA ($n = 4$) (not shown) as reported by others [9]. To further corroborate that the observed current correspond to the opening of I_{CRAC} and not MIC currents, the development of this last current was allowed by omission of internal $MgCl_2$. Under this condition, the MIC current spontaneously opened after a variable period of time (not shown). Addition of spermine (20 μM) to the extracellular solution is known to inhibit this current in a voltage-dependent manner [30]. After addition of 5 μM ceramide a current with a profile essentially identical to the I_{CRAC} developed (Fig. 11C), but larger in magnitude (-5.43 ± 0.34 pA, $n = 3$), probably due to a contamination of the MIC current which is not totally inhibited by spermine at very hyperpolarized potentials [30]. An outward current appears at positive potentials (not shown), which should correspond to the relief of the blockage by spermine on the MIC currents at these potentials, since the inhibition produced by polyamines is partially released at positive potentials [30]. Taken together, these results demonstrated that ceramide induces the opening of I_{CRAC} currents in Jurkat T cells.

Discussion

It has been well recognized that ceramide is an important factor in the induction of apoptosis in many cells

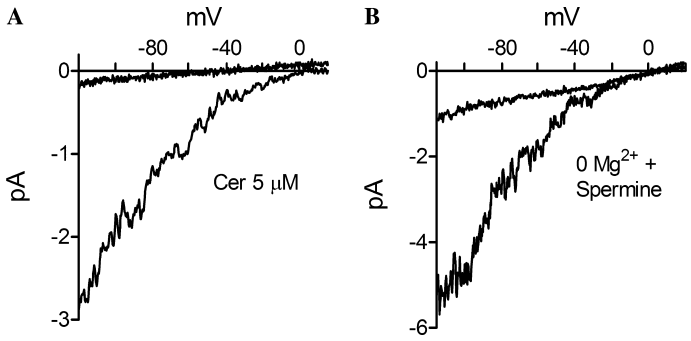

Fig. 11. Development of an inward rectifying current after the application of ceramide in Jurkat T cells. I_{CRAC} currents were measured in whole-cell configuration by applying 100 ms voltage ramps from −120 to +20 mV at a holding potential of 0 mV after an 8 s hyperpolarization prepulse at −120 mV. (A) Effect of ceramide (5 μM). (B) Effect of ceramide (5 μM) in the in the absence of intracellular Mg^{2+} and presence of spermine (20 μM) on MIC and CRAC currents. Currents were measured as in (A), but applying 200 ms voltage ramps from −120 to +20 mV, after a 1 s hyperpolarization prepulse at −120 mV, to observe the development of MIC currents (see text for details). Each figure contains its respective control record. Records were digitally filtered at 1 kHz.

including Jurkat human T cells [31]. However, little is known on the initial triggering of this response, while intracellular Ca^{2+} elevation is known to be one of the main events occurring during T cells activation. In this report we demonstrate that ceramide is able to elevate $[Ca^{2+}]_i$ in Jurkat T cells from an intracellular pool that closely resembles that caused by the well-known specific antibody against the TCR/CD3 complex, OKT3. The lack of additive effect between ceramide and OKT3, in the presence and absence of extracellular calcium support this view.

The use of ceramine, a non-hydrolysable analogue of ceramide, has been proved to be very useful in this work, since this compound has allowed us to ascertain that the effect of ceramide on the $[Ca^{2+}]_i$ is not the result of its enzymatic conversion to sphingosine which could occur very rapidly. Indeed, the rapid metabolism of sphingolipids could drive to wrong conclusions about the particular messenger involved in a given effect, when they are studied in complex systems like whole cells. In this context it is worthwhile to mention that it has been demonstrated that sphingosine could be converted to ceramide by the action of a ceramidase working in the reverse mode [32,33]. However, this does not seem to be the case in this work since we also demonstrate that ceramide and sphingosine act through different mechanisms, as confirmed by the additive effect observed when both sphingolipids are added together. The effect of sphingosine on the $[Ca^{2+}]_i$ in Jurkat T cells had been previously documented [7]. Sphingosine on Jurkat T cells is reported to elevate $[Ca^{2+}]_i$ via stimulation of a diacylglycerol kinase and the subsequent increase in phosphatidic acid, which liberates calcium from an intracellular compartment that is sensitive to thapsigargin but insensitive to Ins(1,4,5)-P_3 [7]. In contrast, the effect of OKT3 on the increase of the $[Ca^{2+}]_i$ is known to occur through PLC stimulation and subsequent induction of release from a compartment that is sensitive to both Ins(1,4,5)-P_3 and thapsigargin. In this perspective it is worthwhile to mention that it has been reported that Jurkat-T lymphocytes possess several intracellular calcium pools [34]. Taken together, our results indicate that ceramide liberates calcium from the same intracellular pool employed by OKT3, which in turn is different to that of sphingosine. This assumption is supported by the fact that thapsigargin is able to release calcium even after the onset of the effect of ceramide (this work) or sphingosine [7]. The experiments done with ionomycin and the calculation of the area under the curve of the Ca^{2+} elevation induced by the different effectors also support this notion, since the value obtained in the presence of ceramide and sphingosine add up to the value obtained with thapsigargin, either in the presence or in the absence of extracellular calcium.

