

Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx

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INTRACELLULAR Ca^{2+} signals that last more than a few minutes after the onset of stimulation depend critically on influx of extracellular Ca^{2+} . Such Ca^{2+} influx can be triggered in many cell types by depletion of intracellular Ca^{2+} stores without detectable elevations of known messengers. The mechanism by which store depletion can control plasma membrane Ca^{2+} permeability remains controversial¹⁻⁷. Here we present evidence for a novel soluble mediator. Calcium depletion of a lymphocyte cell line caused the messenger to be released from intracellular organelles into the cytoplasm and to a much lesser extent into the extracellular medium. The messenger caused Ca^{2+} influx when applied to macrophages, astrocytoma cells, and fibroblasts and was therefore named CIF (for Ca^{2+} -influx factor). CIF appears to have hydroxyls (or hydroxyl and amino groups) on adjacent carbons, a phosphate, and a M_r under 500.

We chose the Jurkat line of human tumour lymphocytes as producers of the putative messenger because they display Ca^{2+} influx that can be triggered by emptying of internal stores^{8,10} and are easily grown in large quantity at high density. Concentrated suspensions of Jurkats were stimulated for 2 min with phytohaemagglutinin (PHA) in zero Ca^{2+} medium, lysed at pH 1 to stop synthesis and degradation of messengers, and centrifuged to remove cell debris. The lymphocyte extracts were neutralized

to physiological pH and tested extracellularly on P388D1 macrophage-related cells, 1321N1 astrocytoma cells, and REF-52 fibroblasts. These three cell lines were physiologically unrelated and insensitive to PHA. To avoid effects of ATP or fatty acids, which increase internal Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in various cell types^{11,12}, the cell extract was treated with hexokinase, glucose, and fatty acid-free bovine serum albumin (BSA, 20 mg ml⁻¹). Control experiments verified that these additions prevented exogenous ATP and fatty acids from having any effect. Typical $[\text{Ca}^{2+}]_i$ responses are shown in Fig. 1. Addition of Jurkat extract triggered a sustained but fluctuating $[\text{Ca}^{2+}]_i$ increase in all three cell types. When the extract was further diluted, the amplitude of the response decreased and the latency increased. Such experiments showed that the P388D1 macrophages were the most sensitive detectors and the REF-52 fibroblasts were the least. Jurkat cells were not the only cells that produced the messenger, because analogous extracts prepared from P388D1 macrophages stimulated with platelet-activating factor caused $[\text{Ca}^{2+}]_i$ elevations in astrocytoma cells similar to those shown in Fig. 1b, yet astrocytoma cells did not respond directly to platelet-activating factor (data not shown).

Further analysis of the $[\text{Ca}^{2+}]_i$ increase induced in astrocytoma cells is presented in Fig. 2. In the presence of external Mn^{2+} (Fig. 2a), Jurkat extract not only elevated $[\text{Ca}^{2+}]_i$ but simultaneously accelerated Mn^{2+} quenching of the fura-2, just as expected in non-excitable cells for Ca^{2+} entry pathways because they usually also conduct Mn^{2+} (refs 6, 8, 12, 13). In Ca^{2+} -free medium (Fig. 2b), the Jurkat extract had no effect on $[\text{Ca}^{2+}]_i$, but readdition of extracellular Ca^{2+} restored the normal $[\text{Ca}^{2+}]_i$ elevation. Econazole, a blocker of stimulated Ca^{2+} and Mn^{2+} entry in various cell types^{6,14}, considerably inhibited the effect of cell extract. Finally, the content of the intracellular Ca^{2+} pools after stimulation with cell extract was compared with results from a blank (Fig. 2c) or the muscarinic agonist carbachol (Fig. 2d). Jurkat extract caused no lasting loss of stored Ca^{2+} , as revealed by the large $[\text{Ca}^{2+}]_i$ transient when those stores were finally dumped by Ca^{2+} ionophore in EGTA medium. By contrast, muscarinic stimulation immediately dumped the internal Ca^{2+} stores. Although the extract and carbachol gave roughly matching levels of Ca^{2+} entry while external Ca^{2+} was present, carbachol kept the stores mostly depleted as

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shown by the final small response to ionomycin in EGTA. These results together confirm that the Jurkat extract applied to astrocytoma cells stimulated Ca^{2+} influx directly rather than as a secondary consequence of triggered release from the stores of those responder cells. The same appeared to hold for REF-52 fibroblasts though they were studied in less detail. But the response of the P388D1 macrophages was more complex and included some transient release of Ca^{2+} from internal stores as well as sustained Ca^{2+} influx.

