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Calcium Release-activated Calcium Current (I_{CRAC}) Is a Direct Target for Sphingosine*

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Whole cell patch-clamp recordings were made to study the regulation of the store-operated calcium release-activated calcium current (I_{CRAC}) by metabolites involved in the sphingomyelin pathway in RBL-2H3 cells. Sphingosine, a regulator of cell growth, inhibits I_{CRAC} completely within 200 s and independently from conversion to either sphingosine 1-phosphate or ceramide. Structural analogs of sphingosine, including *N,N*-dimethylsphingosine, DL-*threo*-dihydrosphingosine, and *N*-acetylsphingosine (C_2-ceramide) also block I_{CRAC}. This effect is always accompanied by an elevation of whole cell membrane capacitance. These sphingolipids appear, therefore, to accumulate in the plasma membrane and directly block I_{CRAC} channels. Sphingosylphosphorylcholine also increases capacitance but does not inhibit I_{CRAC}, demonstrating structural specificity and that the elevation of capacitance is necessary but not sufficient for block. Nerve growth factor, which is known to break down sphingomyelin, inhibits I_{CRAC}, and this inhibition can be antagonized by reducing sphingosine production with L-cycloserine, suggesting that I_{CRAC} is a physiologically relevant and direct target of sphingosine. We propose that sphingosine directly blocks I_{CRAC}, suggesting that the sphingomyelin pathway is involved in I_{CRAC} regulation.

Agonists that stimulate phospholipase C (PLC)[1] and elevate $InsP_3$ levels activate Ca^{2+} entry that is important for refilling depleted stores (for review see Refs. 1–3). In mast cells and T-lymphocytes, this store-operated calcium (SOC) current is highly selective for Ca^{2+} ions and has been termed calcium release-activated-calcium current (I_{CRAC}) (4, 5). I_{CRAC} has also been measured in the mast cell line RBL-2H3, in hepatocytes, in thyrocytes, 3T3 fibroblasts, and HL-60 cells (6). Both protein kinase C (PKC) and a small guanosine triphosphate-binding protein regulate I_{CRAC} (7, 8), although the exact mechanism for activation of I_{CRAC} remains unknown.

One of the difficulties in pin-pointing a definite mechanism for regulation of I_{CRAC} may relate to the fact that PLC activation functions in parallel with other intracellular signaling processes. The sphingomyelin (SM) pathway, for example, "cross-talks" with the PLC pathway (9). Activation of the SM pathway produces important second messengers such as sphingosine, sphingosine 1-phosphate (S1P), and ceramide (10). Generally, sphingosine and S1P mediate cell growth and proliferation, whereas ceramide elevations cause programmed cell death (apoptosis) or cell cycle arrest (9). Although several direct targets have been characterized for ceramide (10), the picture is less clear for sphingosine. PKC has been shown to be a target for sphingosine (11), but the action of sphingosine on other proteins and intracellular processes may be mediated by conversion to S1P and/or ceramide (10, 12, 13).

Intracellular Ca^{2+} elevations may be an important connection for cross-talk between the PLC and SM pathways. Indeed, it has been demonstrated that elevations in $[Ca^{2+}]_i$ can modulate cell growth (14–16), and sphingosine has been shown to alter Ca^{2+} homeostasis in various preparations (17–21). We have therefore investigated the role of the SM pathway in regulating intracellular calcium concentration, focusing on the major Ca^{2+} influx pathway provided by the SOC current I_{CRAC} in the mast cell line RBL-2H3. We surveyed the sphingomyelin pathway for possible regulators of I_{CRAC} and found that sphingosine and structurally related compounds (but not S1P nor ceramides) are inhibitors. None of the agents tested activate I_{CRAC}. A requirement for inhibition of I_{CRAC} is incorporation of the lipid molecule into the plasma membrane. Sphingolipid incorporation was measured as an elevation of the whole cell capacitance, which represents a new approach for monitoring lipid or possibly drug accumulation in the plasma membrane.

To provide a link between cellular sphingosine and inhibition of I_{CRAC}, we tested several growth factors, which are known to activate sphingomyelinase and/or to elevate sphingosine levels (9, 22–27). We observed that nerve growth factor (NGF 2.5 S subunit; 1 μg/ml) inhibits I_{CRAC}. NGF-induced inhibition of I_{CRAC} was reduced by treatment with L-cycloserine (LCS; 2 mM), which lowers SM levels (26) and hinders sphingosine production by NGF. Implications for sphingosine-dependent block of I_{CRAC} are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat basophilic leukemia cells (RBL-2H3) were plated on glass coverslips at low density and incubated at 37 °C with 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 45 mM sodium bicarbonate, 5 mM glucose, 0.12 mg/ml streptomycin, and 0.60 mg/ml penicillin. For the recordings, coverslips

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[1] The abbreviations used are: PLC, phospholipase C; I_{CRAC}, calcium release-activated calcium current; SOC, store-operated calcium; PKC, protein kinase C; SM, sphingomyelin; S1P, sphingosine 1-phosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; NGF, nerve growth factor; CCh, carbamylcholine; LCS, L-cycloserine; BSA, bovine serum albumin; $InsP_3$, inositol 3-phosphate; SMase, sphingomyelinase; DMS, *N,N*-dimethylsphingosine; ATPγS, adenosine 5′-*O*-(thiotriphosphate); pF, picofarads; AA, arachidonic acid; PA, phosphatidic acid; C1P, ceramide 1-phosphate; TTX, tetrodotoxin.

were transferred into the recording chamber containing the desired external solution. RBL-2H3 cells stably transfected with m1 muscarinic receptors (RBL-m1 (28, 29)) were a kind gift from Dr. O. H. Choi (National Institutes of Health, Bethesda). RBL-m1 cells were cultured identically to RBL-2H3 cells, except 400 μg/ml G418 was added to the RBL-m1 growth medium to select for transfected cells. The muscarinic receptor agonist carbamylcholine (CCh; 100 μM) activated calcium release (as measured by fura-2) or I_{CRAC} (in patch-clamp experiments) in every RBL-m1 cell tested.

Whole Cell Patch-Clamp Recording—The tight-seal whole cell configuration was used for patch-clamp. Experiments were conducted at room temperature (20–27 °C) in standard external saline solution containing the following (in mM): 140 NaCl, 2.8 KCl, 10 $CaCl_2$, 2 $MgCl_2$, 10 CsCl, 11 glucose, and 10 HEPES·NaOH (pH 7.2). Sylgard-coated, fire-polished patch pipettes had resistances between 1.5 and 3 megohms after filling with the standard internal solution which contained the following (in mM): 145 Cs·Glu, 8 NaCl, 1 $MgCl_2$, 10 Cs·EGTA, 0.02 $InsP_3$, and 10 mM HEPES·CsOH (pH 7.2). Whole cell break-in with this standard internal solution always led to activation of I_{CRAC}.

Whole cell break-in without activation of I_{CRAC} was achieved by excluding $InsP_3$, clamping intracellular Ca^{2+} to ~80 nM with 2 to 1 ratio of Cs·EGTA/Ca·EGTA, and including nucleotides (4 mM Mg·ATP and 300 μM Na_2·GTP) to maintain intracellular integrity and to prevent depletion of internal Ca^{2+} stores. This internal solution was used when testing for activation of I_{CRAC} by various sphingomyelin pathway intermediates.

High resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9). Currents due to cellular membrane capacitance were recorded and canceled before each voltage ramp using the automatic capacitance compensation of the EPC-9. In this manner, whole cell capacitance was measured throughout the experiment. Series resistance was between 2 and 10 megohms, and inhibition of I_{CRAC} did not correlate with series resistance. Currents were filtered at 3.3 kHz and digitized at 100 μs. Currents elicited by voltage ramps in Fig. 1 were filtered at 1 kHz off-line. Ramps were given every 2 s (−100 to +100 mV in 50 ms) from a holding potential of 0 mV, and the Ca^{2+} current was analyzed at the ramp segment corresponding to −80 mV. This protocol minimized Ca^{2+} entry because of the delay between ramps and because of the relatively small driving force for Ca^{2+} at 0 mV. Minimizing Ca^{2+} entry is important to limit Ca^{2+}-dependent inactivation of I_{CRAC} by local accumulation of Ca^{2+} (30, 31). All currents were leak-subtracted by averaging the first two to six ramps after breaking in and then subtracting this from all subsequent traces. For display purposes, averaged time courses of I_{CRAC} were graphed at slightly lower temporal resolution (*i.e.* 0.25–0.125 Hz). Extracellular solution changes were made by local pressure application from a wide-tipped micropipette placed within 20 μm of the cell. A liquid-junction potential of −10 mV was corrected.

We have eliminated all experiments from our analysis in which nonspecific membrane breakdown by sphingosine occurred within the typical observation window of 5–10 min. During patch-clamp recordings, breakdown was characterized by sudden increases in linear ramp currents, and in fluorescence measurements, increases in $[Ca^{2+}]$ as well as dye leakage occurred. Such breakdown was seen in approximately 50% of the cells, both in patch-clamp and fluorescence experiments.

Intracellular Calcium Concentration—Intracellular $[Ca^{2+}]$ was monitored with a photomultiplier-based system and calculated from the fluorescence ratio (360/390). For single cell Ca^{2+} measurements, coverslips were incubated in a Ringer's solution containing the following (in mM): 140 NaCl, 2.8 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose, and 10 Na·HEPES (pH 7.2), to which 5 μM fura-2/acetoxymethyl ester had been added. Following incubation at 37°C for 15–20 min the coverslips were washed five times in the Ringer's solution. Experiments were done at room temperature.