One of the most interesting branch of the present work concerns the approach to try to identify the mechanism of action of ceramide on the $[Ca^{2+}]_i$ at the biochemical level. It is interesting to mention that it have been reported that ceramide is able to stimulate PLC_β in *Xenopus laevis* oocytes through activation of a G_q protein, and then inducing Ins(1,4,5)-P_3 production and subsequent elevation of the $[Ca^{2+}]_i$ [15]. However, as mentioned before, Jurkat T cells posses PLC_γ , which is activated via tyrosine phosphorylation [6]. Thus, it was conceivable that ceramide also could activate the PLC_γ. This possibility was discarded by the results obtained in this work, since, in contrast to the effect of OKT3 on the Ins(1,4,5)-P_3 production, ceramide did not stimulate phosphoinositide turnover. These results indicates that albeit ceramide can liberate Ca^{2+} from the same intracellular pool that OKT3, as demonstrated by confocal microscopy and other approaches during this work, the sphingolipid acts through a different mechanism of action. Thus, ceramide do not activate the TCR/CD3 receptor, tyrosine kinase activity or the PLC_γ , but some event downstream to the $InsP_3$ produc-

tion. For example, ceramide could activate the IP_3 receptor or the ryanodine receptor directly, or the sphingolipid could increase the level of another messenger able to open these Ca^{2+} channels. Supporting this possibility, ceramide is indeed synthesized in the cytoplasmic surface of the endoplasmic reticulum [35]. Recent works with the fluorescent ceramide analog C_5-DMB-ceramide has also demonstrated synthesis of ceramide de novo in the endoplasmic reticulum [36]. Interestingly, a recent report has presented evidences of the involvement of sphingosine-1-P phosphohydrolase (SPP-1), located in the endoplasmic reticulum, in generating sphingosine as a substrate for ceramide synthase [37], which represents another way to generate ceramide within the cell. These data could imply that the synthesis of ceramide in the endoplasmic reticulum as a consequence of diverse cell signals could be translated in an elevation of $[Ca^{2+}]_i$. In any case, the specific mechanism of action of the ceramide-induced initial elevation of the $[Ca^{2+}]_i$ in Jurkat T cells remains to be elucidated and represents an interesting focus of study.

Another interesting observation obtained in the present work was the decay in the $[Ca^{2+}]_i$ well below the basal level after the ceramide peak when the sphingolipid was added in the absence of extracellular calcium. This could be related to the reported stimulation of the plasma membrane calcium pump induced by ceramide [38]. It has been reported that the plasma membrane calcium pump is the main regulator of the basal $[Ca^{2+}]_i$ in Jurkat T cells [9]. In this context is worthwhile to mention that this $[Ca^{2+}]_i$ decrease was not revealed in the presence of calcium, consequence of the large calcium influx obtained under these conditions.

The transient elevation of the $[Ca^{2+}]_i$ induced by ceramide was followed by a rapid regulation which reached a higher $[Ca^{2+}]_i$ level, when compared to the resting concentration, compatible with the induction of the opening of a capacitative calcium entry. In Jurkat T cells this calcium entrance is known to occur throughout a CRAC channel which has been well characterized in these cells [9,29,39].