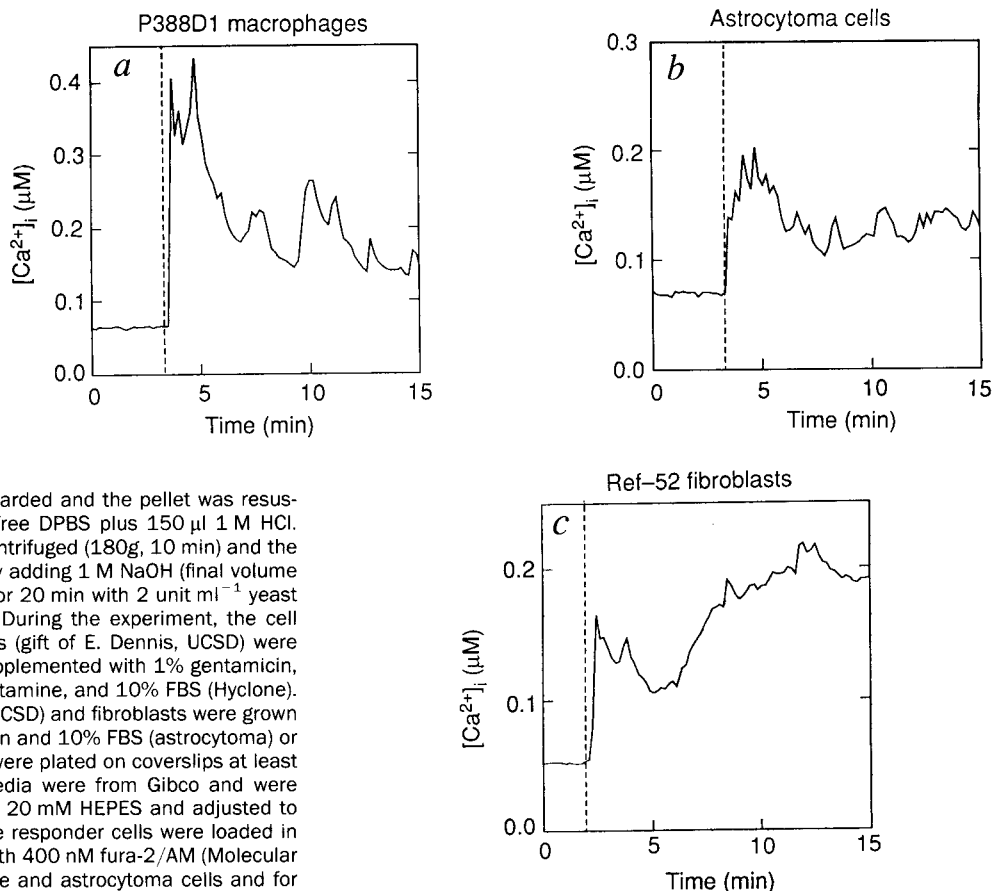
We then examined how the amount and subcellular distribution of the messenger were affected by stimulation of the producer Jurkat cells. Half of a large batch of Jurkats was stimulated with PHA in zero Ca^{2+} for 10 min, and the other half was left unstimulated in normal medium. Each suspension was then separated into supernatant and cells. The latter were subdivided into cytosolic and organellar fractions using digitonin to permeabilize the plasma membranes selectively. After parallel workups to complete lysis, inactivate enzymes, and remove digitonin, ATP, and fatty acids, the fractions were tested on macrophages as detector cells (Fig. 3a). The cell supernatant contained a small but definite amount of Ca^{2+} -elevating activity, which increased by 1.9-fold upon PHA stimulation. PHA stimulation caused much greater (sixfold) enhancement of activity in the cytosol and an almost equal loss of activity from the organelles. The total activity obtained by adding the activities of the three fractions was conserved upon stimulation.

Does release of activity depend on stimulation of surface receptors, elevation of cytosolic Ca^{2+} , or depletion of internal Ca^{2+} stores? Stimulatable release of activity into the cytosol persisted after loading the lymphocytes with an intracellular buffer to suppress even a transient increase in $[\text{Ca}^{2+}]_i$, and could be detected by astrocytoma cells (Fig. 3b). Another comparison (Fig. 3c) was between PHA in the presence or absence of extracellular Ca^{2+} , thapsigargin to inhibit Ca^{2+} -sequestering pumps in the organelles, or simple removal of external Ca^{2+} for long enough to deplete those organelles without any drug treatment or elevation of $[\text{Ca}^{2+}]_i$. Activities were tested in the supernatants of intact cells rather than in digitonin lysates because much less sample handling and cleanup were required, only needing addition of hexokinase to eliminate ATP. PHA and thapsigargin in normal external Ca^{2+} both gave the same ~ 2.5 -fold stimulation of messenger release. Incubation in zero Ca^{2+} was almost as effective by itself and did not further enhance the activity due to PHA. All four conditions of store depletion, using three different mechanisms, gave messenger releases statistically different from the control but not from each other.