Ionic Currents Other Than I_{CRAC}—Inward rectifier potassium currents were measured in RBL-2H3 cells. The external saline solution contained the following (in mM): 140 NaCl, 20 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 11 glucose, and 10 HEPES·NaOH (pH 7.2). The internal saline solution contained the following (in mM): 145 K·Glu, 8 NaCl, 1 $MgCl_2$, 2 Mg·ATP, 0.3 Na_2·GTP, 10 K·EGTA, and 10 HEPES·KOH (pH 7.2). Ramps were given every 2 s (−100 to +100 mV in 50 ms), and cells were held at 0 mV between ramps. Currents were not leak-subtracted. Under these conditions, the K^+ current (measured at −80 mV) did not run down, but in fact the amplitude steadily increased in control experiments over a period of approximately 200 s.

Delayed rectifier potassium currents were measured in rat skeletal myoballs isolated from newborn rats (2–5 days old) as described (32). The external solution contained the following (in mM): 140 NaCl, 2.8 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 0.5 $CdCl_2$, 0.03 TTX, 11 glucose, and 10 HEPES·NaOH (pH 7.2). The internal solution contained the following (in mM): 140 K·Glu, 8 NaCl, 1 $MgCl_2$, 2 Mg·ATP, 0.3 Na_2·GTP, 20 K·EGTA, and 10 HEPES·KOH (pH 7.2). Voltage pulses to +60 mV were applied every 20 s from a holding potential of −70 mV.

N-type Ca^{2+} channel currents were measured in bovine adrenal chromaffin cells isolated as described previously (33). The external solution contained the following (in mM): 135 NaCl, 2.8 KCl, 2 $MgCl_2$, 10 $CaCl_2$, 10 TEA·Cl, 0.03 TTX, and 10 HEPES·NaOH (pH 7.4). The internal solution contained (in mM): 140 Cs·Glu, 8 NaCl, 1 $MgCl_2$, 10 Cs·EGTA, 4 Mg·ATP, 0.3 Na_2·GTP, and 10 HEPES·CsOH (pH 7.4). Voltage pulses to +10 mV were applied every 2 s from a holding potential of −70 mV.

L-type Ca^{2+} channel currents were measured in rat skeletal myoballs. The external solution contained the following (in mM): 140 NaCl, 2.8 KCl, 2 $MgCl_2$, 10 $CaCl_2$, 10 TEA·CL, 0.03 TTX, and 10 HEPES·NaOH (pH 7.2). The internal solution contained the following (in mM): 145 *N*-methyl-D-glucamine, 8 NaCl, 1 $MgCl_2$, 20 Cs·EGTA, 4 Mg·ATP, 0.3 Na_2·GTP, and 10 HEPES·CsOH (pH 7.2). Voltage pulses to 0 mV were applied every 20 s from a holding potential of −70 mV.

Na^+ currents were measured in bovine adrenal chromaffin cells. The external solution contained the following (in mM): 135 NaCl, 2.8 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1 $CdCl_2$, 10 TEA·Cl, and 10 HEPES·NaOH (pH 7.4). The internal solution contained the following (in mM): 140 Cs·Glu, 8 NaCl, 1 $MgCl_2$, 10 Cs·EGTA, 4 Mg·ATP, 0.3 Na_2·GTP, and 10 HEPES·CsOH (pH 7.4). Voltage pulses to +10 mV were applied every 2 s from a holding potential of −70 mV. Currents were filtered at 8 kHz and sampled at 25 μs.

Current records obtained from all experiments in which voltage-activated currents were studied were leak-subtracted by a standard *P/n* procedure, where 4 leak pulses of −0.1× the actual test pulse amplitude were averaged, scaled, and subtracted from the current evoked by the test pulse.

Methods—Palmitoyl-co-enzyme A, sphingomyelinase (*Staphylococcus aureus*), sphingosylphosphorylcholine (lysosphingomyelin), and Long R^3 insulin growth factor were purchased from Sigma. *N*-Acetylsphingosine (C_2-ceramide), *N*-octanoylsphingosine (C_8-ceramide), *N*-octanoylsphingosine 1-phosphate (ceramide 1-phosphate), sphingosine 1-phosphate, phospholipase A_2 (*Trimeresurus flavoviridis*), phospholipase D (*Streptomyces chromofuscus*), sphinganine (D-*erythro*-dihydrosphingosine), sphingomyelin, stem cell factor, platelet-derived growth factor, NGF 2.5S (murine), and tumor necrosis factor-α were from Calbiochem. Sphingosine (D-*erythro*-sphingosine), *N*,*N*-dimethylsphingosine, DL-*threo*-dihydrosphingosine, L-cycloserine, and fumonisin B_1 were from Biomol. All other chemicals were from Sigma.

Sphingosine and palmitoyl coenzyme A were dissolved in dimethyl sulfoxide (Me_2SO) at 10 mM. DL-*threo*-Dihydrosphingosine, sphingosylphosphorylcholine, sphingosine 1-phosphate, and sphingomyelin were dissolved in methanol at 10 mM. C_2-ceramide, C_8-ceramide, and ceramide 1-phosphate were dissolved in either Me_2SO or methanol at 10 mM. In most experiments, the vehicles Me_2SO or methanol were diluted in external saline by a ratio of 1:1000 and neither vehicles significantly altered membrane currents when applied alone (up to 0.5%). Sphingomyelinase, LCS, and phospholipase A_2 were dissolved directly in external saline. Phospholipase D was dissolved in special buffer containing 10 mM Tris·HCl (pH 8.0), 0.05% bovine serum albumin, and 0.1% Triton X-100 to a concentration of 500 units/ml (pH 8.0) and was further diluted in external solution by a factor of 1:1000. Growth factors, including NGF, were dissolved in external saline containing 0.1% essentially fatty acid-free BSA (Sigma). The external solution used for experiments with sphingomyelinase and palmitoyl coenzyme A contained 2 mM Ca^{2+} (instead of the usual 10 mM) to increase the solubility of these molecules.

RESULTS

Block of I_{CRAC} by Exogenous Sphingosine—In RBL cells, $InsP_3$ indirectly activates I_{CRAC} by depleting internal Ca^{2+} stores. Fig. 1*A* shows the time course of I_{CRAC} following whole cell break-in with 20 μM $InsP_3$ in the pipette solution (*left panel*). Voltage ramps (−100 to +100 mV in 50 ms; *right panel*) were applied every 2 s, and I_{CRAC} was measured from the region on the ramp trace corresponding to −80 mV. Averaged values from 22 cells are shown (*left panel*). The Ca^{2+} current activates and peaks in approximately 50 s. Then I_{CRAC} is sustained, decaying only 9.1 ± 2.5% (mean ± S.E.; n = 21) 300 s after break-in. The experiments were done without ATP in the