The results obtained by the use of 2-APB as an I_{CRAC} inhibitor, also support that ceramide indeed open a capacitative calcium entry in Jurkat T cells. It is worthwhile to mention that even though it was previously reported that 2-APB produced an inhibition of the Ins(1,4,5)-P_3 receptor, recent studies [6,8,39,40] demonstrate that, depending on the concentration used, it inhibits I_{CRAC} in Jurkat T cells, in a manner that is not related to the inhibition of the Ins(1,4,5)-P_3 receptor, thus validating its general use as an I_{CRAC} inhibitor. The experiments done by measuring the Fura-2 quench by Mn^{2+} also support this notion, since this divalent cation has been demonstrated to be able to enter the cell by several plasma membrane calcium channels [26]. This proposal was finally corroborated with the patch clamp experiments, where we show that ceramide induce the opening of a calcium channel with the general characteristics of the I_{CRAC}. The experiments done allowing the development of MIC currents and using spermine as a MIC inhibitor also support that the channel opened by the ceramide is indeed $I_{CRAC.}$ It is interesting to point out that ceramide opened I_{CRAC} in ~50 % of the cells studied, similar to the values reported for the opening of I_{CRAC} induced by Ins(1,4,5)-P_3 [41]. Accordingly, in experiments done with isolated cells, ceramide induced an increase in $[Ca^{2+}]_i$ also in approximately half of the cells (combined data 29 out of 50). This means that not all cells respond identically to the action of different messengers, probably due to the lack of growth synchronization.

Concerning a possible physiological role of the sphingolipids used during this work on T cell signaling, it is interesting to mention that it have been reported an increase in sphingolipids upon the activation of T lymphocytes by antigen presenting cells and by other means [42]. Moreover, co-stimulation of the TCR in different T cells lines by specific antibodies increases the activity of a neutral [5] and acidic [43] sphingomyelinase with the subsequent elevation of ceramide, so that a raise in the intracellular concentration of ceramide could be part of the process of T cells activation. In this respect, antibodies against CD3 alone do not produce ceramide elevation, but when used in combination with anti CD28 results in a rapid and transient increase in this sphingolipid. [43]. Both exogenously added acid sphingomyelinase and ceramide in the presence of anti-CD3 antibody enhanced T cell proliferation, associated with the activation of NF-κB and elevated transcription of the gene encoding IL-2 [5]. Moreover, it has been proposed that ceramide might facilitate the cross-talk between CD28 costimulatory and TCR signals through a pathway which is coupled with the activation of a signaling cascade that include p21-activated kinase (PAK) and MEK kinase 1 [44]. It has also been postulated that the rapid elevation of ceramide in response to CD28 might result in enhanced membrane fluidity, which, in turn, might enhance the recruitment of the two receptors (TCR and CD28) into the same lipid raft [5].

Regarding the possible physiological significance of the ceramide-induced calcium fluxes, albeit the role of calcium signaling in T lymphocytes is well documented [6], the relationship between ceramide and intracellular calcium elevation from endoplasmic reticulum is a subject of recent discussion. In this respect, Scorrano et al. [45] have recently reported in a line of mouse embryonic fibroblasts deficient for BAX and BAK (DKO), proteins which are multidomain proapoptotic members from the BCL-2 family, a clear relationship between calcium release from endoplasmic reticulum induced by ceramide (where these proteins are located), and the initiation of apoptosis. These cells have lost the ability to

undergo apoptosis in the presence of ceramide, when compared to the wild- type. The increase in $[Ca^{2+}]_i$ induced by thapsigargin in cell deficient for BAX and BAK is smaller when compared to wild-type cells. When these DKO cells are exposed to a pulse of 2 mM ionomycin in the presence of extracellular calcium, which causes an increase in $[Ca^{2+}]_i$ primarily due to Ca^{2+} influx, the pulse restore killing of these cells by ceramide. Interestingly, the studies on the calcium dependence of physiologic death signal have been extended to T cells through TCR induced apoptosis [45]. Thus, the transfer of calcium from the endoplasmic reticulum to mitochondria is postulated to be required for the initiation of programmed cell death by some (but not all) apoptotic signals [46]. Taken together, this data indicates that the sensitivity to apoptosis depends on the ability of cells to transfer Ca^{2+} from the endoplasmic reticulum to the mitochondria [46]. Accordingly, procedures that enhance the transfer of Ca^{2+} endoplasmic reticulum to mitochondria augment ceramide-induced cell death [47]. All these recent data strongly support a possible physiological role of the ceramide-induced Ca^{2+} release observed in Jurkat T cells during the present work.