Various known messengers were tested to see if they could explain the activity and induce similar $[\text{Ca}^{2+}]_i$ rises in macrophages or astrocytoma cells. If $[\text{Ca}^{2+}]_i$ increases were observed, cross-desensitization experiments were tried, in which macrophages or astrocytoma cells were stimulated several times with the candidate until they became desensitized, after which the

FIG. 1 Increases in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) in P388D1 macrophages (a), 1321N1 astrocytoma cells (b) and REF-52 fibroblasts (c), in response to an extract of stimulated Jurkat lymphocytes.

METHODS. Jurkat T cells were grown in suspension in RPMI 1640, 1% penicillin/streptomycin and 5% heat-inactivated fetal bovine serum (FBS, Gibco). A suspension (50 ml), containing 0.3–0.4 ml packed cell volume, was centrifuged (180g, 4 min) and resuspended in 15 ml of Ca^{2+} -free Dulbecco's phosphate buffered saline (DPBS) with 0.5 mM EGTA. Phytohaemagglutinin (PHA, $20 \mu\text{g ml}^{-1}$) was added. After 2 min stimulation, the cells were centrifuged. The supernatant was discarded and the pellet was resuspended in $700 \mu\text{l}$ of nominally Ca^{2+} -free DPBS plus $150 \mu\text{l}$ 1 M HCl. After 20 min, the suspension was recentrifuged (180g, 10 min) and the supernatant brought back to pH 7.3 by adding 1 M NaOH (final volume 1 ml). The cell extract was then kept for 20 min with 2 unit ml^{-1} yeast hexokinase (Calbiochem) and frozen. During the experiment, the cell extract was kept on ice. P388D1 cells (gift of E. Dennis, UCSD) were cultured in Iscove's DMEM (IMDM) supplemented with 1% gentamicin, 5% non-essential amino acids, 2% glutamine, and 10% FBS (Hyclone). Astrocytoma cells (gift of J. H. Brown, UCSD) and fibroblasts were grown in MEM plus 1% penicillin/streptomycin and 10% FBS (astrocytoma) or 10% calf serum (fibroblasts). All cells were plated on coverslips at least 1 day before the experiments. All media were from Gibco and were supplemented by 11 mM glucose and 20 mM HEPES and adjusted to pH 7.3. For $[\text{Ca}^{2+}]_i$ measurements, the responder cells were loaded in Hank's balanced salt solution (HBS) with 400 nM fura-2/AM (Molecular Probes) for 20–30 min for macrophage and astrocytoma cells and for 45 min for fibroblasts. $[\text{Ca}^{2+}]_i$ was measured at 5-s intervals at room temperature on a ratio imaging system previously described³⁰. The traces are average $[\text{Ca}^{2+}]_i$ values of 6–12 cells in the same field. Where indicated by the dashed lines, $200 \mu\text{l}$ Jurkat extract (derived from 60–80 μl packed cells) supplemented with 20 mg ml^{-1} fatty acid-free



BSA (Calbiochem) and 1.3 mM Ca^{2+} were added to the responder cells in $400 \mu\text{l}$ HBS, which also contained 1.3 mM Ca^{2+} . In c, the volumes of extract and HBS were reversed because REF-52 cells were less sensitive.

TABLE 1 Analysis of CIF

(a) Comparison of known messengers with the new activity				(b) Chemical characterization of CIF	
	[Ca ²⁺] _i rise in macrophages	[Ca ²⁺] _i rise in astrocytoma	Cross- desensitization		Activity (%)
ATP ¹¹ (100 μM)	+++	+	No	Control	100
ADP (100 μM)	++	+	No	After 45 min at room temperature	88
GTP (100 μM)	++	—	No	Heat inactivation 70 °C, 20 min	195*
Ado, Guo, cAMP, cGMP ⁷ , AMP, GMP, cADPR ²⁵ (100 μM)	—	—	—	Protease 5 mg ml ⁻¹ , 30 min	204*
InsP ₃ (50 μM) + InsP ₄ (50 μM)	—	—	No	pH 13, 20 min	156
LPA ²⁶ (50 μg/ml)	++	++	No	Cutoff filter (M _r ≤ 500)	184*
<i>cis</i> -unsaturated fatty acids ¹² (30 μM)	—	—	—	Charcoal column (flow-through)	0*
LTC ₄ ²⁷ (10 μM)	+++	—	No	C18 column	
LTB ₄ (10 μM)	—	—	—	Flow-through	18*
PAF (20 nM)	+++	—	No	20% MeOH	67
Sph(1)P ^{28,29} (50 μM)	—	+	No	100% MeOH	0*
				Flow-through of	
				Mixed bed column	0*
				Cation exchange column	86
				Anion exchange column	7*
				Anion exchange column at pH 2.5	0*
				Alkaline phosphatase, Calf intestine	
				1 unit ml ⁻¹ , 20 min	0*
				Periodate	
				5 mM, 20 min	89
				50 mM, 20 min	0*
				Iodoacetamide 50 mM, 2 h	114
				NaBH ₄ 50 mM, 30 min	126