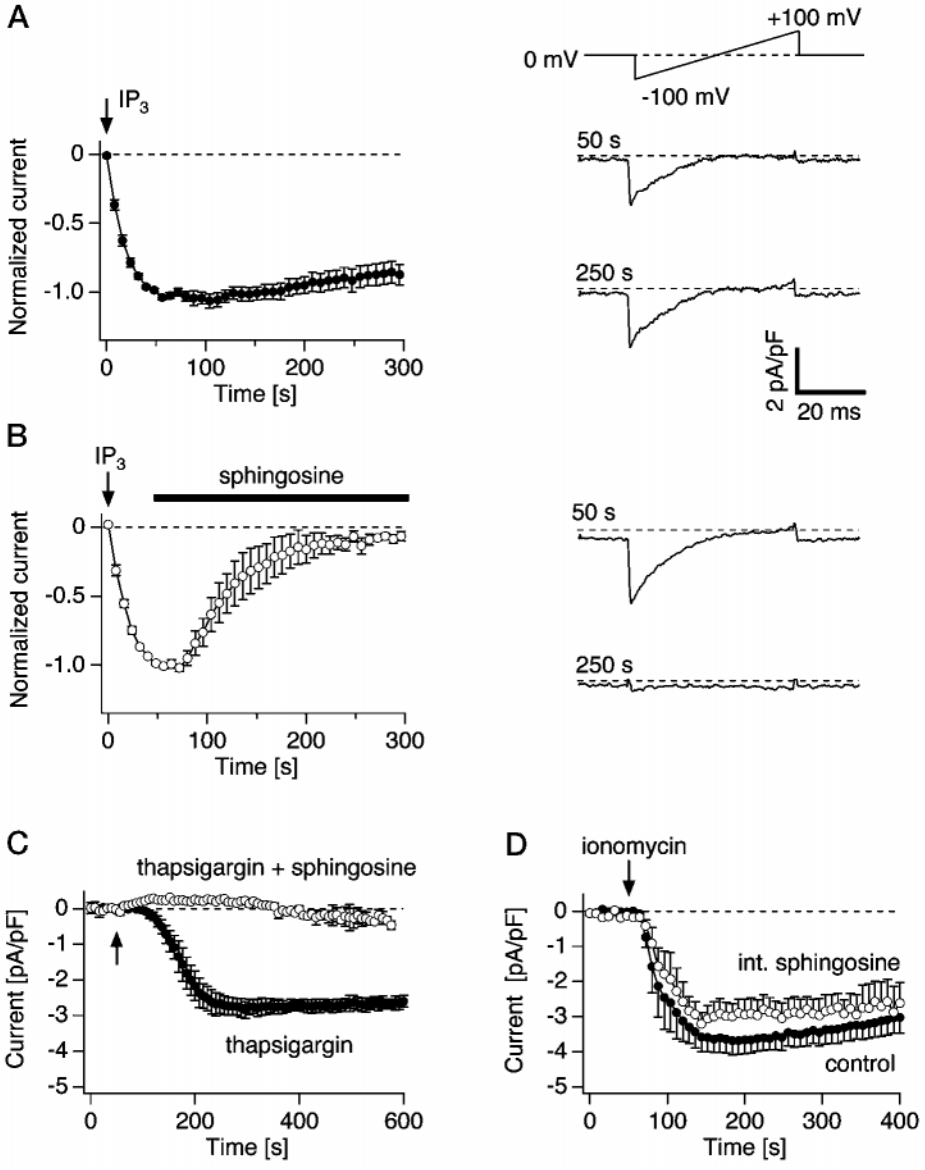


FIG. 1. **Exogenous sphingosine inhibits I_{CRAC}.** Whole cell patch-clamp experiments were conducted, and I_{CRAC} was measured by applying voltage ramps from −100 to +100 mV at a holding potential of 0 mV (see protocol, *upper right*). *A,* following break-in with $InsP_3$ (20 μM) and 10 mM EGTA (see "Experimental Procedures") I_{CRAC} develops and peaks within approximately 50 s (*left panel*). Currents were normalized to the amplitude at 50 s for comparison to experiments with sphingosine or other agents that were always applied at this time in patch-clamp experiments. The average from 22 cells is shown (±S.E.). Examples of ramp currents from a representative cell are shown to the *right* of the graph. There is virtually no change in the I_{CRAC} amplitude, even after 250–300 s following break-in. *B,* sphingosine (5 μM) applied 50 s after break-in inhibits I_{CRAC} following a delay of approximately 15 s. Illustrated in the graph is the average normalized time course from six cells (±S.E.). Ramp currents (*right*) from a representative cell show that I_{CRAC} is almost completely inhibited after 200 s. *C,* sphingosine (5 μM), when co-applied with the calcium pump inhibitor thapsigargin (1 μM) prevents the thapsigargin-induced activation of I_{CRAC} (n = 3, *open symbols*). In control cells, extracellular application of thapsigargin (1 μM) consistently activated I_{CRAC} after a short delay (n = 3, *filled symbols*). In both types of experiments, cells were perfused with the standard intracellular solution containing 10 mM EGTA and no added ATP. *D,* when applied intracellularly, sphingosine (10 μM) does not inhibit I_{CRAC}. Ionomycin (2 μM) was used to activate I_{CRAC} with and without sphingosine in the pipette solution. The graph in the *left panel* shows that no significant difference (p = 0.2; t test) was observed between controls (n = 3; time constant = 1731 ± 459 s) and cells treated with internal sphingosine (n = 4; time constant = 1062 ± 79 s). Currents were normalized to whole cell capacitance.

pipette solution to minimize endogenous kinase-dependent inactivation of I_{CRAC} (8).

Externally applied sphingosine (5 μM) blocks $InsP_3$-evoked I_{CRAC} (Fig. 1*B*). Sphingosine was applied 50 s after whole cell break-in, and the onset of block follows a delay of 18 ± 3 s (n = 6). The delay probably relates to the time taken for sphingosine to accumulate in the plasma membrane (see "Elevation of Membrane Capacitance," below). Complete inhibition of the current occurs within 200 s after addition of sphingosine, and the effect is not reversible within measurement time (5–10 min). Sphingosine also inhibits activation of I_{CRAC} when applied at least 1 min before break-in with $InsP_3$ (n = 2, not shown). Furthermore, sphingosine was also able to suppress I_{CRAC} when activated by thapsigargin, which represents an alternative pool-emptying protocol (Fig. 1*C*). Sphingosine when co-applied with thapsigargin essentially abolished thapsigargin-induced I_{CRAC} (n = 3), whereas control cells that were only stimulated with thapsigargin consistently activated regular-sized I_{CRAC} after a delay of about 60 s (n = 3). In these experiments, cells were perfused with the standard internal solution (buffered to resting $[Ca^{2+}]_i$ and without $InsP_3$), and either thapsigargin alone (1 μM) or in combination with sphingosine (5 μM) were applied externally.

This block of I_{CRAC} by sphingosine seems to be PKC-independent for the following reasons: 1) the PKC inhibitor staurosporine (5 μM; co-applied externally) has no effect on sphingosine block of I_{CRAC} (n = 3, not shown), and 2) exogenous sphingosine inhibits PKC (11). Because PKC inhibitors sustain I_{CRAC} (8), an explanation dependent on PKC inhibition by sphingosine would be opposite to the blocking effect illustrated in Fig. 1*B*. Another possible explanation for inhibition might be that sphingosine maximally activates I_{CRAC}, in a manner similar to ionomycin (4). Maximal activation could, in principle, cause intracellular Ca^{2+} accumulation during long applications and subsequent inhibition of I_{CRAC} channels by calcium-dependent inactivation (30, 31). This possibility was ruled out by the following experiment: whole cell break-in without activation of I_{CRAC} was achieved by excluding $InsP_3$ from the pipette solution, including ATP, and buffering internal Ca^{2+} to ~80 nM. With these conditions, applying sphingosine 50 s after break-in never activated I_{CRAC} (n = 6, not shown), indicating that sphingosine block is not mediated by "over activation." It could still be possible, however, that sphingosine interacts with the $[Ca^{2+}]_i$ inactivation site of CRAC channels thereby mimicking Ca^{2+}-induced inactivation of I_{CRAC}.

Interestingly, sphingosine fails to inhibit I_{CRAC} when applied from the inside (Fig. 1*D*). Whole cell break-in was conducted with or without sphingosine in the pipette solution (omitting

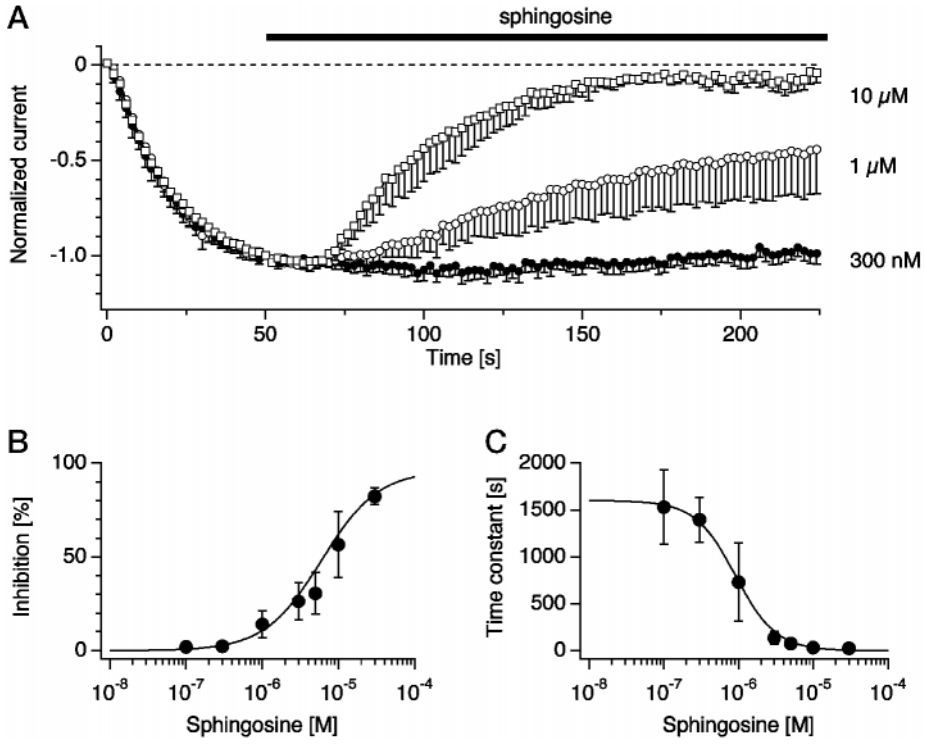


FIG. 2. **Dose-response relationship for sphingosine-mediated block of I_{CRAC}.** *A,* mean $InsP_3$-evoked I_{CRAC} records from experiments with sphingosine at concentrations of 10 μM ($n = 4$), 1 μM ($n = 5$), and 300 nM ($n = 5$). At 10 μM, sphingosine blocks ~100% of the current within 100 s, but at lower concentrations (*i.e.* 300 nM) the effect is not different from controls. *B,* the semi-logarithmic plot shows the inhibition of I_{CRAC} as a function of sphingosine concentration. Data points correspond to the average percentage (± S.E.) inhibition of 100 nM ($n = 4$), 300 nM ($n = 5$), 1 μM ($n = 5$), 3 μM ($n = 9$), 5 μM ($n = 6$), 10 μM ($n = 4$), and 30 μM ($n = 3$) measured 50 s after application of sphingosine. To obtain a dose-response curve, data were fit by Equation 1,

$$\% \text{ inhibition} = \frac{100}{1 + \left(\frac{[\text{sphingosine}]}{IC_{50}}\right)^n} \quad \text{(Eq. 1)}$$

where IC_{50} represents the half-maximal inhibitory concentration, and n is the Hill coefficient. The IC_{50} is at 6 μM and $n = 1.2$. *C,* the dose-response was also measured as the time constant of decay caused by sphingosine block as derived from single exponential fits. These data were then fit by a by a similar Boltzmann equation, giving an IC_{50} value of 1.1 μM and $n = 1.6$.

$InsP_3$ and including 10 mM EGTA), and a short application of ionomycin (2 μM) was used to activate I_{CRAC}. The time course of I_{CRAC} in control cells ($n = 3$) *versus* the time course in cells dialyzed internally with 10 μM sphingosine ($n = 4$) is similar (Fig. 1*D*).

