Signaling between Intracellular Ca^{2+} Stores and Depletion-activated Ca^{2+} Channels Generates $[Ca^{2+}]_i$ Oscillations in T Lymphocytes

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ABSTRACT   Stimulation through the antigen receptor (TCR) of T lymphocytes triggers cytosolic calcium ($[Ca^{2+}]_i$) oscillations that are critically dependent on Ca^{2+} entry across the plasma membrane. We have investigated the roles of Ca^{2+} influx and depletion of intracellular Ca^{2+} stores in the oscillation mechanism, using single-cell Ca^{2+} imaging techniques and agents that deplete the stores. Thapsigargin (TG; 5–25 nM), cyclopiazonic acid (CPA; 5–20 μM), and tert-butylhydroquinone (tBHQ; 80–200 μM), inhibitors of endoplasmic reticulum Ca^{2+}-ATPases, as well as the Ca^{2+} ionophore ionomycin (5–40 nM), elicit $[Ca^{2+}]_i$ oscillations in human T cells. The oscillation frequency is ∼5 mHz (for ATPase inhibitors) to ∼10 mHz (for ionomycin) at 22–24°C. The $[Ca^{2+}]_i$ oscillations resemble those evoked by TCR ligation in terms of their shape, amplitude, and an absolute dependence on Ca^{2+} influx. Ca^{2+}-ATPase inhibitors and ionomycin induce oscillations only within a narrow range of drug concentrations that are expected to cause partial depletion of intracellular stores. Ca^{2+}-induced Ca^{2+} release does not appear to be significantly involved, as rapid removal of extracellular Ca^{2+} elicits the same rate of $[Ca^{2+}]_i$ decline during the rising and falling phases of the oscillation cycle. Both transmembrane Ca^{2+} influx and the content of ionomycin-releasable Ca^{2+} pools fluctuate in oscillating cells. From these data, we propose a model in which $[Ca^{2+}]_i$ oscillations in T cells result from the interaction between intracellular Ca^{2+} stores and depletion-activated Ca^{2+} channels in the plasma membrane.

INTRODUCTION

An increase in the level of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is an essential triggering signal for a variety of cellular processes, including secretion, adhesion, motility, growth, and differentiation. Ca^{2+} signaling in many nonexcitable cells is controlled by cell surface receptors that are linked to the production of inositol 1,4,5-trisphosphate (IP_3) (Berridge, 1993). IP_3 elicits a biphasic rise in $[Ca^{2+}]_i$ by stimulating Ca^{2+} release from the endoplasmic reticulum (ER) and Ca^{2+} entry across

the plasma membrane (Putney, 1990). The IP_3-driven increase in $[Ca^{2+}]_i$ often consists of $[Ca^{2+}]_i$ oscillations at the level of individual cells (for reviews, see Berridge and Galione, 1988; Meyer and Stryer, 1991; Fewtrell, 1993).

The characteristics of $[Ca^{2+}]_i$ oscillations vary widely among different cell types, and it is generally believed that a single mechanism is unlikely to account for the variety of observed responses (Berridge, 1993; Fewtrell, 1993). The mathematical models proposed to explain $[Ca^{2+}]_i$ oscillations differ in many respects, such as whether the IP_3 concentration oscillates, the relative contributions of IP_3 receptors and ryanodine receptors, and the sites of positive and negative feedback required for oscillatory behavior (Meyer and Stryer, 1988; Goldbeter, Dupont, and Berridge, 1990; Cuthbertson and Chay, 1991; De Young and Keizer, 1992). Despite these variations, the common feature of all the models proposed thus far is that oscillations arise from the repetitive release and reuptake of Ca^{2+} from intracellular stores. Ca^{2+} influx generally plays a minor role, serving to trigger Ca^{2+}-induced Ca^{2+} release (CICR) or to maintain the Ca^{2+} content of the stores over prolonged periods of stimulation. This conclusion stems from the common observation that receptor-stimulated $[Ca^{2+}]_i$ oscillations persist for some time following removal of extracellular Ca^{2+} (Woods, Cuthbertson, and Cobbold, 1986; Jacob, Merritt, Hallam, and Rink, 1988; Rooney, Sass, and Thomas, 1989; Wakui, Potter, and Petersen, 1989).

In T lymphocytes, the binding of antigen or polyclonal mitogens to the antigen receptor (TCR) triggers the production of IP_3 and elicits a prolonged rise in $[Ca^{2+}]_i$ (Tsien, Pozzan, and Rink, 1982; Imboden and Stobo, 1985) that is one of the signals leading to cell cycle progression, proliferation, and the acquisition of specific immune functions (Crabtree, 1989). The increase in $[Ca^{2+}]_i$ is manifest in single cells as asynchronous $[Ca^{2+}]_i$ oscillations with a variable amplitude and a period of $\sim 80-120$ s (Lewis and Cahalan, 1989; Donnadieu, Bismuth and Trautmann, 1992a, Donnadieu, Cefai, Tan, Paresys, Bismuth and Trautmann, 1992b; Hess, Oortgiesen, and Cahalan, 1993). These oscillations are unusual because they depend critically on Ca^{2+} influx and therefore cannot be explained by any of the previously proposed models. Oscillations triggered through the TCR are terminated immediately by conditions that inhibit Ca^{2+} influx, including removal of extracellular Ca^{2+}, membrane depolarization with high $[K^+]$, or Ca^{2+} channel blockade with Ni^{2+} (Lewis and Cahalan, 1989; Donnadieu et al., 1992a; Hess et al., 1993). An absolute requirement for Ca^{2+} influx is further supported by whole-cell recordings of the mitogen-activated Ca^{2+} current in T cells stimulated with phytohemagglutinin (PHA). This current oscillates slightly in advance of $[Ca^{2+}]_i$, providing direct evidence that periodic Ca^{2+} influx may generate $[Ca^{2+}]_i$ oscillations in these cells (Lewis and Cahalan, 1989).