In conclusion, the results obtained in the present work are consistent with an activation of an I_{CRAC} induced by calcium stores depletion in Jurkat T cells as a result of ceramide, indicating that this important second messenger is able to integrate the route of calcium signaling with the sphingomyelin cycle. Since ceramide has shown to be also implicated in apoptosis in many cells including Jurkat T lymphocytes [42], and a rise in $[Ca^{2+}]_i$ has also been reported in apoptotic cells, we are tempted to speculate that these two events could be associated. Thus, the rapid elevation of $[Ca^{2+}]_i$ induced by ceramide could constitute a pre-apoptotic condition, necessary for the onset of the apoptotic process, provided that other essential signals are also simultaneously present. However, there is still a large gap between the fast $[Ca^{2+}]_i$ elevation observed upon addition of ceramide during this work (after few seconds) and the onset of the apoptosis which last for few days. The important piece of information that could link this two events remains to be elucidated. Finally, it is also conceivable that the effect of ceramide on $[Ca^{2+}]_i$ in Jurkat T cells could be related to other signaling processes not directly related with apoptosis, as mentioned before for the costimulation of CD3 and CD28, according to the pleiotropic nature of the sphingolipids signaling pathway.

Acknowledgments

This work was supported by grants from Fondo Nacional de Ciencia, Tecnología e Innovación (FONACIT), Venezuela, (S1-99000058 to G.B. and G-2001000637 to G.B. and R.D.) and C.D.C.H.-U.C.V (PI 03-33-4798-00) to G.B.

References

[1] S. Pyne, N.J. Pyne, Biochem. J. 349 (2000) 385–402.
[2] A. Gómez-Muñoz, FEBS Lett. 562 (2004) 5–10.
[3] A.H. Futerman, Y.A. Hannun, EMBO Rep. 5 (2004) 777–782.
[4] B.J. Pettus, C.E. Chalfant, Y.A. Hannun, Biochim. Biophys. Acta 1585 (2002) 114–125.
[5] D. Adam, M. Heinrich, D. Kabelitz, S. Schütze, Trends Immunol. 23 (2002) 1–4.
[6] R.S. Lewis, Annu. Rev. Immunol. 19 (2001) 497–521.
[7] S. Sakano, H. Takemura, K. Yamada, K. Imoto, M. Kaneko, H. Ohshika, J. Biol. Chem. 271 (1996) 11148–11155.
[8] J.W. Putney Jr., L.M. Broad, F.J. Braun, J.P. Lievremont, G.S. Bird, J. Cell Sci. 114 (2001) 2223–2229.
[9] D.M. Bautista, M. Hoth, R.S. Lewis, J. Physiol. 541 (2002) 877–894.
[10] H. Hida, M. Takeda, B. Soliven, J. Neurosci. 18 (1998) 8712–8719.
[11] D.P Relling, K.K. Hintz, J. Ren, Br. J. Pharmacol. 140 (2003) 1163–1168.
[12] A. Lepple-Wienhues, C. Belka, T. Laun, A. Jekle, B. Walter, U. Wieland, M. Welz, L. Heil, J. Kun, G. Busch, M. Weller, M. Bamberg, E. Gulbins, F. Lang, Proc. Natl. Acad. Sci. USA 96 (1999) 13795–13800.
[13] C. Mathes, A. Fleig, R. Penner, J. Biol. Chem. 273 (1998) 25020–25030.
[14] Y. Nakamura, S. Nakashima, K. Ojio, Y. Banno, H. Miyata, Y. Nozawa, J. Immunol. 156 (1996) 256–262.
[15] E. Kobrinsky, A.I. Spielman, S. Rosenweig, A.R. Marks, Am. J. Physiol. 277 (1999) C665–C672.
[16] T. Numakawa, H. Nakayama, S. Suzuki, T. Kubo, F. Nara, Y. Numakawa, D. Yokomaku, T. Araki, T. Ishimoto, A. Ogura, T. Taguchi, J. Biol. Chem. 278 (2003) 41259–41269.
[17] G. Grynkiewicz, M. Poenie, R.Y. Tsien, J. Biol. Chem. 260 (1985) 3440–3450.
[18] M. Mendoza, A. Mijares, H. Rojas, C. Colina, V. Cervino, R. DiPolo, G. Benaim, J. Parasitol. 90 (2004) 1181–1183.
[19] H. Rojas, M. Ramos, A. Mijares, R. DiPolo, Jpn. J. Physiol. 53 (2003) 259–269.
[20] M.J. Berridge, R.M.C. Dawson, C.P. Downes, J.P. Heslop, R.F. Irvine, Biochem. J. 212 (1983) 473–482.
[21] M. del R Garrido, A. Israel, Neuropeptides 38 (2004) 304–310.
[22] O.P. Hamill, A. Marty, E. Neher, B. Sakamann, F.J. Sigworth, Plügers Arch. 391 (1981) 85–100.
[23] N. Karasavvas, R.K. Erukulla, R. Bittman, R. Lockshin, Z. Zakeri, Eur. J. Biochem. 236 (1996) 729–735.
[24] G. Inesi, Y.J. Sagara, J. Membr. Biol. 141 (1994) 1–6.
[25] A. Hussain, G. Inesi, J. Membr. Biol. 172 (1999) 91–99.
[26] M. Hoth, R. Penner, Nature 355 (1992) 353–356.
[27] A.F. Fomina, C.M. Fanger, J.A. Kozak, M.D. Cahalan, J. Cell Biol. 150 (2000) 1435–1444.
[28] C. Fasolato, T.J. Pozzan, Biol. Chem. 264 (1989) 19630–19636.
[29] M. Prakriya, R.S. Lewis, J. Gen. Physiol. 119 (2002) 487–507.
[30] J.A. Kozak, H.H. Kerschbaum, M.D. Cahalan, J. Gen. Physiol. 120 (2002) 221–235.
[31] C.A. Hetz, M. Hunn, P. Rojas, V. Torres, L. Leyton, A.F.G. Quest, J. Cell Sci. 115 (2002) 4671–4683.
[32] S. El Bawab, H. Birbes, P. Roddy, Z.M. Szulc, A. Bielawska, Y.A. Hannun, J. Biol. Chem. 276 (2001) 16758–16766.
[33] N. Okino, X. He, S. Gatt, K. Sandhoff, M. Ito, E.H. Schuchman, J. Biol. Chem. 278 (2003) 29948–29953.