Block of I_{CRAC} by external sphingosine is dose-dependent (Fig. 2). Concentrations between 10 and 30 μM block ~100% of the Ca^{2+} current within 100 s. Lower concentrations inhibit more slowly. For example, 1 μM sphingosine blocks approximately 70% of I_{CRAC} in 400 s. Average I_{CRAC} time courses during application of 300 nM ($n = 5$), 1 μM ($n = 5$), and 10 μM ($n = 4$) sphingosine are plotted in Fig. 2*A*. The average I_{CRAC} time course during treatment with 300 nM sphingosine is indistinguishable from the control (*cf.* Fig. 1*A*). The large variability observed at 1 μM is due to variations of endogenous decay of I_{CRAC}. Concentrations of sphingosine between 100 nM and 30 μM were tested, and a dose-response curve was constructed by measuring the percentage of inhibition at 50 s after application (Fig. 2*B*). The corresponding apparent half-maximal inhibitory concentration (IC_{50}) for sphingosine is at 6 μM.

Because full block occurs at lower concentrations, but over a much longer time, we also constructed a dose-response curve using the time course of decay as the dependent variable (Fig. 2*C*). To measure the blocking time, single exponentials were fit to the I_{CRAC} time course during inhibition by sphingosine. The time constants of inhibition ranged from 24 ± 4 s with 30 μM sphingosine ($n = 3$) to 1,529 ± 400 s with 100 nM sphingosine ($n = 4$). The apparent IC_{50} using this method is at 1 μM.

To determine whether sphingosine blocks I_{CRAC} activated by agonist, we used muscarinic type 1 receptors stably transfected in RBL-2H3 cells (RBL-m1). Muscarinic receptor activation with carbamylcholine (CCh) elevates $InsP_3$ levels which release Ca^{2+} from internal stores (Fig. 3*B* (28, 29)). Following store depletion, I_{CRAC} activates and provides a pathway for Ca^{2+} entry. In whole cell recordings, CCh (100 μM) activated I_{CRAC} in 9 of 9 cells tested (Fig. 3*A*). Co-application of sphingosine (5 μM) and CCh decreased the amplitude of I_{CRAC} by greater than 50%, and the current decayed to base line with a time constant of 105 ± 53 s ($n = 5$). This rate of inhibition is similar to the rate measured for sphingosine-mediated block of $InsP_3$-evoked I_{CRAC} at the same concentration (76 ± 20 s; $n = 6$). Therefore, sphingosine can block I_{CRAC} activated by a physiologically relevant stimulus.

These results were confirmed by measuring intracellular calcium concentration ($[Ca^{2+}]_i$) changes in single RBL-m1 cells loaded with fura-2/acetoxymethyl ester. Fig. 3*B* shows averaged muscarinic receptor-mediated Ca^{2+} signals in the presence of 10 mM extracellular Ca^{2+} (control), 0 Ca^{2+}, or 10 mM Ca^{2+} + 5 μM sphingosine. In the control cells, CCh elevates Ca^{2+} levels to 1.3 ± 0.1 μM ($n = 17$) following a short delay. The $[Ca^{2+}]_i$ remained elevated for longer than 100 s before declining slightly. I_{CRAC} contributes to this sustained elevation of Ca^{2+}. Following removal of CCh the $[Ca^{2+}]_i$ returns toward base line (not shown). The Ca^{2+} dependence of the sustained phase is demonstrated by similar experiments in nominally Ca^{2+}-free external saline where the $[Ca^{2+}]_i$ returns quickly

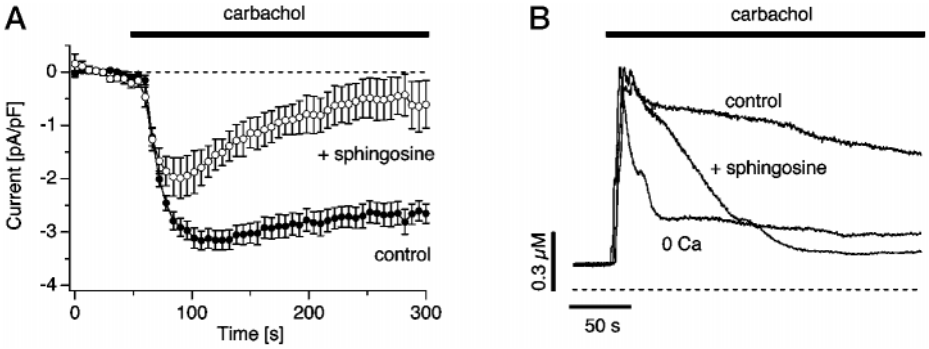


FIG. 3. **Sphingosine blocks I_{CRAC} activated by CCh in RBL-2H3 cells stably transfected with m1 muscarinic receptors.** *A,* a saturating concentration of CCh (100 μM) was applied to patch-clamped cells with and without sphingosine (5 μM). In control cells, application of CCh activated I_{CRAC} without much delay, to a peak amplitude of approximately −3 pA/pF ($n = 9$). Sphingosine coapplication inhibited the amplitude by ~50% and caused the current to decay back toward base-line values. The time constant of decay during sphingosine treatment, 105 ± 53 s (mean ± S.E.; $n = 5$), is significantly different from control values, 1278 ± 204 s ($n = 9$), as assessed by a *t* test ($p < 0.01$). *B,* averaged single cell calcium measurements ($n = 5$–17) from intact cells confirm the patch-clamp results. In each case, 10 mM CsCl was included in the bath solution to mimic the solution used in patch experiments and to prevent changes in membrane potential caused by alterations of the inward rectifier potassium conductance. Sphingosine inhibits the component of the plateau phase of the CCh-activated Ca^{2+} signal, in a manner similar to that of removal of external Ca^{2+} (0 Ca^{2+} = nominally Ca^{2+}-free, *i.e.* no Ca^{2+} or EGTA added).

toward base-line values following the peak elevation (1.3 ± 0.1 μM; $n = 5$). When sphingosine was co-applied with CCh, the sustained phase was dramatically reduced ($n = 5$). Clearly, the time course of sphingosine inhibition is similar to the time course of I_{CRAC} block shown in Fig. 3*A*.

Assessment of the mechanism by which sphingosine blocks I_{CRAC} depends on whether this sphingolipid alters calcium homeostasis; however, the reported effects of sphingosine on intracellular calcium homeostasis are highly variable. In some preparations, sphingosine elevates intracellular $InsP_3$ (34, 35) and $[Ca^{2+}]_i$ (17–20). In other cell types, exogenous sphingosine has no effect on resting Ca^{2+} levels (21, 36). We observed variability within the population of RBL cells tested. In 15 of 21 cells tested, sphingosine (5 μM) by itself elevated Ca^{2+} levels following a delay of 81 ± 11 s (data not shown). In nominally Ca^{2+}-free external solution, sphingosine elevated Ca^{2+} levels in 8 of 16 cells after a delay of 49 ± 10 s. However, including 0.1% fatty acid-free bovine serum albumin (BSA) to the perfusion solution along with sphingosine prevented $[Ca^{2+}]_i$ changes in Ca^{2+}-containing ($n = 7$) and Ca^{2+}-free ($n = 8$) solutions. BSA had no effect on sphingosine-mediated block of I_{CRAC} ($n = 2$). When co-applied with sphingosine, BSA prevents cell permeabilization and subsequent dye leakage (37), which would appear as an increase in Ca^{2+} levels. The approximately 50% occurrence of a sphingosine effect on $[Ca^{2+}]_i$ correlated well with the percentage of patch-clamped cells that showed evidence of membrane breakdown (*i.e.* seal loss or large nonspecific ramp currents) in the presence of sphingosine (see "Experimental Procedures"). This supports a concept where exogenous sphingosine by itself has no specific effect on Ca^{2+} levels in RBL cells.

Sphingosine Analogs and Related Agents—We investigated the mechanism of block by testing sphingosine analogs and intermediates from the sphingomyelin pathway. I_{CRAC} was evoked with $InsP_3$ in the patch pipette solution, and various sphingolipids or related agents were applied externally 50 s after break-in. Fig. 4*A* shows that dimethylsphingosine (DMS; 5 μM) inhibits I_{CRAC} with a time constant of 42 ± 4 s ($n = 6$) and by 96 ± 3% after 250 s. The sphingosine kinase inhibitor, DL-*threo*-dihydrosphingosine (38), also inhibits I_{CRAC} at 5 μM, although with slightly less potency (see Table I for inhibition profile). D-*erythro*-Dihydrosphingosine (sphinganine; 5 μM) is another sphingosine analog that inhibits I_{CRAC} with similar potency to sphingosine ($n = 3$, not shown). However, sphingosylphosphorylcholine (lysosphingomyelin), a sphingolipid with less structural similarity to sphingosine, has no effect on the calcium current (see also Table I).

Spingosine 1-phosphate (S1P) is an important intermediate because it is formed from sphingosine by endogenous sphingosine kinase. Interestingly, S1P at 10 μM had no effect on I_{CRAC} (Fig. 4*A*; $n = 5$), and it failed to do so even at a higher concentration of 50 μM ($n = 3$; not shown). Moreover, the sphingosine kinase inhibitor DL-*threo*-dihydrosphingosine blocks I_{CRAC} in a manner similar to that of sphingosine (Fig. 4*A*). Since DMS inhibits both I_{CRAC} and sphingosine kinase (39), and since S1P fails to block I_{CRAC}, while inhibitors of S1P formation readily block, we conclude that sphingosine inhibition does not seem to be mediated by conversion to S1P.