a, For each compound, the maximal dose tested is indicated. When a candidate was able to induce [Ca²⁺]_i increases in macrophages and astrocytoma cells, cross-desensitization experiments were done where indicated. *Cis*-unsaturated fatty acids (all from Cayman Chemical) were arachidonic, docosahexaenoic, linoleic, γ -linoleic, and oleic acids. Sphingosine-1-phosphate, Sph(1)P, was a gift of T. Ghosh and D. Gill²⁸. Cyclic ADP-ribose (cADPR) was a gift of H. C. Lee²⁵. In addition to the lack of cross-desensitization with CIF, further evidence against CIF being Sph(1)P was obtained from tests on REF52 fibroblasts. Unlike CIF, Sph(1)P caused [Ca²⁺]_i increases in fibroblasts at much lower doses (~1 μM) than required for the astrocytoma cells (~50 μM), and the fibroblast responses were almost entirely due to release from internal Ca²⁺ stores, in agreement with previous reports²⁹. b, Lymphocyte supernatant was prepared using PHA stimulation in zero Ca²⁺, subjected to the indicated manipulations and finally assayed on macrophages as described in Fig. 3. The values correspond to the mean [Ca²⁺]_i increase induced in two to five dishes and have been normalized to appropriate controls as follows. For cutoff filters, C₁₈ reverse-phase, charcoal and ion exchange columns, the control was the activity of the supernatant before the filter or column. Methanolic fractions were evaporated to dryness before reconstitution in the same volume of HBS. For stability tests other than pH extremes, the control activity was measured right after supernatant isolation. For pH stability tests, the same amount of NaOH and HCl were premixed and added to the control to mimic the dilution. In all other cases, the supernatant was split into two fractions; one was treated as indicated and the other one, the control, was kept at room temperature at the same pH for the same amount of time. Iodoacetamide treatment was for 2 h at pH 8, followed by quenching with 60 mM glutathione for 20 min, then neutralization to pH 7.3 with HCl. Both treated and control fractions were then passed through a C₁₈ reverse-phase column, which retained the activity and allowed excess reagents to flow through. The fraction eluted by 20% MeOH was then analysed. Excess periodate was similarly removed by reverse phase columns. C₁₈ reverse phase columns were 3 cm long, packed with 40 μm prep LC packing (Bakerbond Octadecyl, J. T. Baker) and washed with 7–10 ml buffer. Excess borohydride was removed by two additions and evaporations of methanol. Cutoff filters were Centri/Por microconcentrators, MWCO 500, Spectrum. Mixed-bed, cation, and anion exchangers were AG501-X8(D), Dowex 50W-X8 (H⁺ form), and AG1-X8 (formate form), respectively (BioRad). Protease was type IX bacterial from *Bacillus polymyxa*, Sigma P-6141. Calf intestinal alkaline phosphatase was from Boehringer. The values statistically different from the control (Student's *t*-test, *P* < 0.05) are indicated by asterisks.

Jurkat supernatant or extract was applied. Table 1a summarizes results arguing that the supernatant activity could not be attributed to any of the candidates listed. Though some ATP was detectable in raw supernatants, activity survived hexokinase/glucose treatment as applied in all the previous traces. This remaining activity was unaffected by prior desensitization of the ATP response. ADP and GTP could be excluded because they activate the same receptors on macrophages as ATP¹⁵ and show cross-desensitization. Many other candidates were excluded by the above criteria. Also, the mediator was probably not an eicosanoid or other metabolite of arachidonic acid, because supernatant activity continued despite 15 min pretreatment of the Jurkat cells with the phospholipase A₂ inhibitor 4-bromophenacyl bromide (20 μM) or blockage of eicosanoid production with nordihydroguaiaretic acid (20 μM), 5,8,11,14-eicosatetraenoic acid (50 μM)⁶, or indomethacin (25 μM). Putative inhibitors of cytochrome P450^{6,16} such as econazole (20 μM) or carbon monoxide (saturated at 1 atm) also failed to prevent Jurkats from releasing activity into the supernatant.