[34] A.H. Guse, E. Roth, F. Emmrich, Biochem. J. 291 (1993) 447–451.
[35] K. Hirschberg, J. Rodger, A.H. Futerman, Biochem. J. 290 (1993) 751–757.
[36] R.J. Perry, N.D.J. Ridgway, J. Lipid Res. 45 (2004) 164–173.
[37] H. Le Stunff, I. Galve-Roperh, C. Peterson, S. Milstien, S. Spiegel, J. Cell Biol. 158 (2002) 1039–1049.
[38] C. Colina, V. Cervino, G. Benaim, Biochem. J. 362 (2002) 247–251.
[39] M. Prakriya, R.S. Lewis, J. Physiol. 536 (2001) 3–19.
[40] M.D. Bootman, T.J. Collins, L. Mackenzie, H.L. Roderick, M.J. Berridge, C.M. Peppiatt, FASEB J. 16 (2002) 1145–1150.
[41] A.V. Yeromin, J. Roos, K.A. Stauderman, M.D. Cahalan, J. Gen. Physiol. 123 (2004) 167–182.
[42] E.A. Martinova, Biochemistry (Moscu) 63 (1998) 102–110.
[43] L.M. Boucher, K. Wiegmann, A. Fütterer, K. Pfeffer, T. Machleidt, S. Schütze, T.W. Mak, M. Krönke, J. Exp. Med. 181 (1995) 2059–2068.
[44] S. Kaga, S. Ragg, K.A. Rogers, A. Ochi, J. Immunol. 160 (1998) 4182–4189.
[45] L. Scorrano, S.A. Oakes, J.T. Opferman, E.H. Cheng, M.D. Sorcinelli, T. Pozzan, S.J. Korsmeyer, Science 300 (2003) 135–139.
[46] N. Demaurex, C. Distelhorst, Science 300 (2003) 65–67.
[47] E. Rapizzi, P. Pinton, G. Szabadkai, M.R. Wieckowski, G. Vandecasteele, G. Baird, R.A. Tuft, K.E. Fogarty, R. Rizzuto, J. Cell Biol. 159 (2002) 613–624.