Calcium measurements were conducted to supplement the results obtained from direct patch-clamp recordings of I_{CRAC} inhibition by sphingosine analogs. Calcium signals were activated by CCh (100 μM) in RBL-m1 cells. When co-applied with CCh, none of these sphingolipids alter peak amplitudes of the intracellular calcium signals (~1.5 μM) due to release from internal stores (Fig. 4*B*). However, DMS and DL-*threo*-dihydrosphingosine dramatically inhibit the plateau phase, in the same order of potency observed with patch-clamp. On the other hand, neither sphingosylphosphorylcholine ($n = 9$; Fig. 4*B*) nor sphingosine 1-phosphate ($n = 5$; not shown) significantly affect the sustained phase of the Ca^{2+} signal, consistent with the observation that both compounds do not alter I_{CRAC} (Fig. 4*A*).

We also tested short chain ceramide analogs, which possess a sphingosine backbone thus sharing structural features with sphingosine. One of these analogs, C_2-ceramide (*N*-acetylsphingosine), is similar to sphingosine, yet its biological effects can differ from those of sphingosine. For example, it has been reported that exogenous C_2-ceramide does not inhibit PKC (40). Nevertheless, we observed that 10 μM C_2-ceramide inhibits I_{CRAC} slowly, with a time constant of 408 ± 114 s ($n = 6$; Fig. 5*A*). A 250-s treatment of C_2-ceramide inhibits 47 ± 10% of I_{CRAC} (Table I). Since there is no evidence that exogenous C_2-ceramide converts to sphingosine in cells (41), it seems that C_2-ceramide blocks I_{CRAC} independently from sphingosine formation.

A longer chain ceramide analog (C_8-ceramide) has no effect on the calcium current even at a higher concentration of 50 μM ($n = 6$; Fig. 5*B*). Interestingly, however, the phosphorylated form of C_8-ceramide (C1P) inhibits I_{CRAC} at 50 μM ($n = 6$; Fig. 5*B*). The inhibition by C1P is slow and has a similar profile to that of C_2-ceramide (Table I).

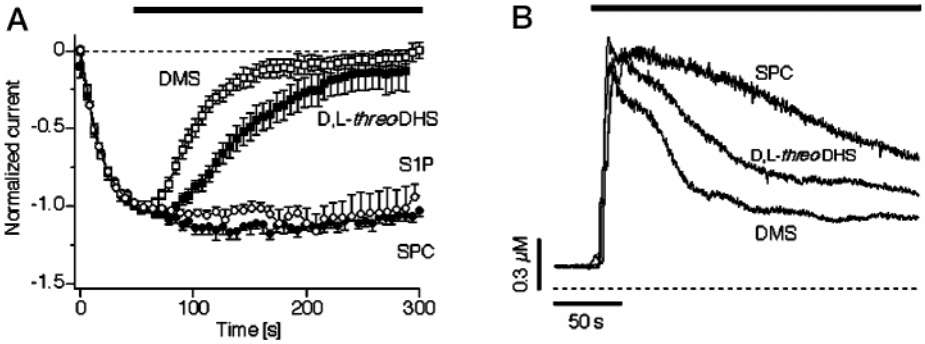


FIG. 4. **Sphingosine analogs inhibit I_{CRAC}.** *A,* DMS and DL-*threo*-dihydrosphingosine (*DL-threo-DHS*) inhibit $InsP_3$-evoked I_{CRAC}, whereas sphingosylphosphorylcholine (*SPC*) and S1P have no effect. DMS inhibits I_{CRAC} by 96 ± 3% (mean ± S.E.; $n = 5$) 250 s after addition, with a time constant of 42 ± 4 s ($n = 6$). DL-*threo*-DHS is slightly less potent, inhibiting I_{CRAC} by 82 ± 13% ($n = 5$) after 250 s, with a time constant of 96 ± 15 s ($n = 6$). For a comparison of structures for these compounds see Table I. *B,* single cell Ca^{2+} measurements with RBL-m1 cells confirmed the patch-clamp results. Averaged Ca^{2+} signals are shown. SPC added together with 100 μM CCh ($n = 9$) had no significant effect on the Ca^{2+} signal compared with controls (see Fig. 3*B*). However, DMS ($n = 8$) and DL-*threo*-DHS inhibited the plateau phase of the Ca^{2+} signal with the same order of potency as in the patch-clamp experiments (*i.e.* DMS > DL-*threo*-DHS). None of these compounds significantly altered the peak elevation of Ca^{2+} due to $InsP_3$-mediated Ca^{2+} release from internal stores.

Accumulation of charged lipids at the plasma membrane and the subsequent alteration of the voltage field across the bilayer can modulate ion channels (42, 43). If charge-screening mediated block of I_{CRAC}, we would expect opposite effects of positively and negatively charged sphingolipids. This was not the case. Both positively charged C_2-ceramide and sphingosine as well as negatively charged C1P inhibit I_{CRAC}. Moreover, negatively charged palmitoyl coenzyme A accumulates in the plasma membrane, as measured by an increase in capacitance, but it does not inhibit nor activate ($n = 3$) I_{CRAC} (Table I).

To complete the profile of inhibition, we tested agents related to sphingosine or the SM pathway for their ability to block I_{CRAC} (Table I). The sphingosine precursors sphingomyelin and palmitoyl coenzyme A have no effect on I_{CRAC}, suggesting that breakdown of sphingosine or conversion to sphingomyelin cannot account for block. In support of this conclusion is our observation that sphingomyelinase (SMase; 0.1 units/ml) does not significantly alter I_{CRAC} ($n = 3$).

Since exogenous SMase preferentially elevates ceramide levels (44, 45), the fact that SMase does not block I_{CRAC} also supports the assertion that sphingosine blocks I_{CRAC} independently from ceramide formation. To confirm further that sphingosine acts separately from ceramide, we used fumonisin B_1, an inhibitor of ceramide synthase (46). Fumonisin B_1 has no effect on sphingosine block of I_{CRAC} when applied from the patch pipette (*i.e.* intracellularly) or together with sphingosine from the outside ($n = 3$; not shown).

Sphingosine could potentially block I_{CRAC} through pathways involving arachidonic acid (AA) and/or phosphatidic acid (PA). Therefore, we tested phospholipase A_2 (0.5 units/ml) and phospholipase D (0.5 units/ml) which elevate AA and phosphatidic acid, respectively. These enzymes neither block (Table I) nor activate I_{CRAC}, indicating that sphingosine does not mediate its effect via accumulation of AA nor PA, although in other preparations, elevation of AA and PA levels follow sphingosine or S1P treatment (9).

Elevation of Membrane Capacitance—Sphingosine and all of the analogs tested in this study that inhibit I_{CRAC} also elevate membrane capacitance by approximately 10% (in 150–250 s). We interpret this effect as accumulation of the lipid in the plasma membrane. Membrane capacitance does not change in control cells, even after long, 10-min experiments. In control cells, the average membrane capacitance at the beginning of the experiment (just following whole cell break-in) is 16.9 ± 0.4 pF ($n = 21$) and does not change significantly after 250 s (17.0 ± 0.4 pF). On the contrary, sphingosine (5 μM) elevates membrane capacitance by 11.0 ± 2.0% after 250 s, which is significantly different from controls (0.8 ± 0.6%; $p \ll 0.001$; *t* test). Fig. 6*A* shows an example from a representative experiment. Higher concentrations of sphingosine lead to larger increases in capacitance, and the lowest concentration of 100 nM has no significant effect (Fig. 6*B*). Sphingosine also elevates capacitance when co-applied with CCh to RBL-m1 cells (by 7.2 ± 0.9% after 250 s; $n = 3$).

Nonspecific effects are unlikely to account for the increase in capacitance, since two of the lipids that do not block I_{CRAC} (sphingosylphosphorylcholine and palmitoyl Co-enzyme A) also elevate membrane capacitance (Table I). Fig. 6*C* shows a representative experiment in which sphingosylphosphorylcholine increases membrane capacitance without altering I_{CRAC}. Furthermore, sphingosine did not lead to blebbing of RBL cells, which would indicate cytotoxic effects. Taken together, these results suggest that sphingosine accumulates in the plasma membrane, a finding consistent with the notion that exogenous sphingosine directly blocks I_{CRAC}.

Interestingly, S1P fails to inhibit I_{CRAC} or elevate membrane capacitance (Table I). This could be due to the fact that S1P is highly insoluble in the absence of BSA, preventing incorporation into the plasma membrane. Thus we applied S1P in the presence of 0.1% fatty acid-free BSA. This, however, neither increased the whole cell capacitance nor enabled S1P to block I_{CRAC} ($n = 3$). A lack of accumulation in the plasma membrane could indicate rapid metabolism of S1P and/or possible transport to different intracellular compartments.