The chemistry of the Ca²⁺-influx factor or CIF was then further characterized (Table 1b) starting from cell supernatant

because it should be cleaner than whole-cell extracts. CIF seemed stable and not protein-like, because heat or protease treatment or passage through a M_r 500 cutoff filter enhanced the activity. Such enhancement might be explained if the activity in crude supernatant had been weakened by partial binding to protein. But activity of heat-treated supernatant could not be reduced by BSA, so any such protein binding did not seem totally nonspecific. The small size of CIF was confirmed by size exclusion chromatography through Bio-Gel P-2 polyacrylamide. Even though the activity passed through the small short cartridges of reverse-phase silica used to filter out digitonin, CIF did seem to have some hydrophobic portions, because it was mostly retained on larger and longer columns of chromatographic-grade C₁₈ reverse-phase silica. Elution of such columns with buffer containing 20% methanol allowed most of the activity to be recovered, but no further activity could be eluted with 100% methanol. Charcoal columns also filtered out all the activity. Although these retentions suggested at least some hydrophobicity, extraction of supernatants with chloroform-methanol (1 : 1 v/v) left all the activity in the aqueous phase, even when the latter was acidified to pH 2.5 to protonate carboxylate groups. Cation

exchange columns let the activity through whereas anion exchange or mixed bed columns trapped it, suggesting that the messenger was negatively charged. Acidification to pH 2.5 did not prevent retention by the anion exchange column, suggesting again that the relevant charge was more likely to reside on a phosphate (pK_a typically 1–2) than a carboxylate (pK_a typically 4–5). The supernatant activity completely disappeared after treatment with alkaline phosphatase (Table 1b). The sensitivity to alkaline phosphatase and the extraction and ion-exchange behaviour argue for an essential phosphate in the molecule. Exposure to pH 13 for 20 min did not significantly affect the activity, suggesting the absence or unimportance of readily hydrolysable ester linkages. Periodate (50 mM), which cleaves vicin-diol or aminoalcohols to carbonyl compounds and oxidizes thiols or thioethers, completely destroyed the activity in 20 min. This effect of periodate was probably not due to thiol groups

because the activity was insensitive to iodoacetamide, which should alkylate thiols. Finally, insensitivity to borohydride suggested that aldehydes, ketones, or reducing sugars were absent or non-essential.

The main short-term effect of depletion of Ca^{2+} stores seems not to be fresh biosynthesis of the CIF but rather its movement from organelles into the digitonin-releasable fraction, the cytosol. The mechanism for such release from storage is unknown but unlikely to be through the inositol 1,4,5-trisphosphate ($InsP_3$)-activated Ca^{2+} channels, because the latter are believed to be cation-selective and are not activated by thapsigargin or by Ca^{2+} -depletion of the cytosol. Precedent exists for Ca^{2+} -dependent release of anions from the $InsP_3$ -sensitive stores. Recently, Fulceri *et al.*¹⁷ have shown that inorganic phosphate accumulates in microsomal vesicles when the latter are allowed to take up Ca^{2+} ; release of the microsomal Ca^{2+} by $InsP_3$, Ca^{2+} -