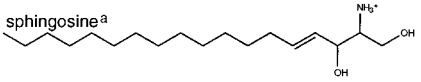
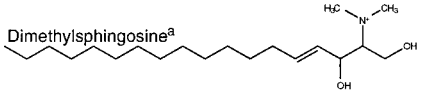
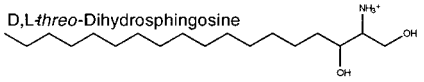
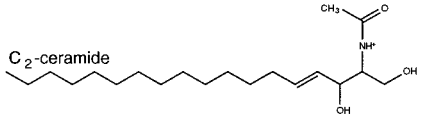
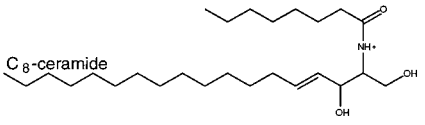
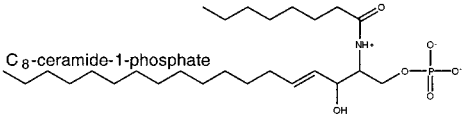
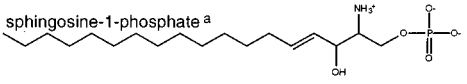
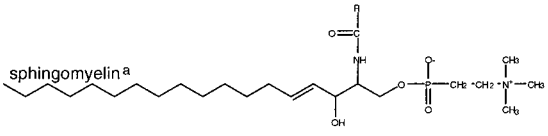
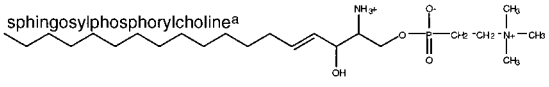
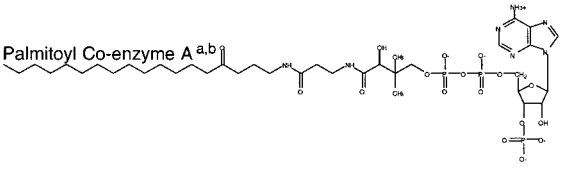
Sphingosine and Other Ionic Currents—We further investigated possible effects of sphingosine on K^+, Ca^{2+}, or Na^+ currents in various preparations (Table II). Sphingosine (5 μM) inhibits endogenous inward rectifier potassium currents in RBL-2H3 cells, with a potency similar to that for block of I_{CRAC}. In skeletal myoballs, however, sphingosine has no effect on voltage-gated, delayed rectifier K^+ currents. Sphingosine also differentially affected voltage-gated Ca^{2+} channels in two preparations. In chromaffin cells, sphingosine inhibits N-type Ca^{2+} currents, with a potency greater than for block of I_{CRAC} in RBL cells. With L-type Ca^{2+} currents in skeletal myoballs, however, there was no effect. Finally, sphingosine strongly inhibits Na^+ currents measured in chromaffin cells. Table II summarizes these results and compares them to block of I_{CRAC} by sphingosine.

In all cases where block occurred, sphingosine also elevated membrane capacitance (Table II). Only in rat skeletal myoballs did sphingosine fail to block the two channel types tested or to

TABLE I

Profile of sphingosine and related compounds, block of I_{CRAC} and elevation of membrane capacitance

NS, not significant ($p > 0.2$) compared to controls (t test). *, statistically significant ($p < 0.01$) compared to controls (t test). Data are mean values ± S.E.

Agent	Concentration	No. cells	Time constant (s)	% block (at 250 s)	% capacitance increase (at 250 s)
 sphingosine[a]	5 μM	4	76 ± 20	82 ± 8	11.0 ± 2.0 *
 Dimethylsphingosine[a]	5 μM	5	42 ± 4	96 ± 3	12.0 ± 1.6 *
 D,L-*threo*-Dihydrosphingosine	5 μM	5	96 ± 15	82 ± 13	6.4 ± 1.4 *
 C_2-ceramide	10 μM	4	408 ± 114	47 ± 10	5.1 ± 1.2 *
 C_8-ceramide	50 μM	5	2272 ± 114	12 ± 5	0.6 ± 0.9 NS
 C_8-ceramide-1-phosphate	50 μM	8	605 ± 86	45 ± 4	6.8 ± 1.2 *
 sphingosine-1-phosphate[a]	10 μM	3	2044 ± 346	7 ± 6	2.7 ± 0.5 NS
 sphingomyelin[a]	50 μM	2	3928 ± 2072	5 ± 2	1.0 ± 0.2 NS
 sphingosylphosphorylcholine[a]	5 μM	4	1691 ± 358	1 ± 1	6.5 ± 1.0 *
 Palmitoyl Co-enzyme A[a,b]	10 μM	3	1073 ± 321	16 ± 9	5.6 ± 1.8 *
Sphingomyelinase[b]	0.1 U/ml	3	1145 ± 99	6 ± 3	0.0 ± 0.7 NS
Phospholipase A2	0.5 U/ml	3	3813 ± 736	5 ± 4	1.4 ± 0.2 NS
Phospholipase D	0.5 U/ml	3	4700 ± 355	0 ± 0	1.1 ± 0.6 NS

[a] Endogenous lipid molecule.

[b] Experiments done in 2 mM Ca^{2+} external saline.

elevate membrane capacitance.

A Link between Cellular Sphingosine and I_{CRAC}—In order to determine whether a connection exists between cellular sphingosine and inhibition of I_{CRAC}, we tested several growth factors known to activate SMase and/or to elevate sphingosine levels (9, 22–27). Insulin growth factor (1 μg/ml), platelet-derived

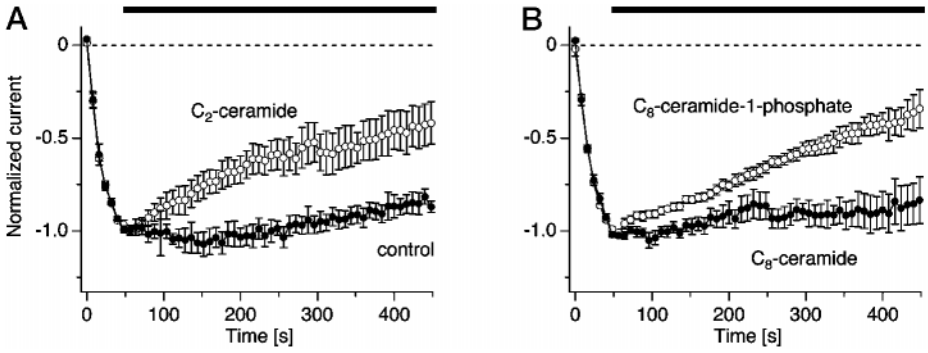


FIG. 5. **Slow inhibition of I_{CRAC} by a short chain ceramide analog.** *A,* *N*-acetylsphingosine (C_2-ceramide; 10 μM) inhibits $InsP_3$-evoked I_{CRAC}, albeit slowly ($n = 6$), compared with controls done in parallel ($n = 3$). *B,* C1P, 50 μM, significantly and slowly inhibits I_{CRAC} activated by $InsP_3$ ($n = 8$). However, *N*-octanoylsphingosine (C_8-ceramide; 50 μM) has no effect on I_{CRAC} ($n = 6$).

growth factor (1 μg/ml), stem cell factor (1 μg/ml), or tumor necrosis factor (1 μg/ml) had no significant effect on I_{CRAC} in RBL-2H3 cells, when tested for over 10 min. Mast cells also express NGF receptors (47, 48), and although we are unaware of a report with measurement of sphingosine levels following NGF treatment in mast cells, this growth factor is known to stimulate SM breakdown in other systems (22, 23).

As shown in Fig. 7, NGF inhibited I_{CRAC} by 71 ± 6% after 450 s (mean ± S.E.; $n = 10$). The time constant for inhibition by NGF is 490 ± 76 s. These values are statistically significant compared with vehicle (0.1% BSA) control values (7 ± 4% inhibition after 450 s; time constant = 3892 ± 594; $n = 5$; $p < 0.01$; *t* test). When compared with exogenously applied sphingosine, 1 μg/ml NGF is approximately as potent as 1 μM sphingosine (see Fig. 2). Unlike exogenous sphingosine, however, NGF did not elevate membrane capacitance.

NGF could potentially act on I_{CRAC} by elevating ceramide, sphingosine, and/or S1P levels. Because, however, S1P and ceramide do not mediate block of I_{CRAC} by sphingosine (Table I), it seems that NGF inhibits I_{CRAC} by elevating sphingosine levels, especially since other known effects of NGF could be ruled out through the following experimental observations. (i) Inhibition of I_{CRAC} by NGF is not mediated by alterations in Ca^{2+} store content, as NGF failed to release Ca^{2+} from internal stores in fura-2/acetoxymethyl ester experiments with intact cells ($n = 4$). (ii) Inhibition by NGF is not mediated by PKC, the only other inhibitory modulator of I_{CRAC} currently known in these cells, as the selective PKC inhibitor bisindolylmaleimide (1 μM; $n = 9$) failed to significantly alter NGF-mediated inhibition of I_{CRAC}. (iii) Inhibition by NGF is unlikely to result from phosphorylation events through other kinases either, since high concentrations of the less selective kinase inhibitor staurosporine (5 μM; $n = 12$) also failed to affect NGF-mediated inhibition of I_{CRAC}. In addition, experiments in which ATPγS (1 mM) was perfused into the cell revealed that the inhibition by ATPγS was indistinguishable in control ($n = 11$) and LCS-treated cells (16–24 h, 2 mM, see below; $n = 9$; data not shown). Together, these results suggest that NGF most likely inhibits I_{CRAC} via elevation of cellular sphingosine levels.

To test this notion further, we employed LCS which reduces SM levels (26) and should accordingly prevent NGF from maximally stimulating SMase and ceramidase (converting ceramide to sphingosine). Indeed, when treating cells for 16–24 h with 2 mM LCS, the NGF-mediated inhibition of I_{CRAC} was reduced by about half (Fig. 7). This reduction of the NGF effect by LCS is statistically significant (45 ± 6% inhibition after 450 s; time constant = 1552 ± 285 s; $n = 10$; $p < 0.01$; *t* test). The fact that LCS reduces the NGF effect on I_{CRAC} by ~40% is consistent with the previously reported magnitude of reduction in sphingosine levels by about 50% in LCS-treated cells stimulated by platelet-derived growth factor (26).

DISCUSSION

The main conclusion of this paper is that sphingosine directly blocks I_{CRAC}. The following lines of evidence support a direct block by sphingosine: neither ceramide, nor S1P, nor other SM pathway metabolites mediate the blocking effect of sphingosine. Furthermore, structural analogs of sphingosine (C_2-ceramide, DMS, DL-*threo*-dihydrosphingosine), which can differentially modulate SM pathway intermediates, inhibit I_{CRAC}. Also, our results strongly support the notion that sphingosine accumulates in the plasma membrane (which in and of itself does not cause block), and, last but not least, sphingosine blocks other ion channels. Therefore, it appears that both the sphingomyelin and phosphoinositide pathways can regulate I_{CRAC}. The observation that NGF inhibits I_{CRAC} by elevating sphingosine levels provides an important link between cellular sphingosine and I_{CRAC}. Furthermore, we demonstrate that whole cell capacitance measurements provide an excellent indication of exogenously applied lipid accumulation in the plasma membrane.

Sphingosine block of I_{CRAC} behaves in a dose-dependent and time-dependent manner. That is, lower concentrations of sphingosine inhibit more slowly, albeit completely, and assessing the potency of inhibition at a fixed point in time will underestimate the actual potency of sphingosine. To solve this problem we used both the amount of block at a fixed point in time (50 s of sphingosine application) and the time course of decay during application to assess the the value of half-maximal inhibition. Consideration of both methods of analyzing the dose-response relationship gives an IC_{50} value of ~4 μM. This value agrees well with estimates from previous reports of sphingosine-dependent inhibition of calcium signals in Jurkat T-lymphocytes (21), pancreatic duct adenocarcinoma cells (49), and sphingosine-mediated enhancement of epidermal growth factor-evoked Ca^{2+} signals in human epidermoid carcinoma A431 cells (36).

Surprisingly, intracellularly applied sphingosine did not exhibit any inhibitory effect on I_{CRAC}. However, since there was no change in the membrane capacitance, sphingosine applied in this manner presumably does not accumulate in the plasma membrane. Internally applied sphingosine may not accumulate in the plasma membrane because 1) it becomes quickly metabolized, 2) it traverses the plasma membrane too easily and escapes into the bath, or 3) it cannot assimilate into the inner leaflet of the membrane. The latter two explanations are unlikely, since sphingosine inhibits voltage-independent potassium channels when applied to either cell-attached, inside-out, or outside-out patches in smooth muscle cells (50). We suggest, therefore, that internally applied sphingosine may become metabolized before it can incorporate into the plasma membrane of RBL-2H3 cells. We cannot entirely discard the other explanations, since we also observed that membrane incorporation of sphingosine (as evidenced by capacitance changes) can be vari-

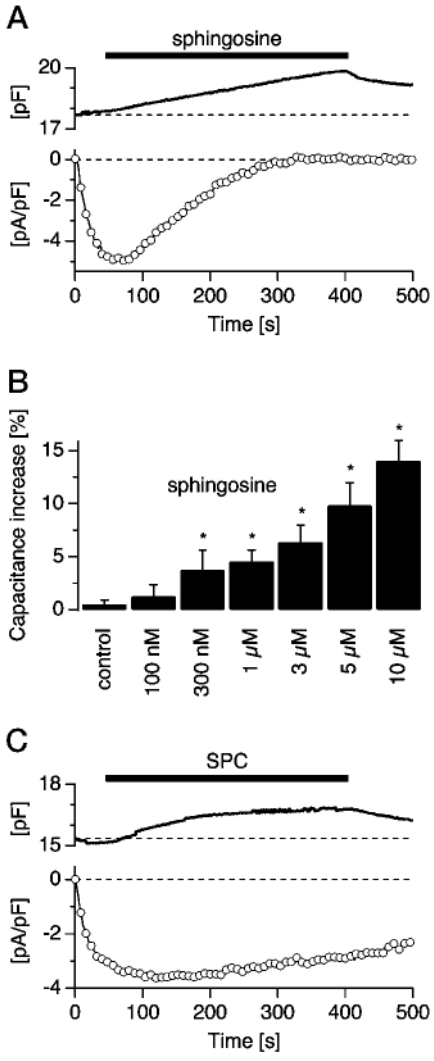


FIG. 6. **Sphingosine elevates whole cell membrane capacitance.** I_{CRAC} was activated by whole cell break-in with $InsP_3$ at time 0. I_{CRAC} ramps and whole cell capacitance were measured simultaneously. *A,* a long sphingosine application (350 s) is shown from a representative cell. Inhibition of I_{CRAC} by sphingosine followed a delay (below). The delay coincides with the time taken for a noticeable increase in capacitance (above). Throughout the sphingosine application, membrane capacitance increased slowly to a maximum of ~10% of the original value by the time sphingosine was removed. Note that I_{CRAC} does not recover, but the membrane capacitance does slightly. *B,* a histogram plot of sphingosine concentration *versus* the average percent capacitance increase (±S.E.) after 150 s shows that the elevation of capacitance is concentration-dependent. Importantly, there was no significant increase ($p = 0.46$; *t* test) in capacitance at the lowest concentration of 100 nM sphingosine (*i.e.* the same as in control experiments). The increase in capacitance is nearly 15% with 10 μM sphingosine. The *asterisk* indicates statistical significance compared with the control mean ($p < 0.01$; *t* test). *C,* sphingosylphosphorylcholine (*SPC*) application (5 μM) increases membrane capacitance without altering $InsP_3$-evoked I_{CRAC}. An experiment from a representative cell is shown.

able in different cell types (see below).

Assessment of the mechanism by which sphingosine blocks I_{CRAC} depends on whether this sphingolipid alters calcium homeostasis. The possibility that sphingosine inhibits I_{CRAC} indirectly by promoting internal Ca^{2+} store refilling seems to be unlikely. If sphingosine promoted refilling by enhancing microsomal Ca^{2+}-ATPase activity then it should lower resting Ca^{2+} levels, and we never observed this effect ($n = 37$). Moreover, sphingosine has been shown to inhibit microsomal Ca^{2+}-ATPase activity (18) and does not promote refilling in Jurkat T cells (21).

A second possibility is that sphingosine might block the $InsP_3$ receptor, thereby interfering with store depletion. Note, however, that in a previous report sphingosine failed to modify epidermal growth factor-stimulated calcium release in A431 cells (36). In our experiments with both clamped and unclamped (fura-2 experiments) cells, the Ca^{2+} stores are rapidly depleted by $InsP_3$, and sphingosine is acting after store depletion has occurred. We have previously demonstrated that under our experimental conditions (no internal ATP and 10 mM EGTA intracellularly), even a complete block of $InsP_3$ receptors (*e.g.* by heparin) would not turn off I_{CRAC} (4). Furthermore, sphingosine remained an effective inhibitor of I_{CRAC} when stores were depleted by thapsigargin. We therefore conclude that sphingosine is not blocking I_{CRAC} indirectly via alteration of internal Ca^{2+} stores (although such an effect remains a possibility when sphingosine is applied before depleting stores; we are currently investigating this possibility). If indeed sphingosine also affected the $InsP_3$ receptor, both effects could synergistically lead to an even stronger suppression of Ca^{2+} influx. The present article, however, clearly demonstrates that sphingosine can potently and completely inhibit I_{CRAC} directly after stores have been depleted.

To elucidate the mechanism of I_{CRAC} inhibition induced by sphingosine, we tested sphingosine analogs and intermediates from the sphingomyelin pathway. Although structural analogs to sphingosine are similarly potent in inhibiting I_{CRAC}, analogs derived from ceramide, and thus containing a sphingosine backbone, were either far less potent or had no effect at all (see Table I). These results obtained from patch-clamp measurements corroborate the Ca^{2+} measurements from intact cells, and together the results demonstrate that only sphingolipids with structural similarity to sphingosine seem to be able to inhibit I_{CRAC} (with the notable exception of S1P).

Interestingly, the phosphorylated form of C_8-ceramide (C1P) does have an effect on I_{CRAC} although with much less efficiency than C_2-ceramide or even sphingosine itself (Table I). Why then does C_8-ceramide fail to inhibit I_{CRAC}? It seems tempting to relate the inability of C_8-ceramide to block I_{CRAC} to its lack of incorporation in the plasma membrane, since application of both C1P and C_2-ceramide elevate membrane capacitance, whereas C_8-ceramide fails to do so (see "Elevation of Membrane Capacitance" under "Results"). Still, C1P is five times less efficient in blocking I_{CRAC} than C_2-ceramide. Moreover, C1P is structurally less similar to sphingosine than C_2-ceramide, and C_8-ceramide has no effect. Therefore, we favor the view that structural similarity to sphingosine, and not ceramide itself, mediates the slow inhibition by the ceramide analogs C_2-ceramide and C1P.

The fact that short-chained ceramide analogs (C_2-ceramide and C_8-ceramide 1-phosphate) inhibit I_{CRAC} may have important implications for studies using short-chained ceramide analogs to mimic the actions of endogenous ceramides. For example, it was shown that C_8-ceramide 1-phosphate stimulates DNA synthesis and cell division (41), an effect typically associated with sphingosine or S1P. However, our data support the idea that some effects of permeable ceramide analogs may occur because they mimic sphingosine actions.

In vivo, sphingosine is a secondary product derived from the membrane lipid sphingomyelin via ceramide formation. In principle, block of I_{CRAC} could be mediated by sphingosine precursors, breakdown products, or enzymes involved in the SM cycle rather than by sphingosine itself. However, the experiments with sphingomyelin, palmitoyl coenzyme A, S1P, or fumonisin B_1 clearly show no effect of these substances on I_{CRAC}. Even exogenous SMase does not affect calcium influx, and therefore, whereas some sphingosine may indeed become

TABLE II
Differential block of ion channels by exogenous sphingosine (5 μM)
NA, not applicable, *i.e.* no block. Data are mean values ± S.E.