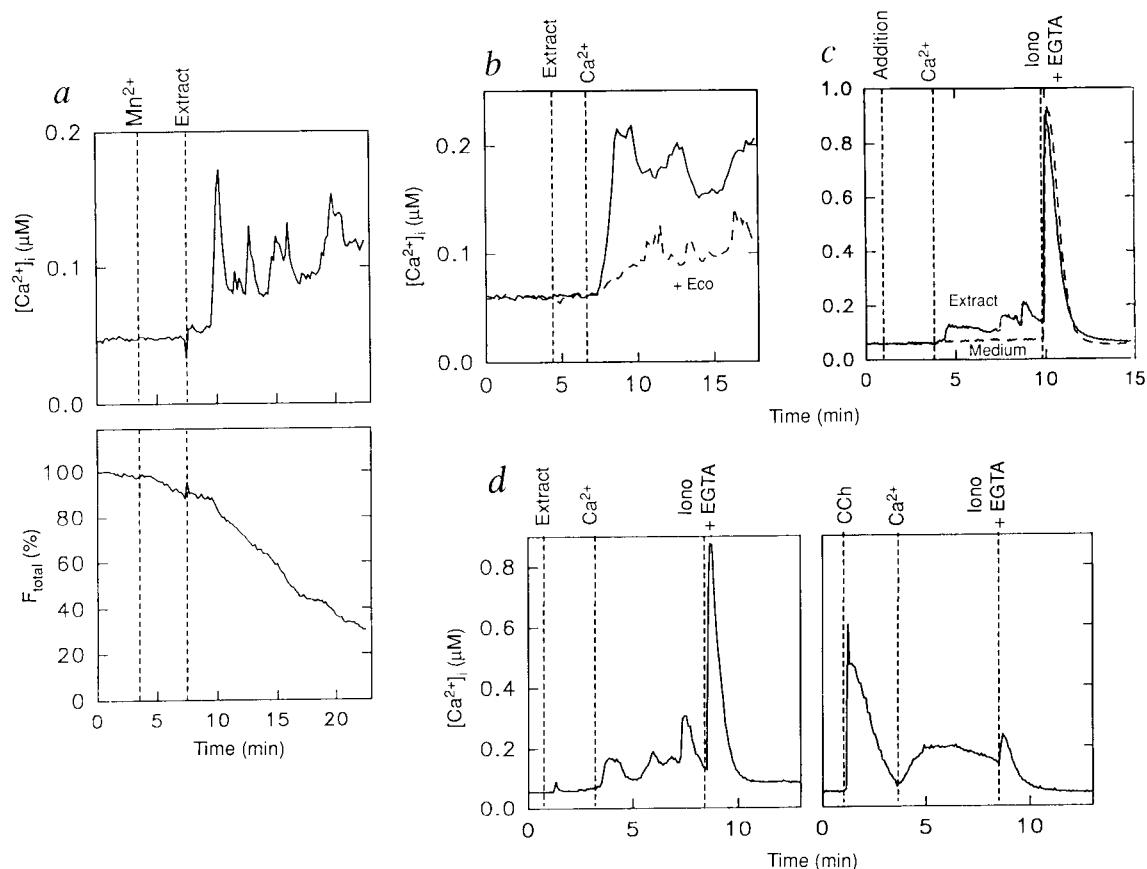


FIG. 2 Lymphocyte extract causes Ca^{2+} influx but not release of internal Ca^{2+} stores in astrocytoma cells. a, $[Ca^{2+}]_i$ (top) and total Ca^{2+} -independent fluorescence (F_{total} , bottom) were simultaneously measured in astrocytoma cells in 1.3 mM external Ca^{2+} after addition of 0.4 mM Mn^{2+} and lymphocyte extract. The latter induced a $[Ca^{2+}]_i$ increase in parallel with an acceleration of Mn^{2+} -quenching of F_{total} . b, In Ca^{2+} -free DPBS medium with 0.5 mM EGTA, lymphocyte extract did not increase $[Ca^{2+}]_i$ (solid line) until external Ca^{2+} (2 mM) was readmitted. Econazole (5 μ M, applied 3 min before extract) blunted the $[Ca^{2+}]_i$ increase (dashed line) after external Ca^{2+} readmission. c, Lymphocyte extract (solid line) or control buffer (dashed line) was added to astrocytoma cells in DPBS with no added Ca^{2+} and 0.5 mM EGTA. Ca^{2+} (2 mM) was added, followed by 2 μ M ionomycin and 10 mM EGTA. d, The astrocytoma cells were treated as in c with lymphocyte extract (left) or 100 μ M carbachol (right). The sizeable fluctuations in average $[Ca^{2+}]_i$ in response to lymphocyte extract (Figs 1 and 2) reflect even larger fluctuations in single responder cells and may be due to occasional Ca^{2+} -induced Ca^{2+} -release from their fully loaded Ca^{2+} stores. b, d,

Show that any such release from internal stores is secondary to the Ca^{2+} influx. Analogous fluctuations (data not shown) are generated by a threshold dose of digitonin, another treatment that should likewise induce Ca^{2+} entry without directly discharging internal Ca^{2+} stores. By contrast, the Ca^{2+} influx generated by carbachol in astrocytoma cells (Fig. 2d) is smoother and less prone to amplification because the Ca^{2+} stores have already been depleted as a primary step.