Channel	Preparation	No. cells	Time constant	% block (at 50 s)	% capacitance increase (at 50 s)
			s		
I_{CRAC}	RBL cells	6	76 ± 20	31 ± 11	3.6 ± 1.2
Inward rectifier K	RBL cells	3	81 ± 1	30 ± 6[a]	6.8 ± 2.7[a]
Delayed rectifier K	Rat skeletal myoballs	8	NA	8 ± 3[b]	0.2 ± 0.3[b]
N type Ca^{2+}	Bovine chromaffin cells	3	22 ± 2	87 ± 3	8.6 ± 1.0
L type Ca^{2+}	Rat skeletal myoballs	8	NA	9 ± 6[c]	0.2 ± 0.3[c]
Na^+	Bovine chromaffin cells	3	24 ± 7	78 ± 9	11.3 ± 0.7

[a] 79 ± 3% block of inward rectifier K^+ current and a 9.4 ± 2.5% increase in membrane capacitance occurred 150 s after adding sphingosine.
[b] 17 ± 3% block of delayed rectifier K^+ current and a 0.2 ± 4% increase in membrane capacitance occurred 150 s after adding sphingosine (n = 7).
[c] 18 ± 10% block of L type Ca current and a 0.5 ± 7% increase in membrane capacitance occurred 100 s after adding sphingosine.

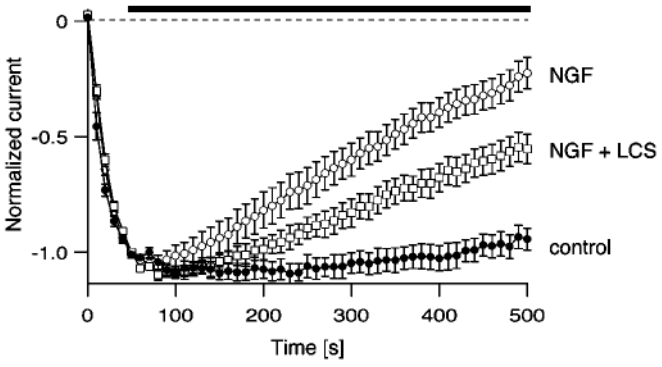


FIG. 7. **Nerve growth factor 2.5 S subunit (NGF) inhibits I_{CRAC}.** When applied 50 s after break-in with $InsP_3$, NGF reduces I_{CRAC} by 71 ± 6% (n = 10) in 450 s. Cells treated with LCS (2 mM) showed 24% less inhibition in response to NGF. LCS treatment involved incubation in growth medium for 16–24 h and continued presence in the experimental and application pipette solutions. Application of the vehicle (control), 0.1% BSA, had no significant effect on I_{CRAC} (7 ± 4% inhibition after 450 s; n = 5). Vehicle controls with LCS-treated cells (n = 7) were not significantly different from untreated cells.

ceramide in the plasma membrane induced by SMase (10), ceramide does not appear to mediate block of I_{CRAC} by exogenous sphingosine. The exception would be if ceramide took the form C_2-ceramide, which inhibits I_{CRAC} and can occur *in vivo* (51).

These results (summarized in Table I) thus demonstrate that sphingosine blocks I_{CRAC} independently from ceramide, sphingosine 1-phosphate, or other sphingomyelin pathway intermediates. The only other reasonable alternative is that sphingosine itself blocks I_{CRAC} directly or via a closely associated protein that interacts with I_{CRAC}. This supports the established notion that fatty acids directly regulate ion channels (52). For example, it has been shown that AA modulates voltage-gated calcium channels (53, 54). Moreover, fatty acids, lysophosphatidate, acyl coenzyme A, and sphingosine differentially regulate a voltage-independent potassium channel in smooth muscle cells (50). Concerning sphingosine, the effect is independent of soluble messengers, particularly in the case of excised-patch experiments. However, the report by Petrou *et al.* (50) did not rule out involvement of S1P or ceramide in the plasma membrane. We demonstrate that block by sphingosine is independent from ceramide and S1P, and the previous reports described above support our conclusion that sphingosine acts directly on I_{CRAC} channels (or a closely associated protein). Our results differ, however, from those of Petrou *et al.* (50) for two reasons: 1) we did not observe opposite effects of positively and negatively charged lipids (*i.e.* in RBL cells we observe that palmitoyl coenzyme A, which is negatively charged, has no effect on I_{CRAC}, despite the fact that it elevates membrane capacitance), and 2) unlike Petrou *et al.* (50) we observed that the inhibition of I_{CRAC} by sphingosine is irreversible within tens of minutes. These differences suggest that sphingosine can differentially block ion channels in various preparations.

Effects on Membrane Capacitance—All the substances investigated in this study that inhibited calcium influx also significantly elevated membrane capacitance, indicating accumulation of these substances in the plasma membrane itself. Concomitant increase in capacitance does not seem to be a nonspecific effect since, as already stated under "Results," neither sphingosylphosphorylcholine nor palmitoyl coenzyme A block I_{CRAC} although elevating capacitance.

Further evidence favoring a positive correlation between the ability of sphingosine to block calcium influx and its membrane incorporation emanates from our experiments assessing sphingosine effects on other ion channels in various tissues. Although all the channel types investigated in RBL cells or chromaffin cells showed significant block and capacitance increase upon exogenous sphingosine application, this sphingolipid failed both to block the channel types tested and to elevate membrane capacitance in rat skeletal myoballs (Table II). For the latter case, it seems that sphingosine either becomes rapidly metabolized or quickly transferred to a different intracellular compartment. Therefore, the extent of sphingosine accumulation in the plasma membrane may have a tremendous impact on block of ion channels. It would imply that sphingosine potentially could block all ion channels if allowed to accumulate in the plasma membrane, which, however, remains to be determined. A differential accumulation of sphingosine in different cell types might help explain the variability and differences in effects of sphingosine between preparations. The whole cell patch-clamp technique provides an excellent tool to measure whether or not sphingosine accumulates in the plasma membrane, important information regarding the site of action of this sphingolipid.

The ability of sphingosine to block many different ion channels may prove to have far-reaching physiological relevance. Along with preventing Ca^{2+} influx, which would modulate cell growth, blocking other channels would decrease cellular excitability and could have various effects on membrane potential depending on the channel constituents in a particular cell type. Blocking all channels may actually be desirable at various times during the life of a cell. Whether or not a reduction in excitability (or an increase in membrane input resistance) is important for cellular growth and proliferation remains to be determined (55).

Physiological Significance—To establish a physiological relevance of I_{CRAC} inhibition by sphingosine, we tested several growth factors known to activate SMase and/or to elevate sphingosine levels (9, 22–27). However, we only found NGF to have significant inhibitory action regarding I_{CRAC}, and this inhibition can be significantly prevented by LCS treatment.

Our experiments show that neither PKC nor phosphorylation or alterations in Ca^{2+} store content can account for the NGF effect on I_{CRAC}, thus strongly favoring the notion that NGF inhibits I_{CRAC} via elevation of cellular sphingosine levels.

NGF promotes mast cell survival (47, 56), instead of proliferation which is the case for many growth factors (9). Mast cells produce NGF themselves or receive it from nervous, endocrine, or immune input (56). Inhibition of I_{CRAC} by NGF may be an important signal in the process that leads to increased survival of mast cells. This places I_{CRAC} in a favorable position as a potentially important ion channel for regulating mast cell growth and may also regulate growth in other cell types. Pharmacological tools aimed at inhibition of I_{CRAC} may be useful, therefore, for enabling cell proliferation, and activators of I_{CRAC} may mediate apoptosis. Such agents are still forthcoming.

Our observation that NGF inhibits I_{CRAC} presumably via elevation of cellular sphingosine levels suggests that block of this SOC current contributes to cellular events attributed to sphingosine, such as proliferation and survival. Increases in intracellular calcium can inhibit cell growth (14–16, 57), and therefore, the inhibition of I_{CRAC} may be an important signal to prevent elevation of intracellular Ca^{2+} levels by I_{CRAC} in a situation where cell growth or division is necessary.

It is noteworthy that when the PLC and SM pathways act in parallel, the end result is inhibition of I_{CRAC}. Whole cell break-in with $InsP_3$ followed by NGF application mimics coactivation of these pathways. In regard to regulation of I_{CRAC}, the PLC and SM pathways may operate in parallel or at different times in the life of a cell. For example, agonists that stimulate only the PLC pathway can regulate I_{CRAC} by activation of PKC (8). Activation of both the PLC and SM pathways by multiple agonists is necessary for sphingosine block of I_{CRAC} (*i.e.* when sphingosine production becomes stimulated by external stimuli, such as NGF, following receptor-mediated Ca^{2+} release and activation of I_{CRAC}).

Finally, we speculate that sphingosine might be involved in the activation mechanism of I_{CRAC}. In the resting state, sphingosine could act as a blocker of I_{CRAC}. Upon depletion of internal stores, metabolism of sphingosine, possibly by conversion to sphingosine 1-phosphate by sphingosine kinase, could lower the sphingosine levels and lead to the disinhibition of I_{CRAC}. This would be compatible with the observed elevations in $[Ca^{2+}]_i$ associated with antigen-induced stimulation of sphingosine kinase and elevation of S1P (58).

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Calcium Release-activated Calcium Current (I_{CRAC}) Is a Direct Target for Sphingosine

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