METHODS. Lymphocyte extract was prepared and added to the astrocytoma cells as described in Fig. 1 except that 0.5 mM EGTA was included in the extract for (b, d) instead of 1.3 mM Ca^{2+} for a. Each trace shows the average $[Ca^{2+}]_i$ of 3–12 astrocytoma cells in the same dish. F_{total} was defined as (fluorescence at 350 nm excitation + 0.22 \times fluorescence at 385 nm excitation); the coefficient 0.22 was determined in control experiments to simulate the isoexcitation wavelength and make F_{total} insensitive to $[Ca^{2+}]_i$ changes imposed by ionomycin, as described previously³¹. The F_{total} values have been normalized to 100% corresponding to the mean fluorescence during the first 2 min of recording.

ionophore, or chelation of external Ca^{2+} causes parallel release of the phosphate with no discernable additional time delay, a few seconds or less in the case of InsP_3 or ionophore. A similar Ca^{2+} -dependent release of CIF, which is a phosphate-containing anion, would be an economical mechanism for gating the signal to activate Ca^{2+} influx. But the precise timing of CIF release is unknown, so more complex mechanisms involving enzymatic steps cannot be excluded. Also, Ca^{2+} influx cannot depend entirely on prestored CIF, because depletion-induced Ca^{2+} permeability can be quite long lasting. Under those conditions, CIF may be continually produced in the organelles and released into the cytosol. In addition, the present results do not rule out other mechanisms^{3,7,18,22} that might trigger Ca^{2+} influx in parallel. CIF seems to have some ability to cross intact plasma membranes, because perhaps 10–20% of the cytosolic CIF appears in the supernatant of stores-depleted cells even without digitonin permeabilization. This fraction is larger than would be readily explained by the few per cent of leaky cells. CIF showed detect-

able activity when applied extracellularly to three unrelated cell types, macrophages, fibroblasts, and astrocytoma cells, showing that its action may have some generality. Present evidence does not show whether it acts from the outside or inside the target cells. A receptor on the intracellular face of the plasma membrane would make more sense for a signal released from organelles, but may seem difficult to reconcile with the observed effectiveness of an anionic, phosphate-containing messenger administered outside. But translocases might be present not only in the organellar membranes but also the plasma membranes both of generator and of responder cells. The modest absolute amplitude of the $[\text{Ca}^{2+}]_i$ increases in our responder cells may well reflect administration on the 'wrong' side of a permeability barrier, and the variations in sensitivity of different responder cell types might well reflect differences in permeability rather than receptor affinity. Direct intracellular delivery of CIF by whole-cell patch clamp should be informative. Nevertheless, because CIF shows detectable extracellular potency and might

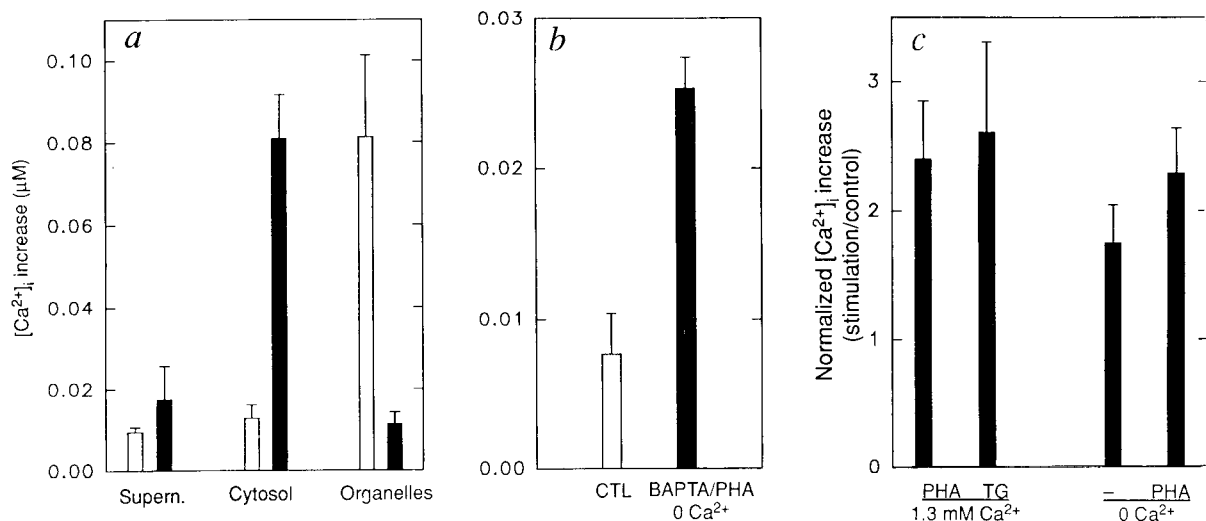


FIG. 3 Location of the messenger within Jurkat lymphocytes and ways of stimulating its release. *a*, $[\text{Ca}^{2+}]_i$ -elevating activities in the supernatant, the cytosol and the organelles of nonstimulated (open bars) or stimulated ($20 \mu\text{g ml}^{-1}$ PHA in Ca^{2+} -free DPBS plus 0.5 mM EGTA for 10 min, solid bars) lymphocytes were assayed on macrophages. Each suspension of lymphocytes (10% cytocrit) was fractionated by centrifugation, removal of the supernatant, and resuspension in a matching volume of nominally Ca^{2+} -free DPBS with $50 \mu\text{M}$ digitonin to permeabilize the plasma membranes selectively. Control experiments showed that $50 \mu\text{M}$ was the minimum dose of digitonin for complete permeabilization as assessed by trypan blue. This high dose probably reflects depletion of the digitonin by the high concentration of cells. The soluble cytosolic contents thus released were separated from intracellular organelles and insoluble residues by recentrifugation after 5 min; the pellet was resuspended in the same volume of nominally Ca^{2+} -free DPBS. All fractions were immediately acidified to pH 2.5, kept for 20 min to inactivate breakdown enzymes (and to extract the final pellet), filtered through two small cartridges of reverse-phase octadecyl silica (SPICE C₁₈, Rainin) to remove digitonin and fatty acids, neutralized to pH 7.3, treated with 2 units ml^{-1} hexokinase for 20 min, and supplemented with 1.3 mM Ca^{2+} . $[\text{Ca}^{2+}]_i$ increases were determined on macrophages as the difference between the resting level of $[\text{Ca}^{2+}]_i$ and the maximal $[\text{Ca}^{2+}]_i$ value reached within 7 min after addition of extract (diluted 1:1 with HBS to a final concentration equivalent to the contents of 30–40 μl packed Jurkat cells in 600 μl medium). Each value corresponds to the average \pm s.e. of responses obtained in 3 to 5 different dishes with 12 cells measured per dish. Macrophages were used as detector cells in these experiments because they had the highest sensitivity to the messenger, despite the greater complexity of their response. In the experiment shown, the total activity obtained by summing the three fractions from stimulated cells was 99% of the total activity of unstimu-

lated cells. In 4 such experiments, the corresponding ratio was $110 \pm 7\%$ (mean \pm s.e.), confirming rough conservation of total activity over 10 min of stimulation. *b*, Activities appearing in the cytosol of nonstimulated (open bar) or BAPTA-loaded and stimulated cells (closed bar) measured on astrocytoma cells. This experiment was similar to *a* except that the lymphocytes to be stimulated had been loaded with BAPTA by pre-incubation with $50 \mu\text{M}$ BAPTA/AM for 20 min in HBS with 1.3 mM Ca^{2+} before changing them into the Ca^{2+} -free DPBS (0.5 mM EGTA) and adding PHA. Also, the astrocytoma cells were allowed 15 min to reach their maximal $[\text{Ca}^{2+}]_i$ values. *c*, Activities of the supernatants from lymphocytes stimulated at 10% cytocrit in 1.3 mM Ca^{2+} with $20 \mu\text{g ml}^{-1}$ PHA or $50 \mu\text{M}$ thapsigargin, or held in Ca^{2+} -free medium plus 0.5 mM EGTA without or with PHA, all for 10 min. The cells were then centrifuged. The supernatant was treated with 2 units ml^{-1} hexokinase and added to macrophages as in *a*. Because each condition was tested on a different day, each $[\text{Ca}^{2+}]_i$ increase was normalized to a matching control obtained the same day from unstimulated cells kept for 10 min in $1.3 \text{ mM external Ca}^{2+}$. These control amplitudes were 24.5, 21, 19 and 19 nM for the four conditions, respectively. The sample activities were all significantly different from their controls (Student's *t*-test gave $P < 0.05$, except $P < 0.09$ for thapsigargin), but not different from each other ($P > 0.2$). The thapsigargin experiments required special care. Control experiments showed that $50 \mu\text{M}$ thapsigargin was necessary to counteract the strong binding to the high concentration of cells and maintain enough free drug to be bioassayable in the supernatant. To measure the messenger activity, the supernatants from both control and thapsigargin-treated cells were titrated to pH 13 with NaOH to hydrolyse thapsigargin, which contains multiple ester groups. After 20 min, the pH was neutralized to 7.3 with HCl. Control experiments verified that this alkaline treatment completely destroyed $50 \mu\text{M}$ thapsigargin as judged by its ability to elevate $[\text{Ca}^{2+}]_i$.

pass through gap junctions, it could conceivably have paracrine functions in cell clusters. Also, depletion of Ca^{2+} stores is known to trigger transcription of certain genes^{23,24}, so factors for carrying information from the lumen of Ca^{2+} -sequestering organelles to the cytosol or nucleus may have functions beyond control of Ca^{2+} entry. □

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