Given the critical role of Ca^{2+} influx in driving $[Ca^{2+}]_i$ oscillations in T cells, it is essential to understand how mitogen-regulated Ca^{2+} channels are activated. A growing body of evidence suggests that the depletion of internal Ca^{2+} stores triggers Ca^{2+} influx in T cells and other non-excitable cells by a process referred to as capacitative Ca^{2+} entry (Putney, 1990). Inhibition of ER-Ca^{2+}-ATPases by thapsigargin (TG), cyclopiazonic acid (CPA), or tert-butylhydroquinone (tBHQ) depletes Ca^{2+} stores by blocking uptake, unmasking an endogenous leak for Ca^{2+}. Store depletion by these compounds can trigger pronounced Ca^{2+} influx, without significant IP_3 generation (Takemura, Hughes, Thastrup, and Putney, 1989; Gouy, Cefai, Chris-

tensen, Debre, and Bismuth, 1990; Putney, 1990; Foskett, Roifman, and Wong, 1991; Mason, Garcia-Rodriguez, and Grinstein, 1991a). Pharmacological similarities and the lack of additivity between TCR- and TG-stimulated Ca^{2+} entry pathways suggest the two are identical (Mason, Mahaut-Smith, and Grinstein, 1991b). The Ca^{2+} current activated by TG was recently characterized in T cells using whole-cell and perforated-patch recording (Zweifach and Lewis, 1993). This current was shown to be identical in its biophysical properties to the TCR-activated current described previously (Lewis and Cahalan, 1989): both are highly Ca^{2+}-selective and show inward rectification, voltage-independent gating, and a unitary conductance of ~ 24 fS in isotonic Ca^{2+}. The TG- and TCR-activated currents appear to be identical to the Ca^{2+}-release activated Ca^{2+} current (I_{CRAC}) described in rat mast cells (Hoth and Penner, 1992, 1993). Taken together, these results argue strongly that Ca^{2+} influx, and hence $[Ca^{2+}]_i$ oscillations, involve depletion-activated Ca^{2+} channels in the plasma membrane of T cells.

The study reported here was undertaken to assess more directly the roles of intracellular Ca^{2+} stores and depletion-activated Ca^{2+} channels in generating $[Ca^{2+}]_i$ oscillations in human T cells. We report that low concentrations of the ER-Ca^{2+}-ATPase inhibitors TG, CPA, or tBHQ, as well as the Ca^{2+} ionophore ionomycin, can trigger $[Ca^{2+}]_i$ oscillations similar in many ways to those stimulated through the TCR. Moreover, the store content and Ca^{2+} influx fluctuate during each oscillation cycle. This study provides the first experimental evidence in support of a novel mechanism in which communication between intracellular Ca^{2+} stores and depletion-activated Ca^{2+} channels generates $[Ca^{2+}]_i$ oscillations.

MATERIALS AND METHODS

Cells

T cells were isolated from the blood of healthy human volunteers by centrifugation on Ficoll Paque (Pharmacia, Uppsala, Sweden), followed by passage through a nylon wool column as described previously (Hess et al., 1993). The cell population contained $\sim 80\%$ CD4⁺ or CD8⁺ T cells as determined by flow cytometry with the remainder being mostly B cells. Cells were maintained at a density of 2×10^6 cells/ml in culture media containing RPMI 1640, 10% fetal calf serum and 1% glutamine at 22°C or at 37°C in an atmosphere of 95% air and 5% CO_2, and were used within 10 h after isolation.

Solutions

Normal Ringer's solution contained (in mM) 155 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 5 HEPES (pH 7.4 with NaOH). Ca^{2+}-free Ringer's solution was made by replacing $CaCl_2$ with $MgCl_2$, resulting in a free $[Ca^{2+}]_o$ of 5–10 μM. 1 mM EGTA was added where noted. K⁺ Ringer's was made by equimolar substitution of K⁺ for Na⁺. Thapsigargin (LC Biochemicals, Woburn, MA) was diluted from a 1 mM stock in DMSO; cyclopiazonic acid and *tert*-butylhydroquinone were prepared from a 100 mM DMSO stock; ryanodine (Calbiochem Corp., La Jolla, CA) was dissolved in 50% ethanol at a concentration of 100 mM and $NiCl_2$ was added to Ringer's solution from a 1 M stock in H_2O. Cyclopiazonic acid, *tert*-butylhydroquinone, and caffeine were purchased from Sigma Chemical Co. (St. Louis, MO).

Single-Cell [Ca²⁺]ᵢ Measurements

T cells at a density of 2 × 10⁶ cells/ml were incubated at 20–24°C for 30 min in culture medium containing 0.5 μM fura-2 acetoxymethylester (Molecular Probes Inc., Eugene, OR) and 100 μM chloroquine (Sigma Chemical Co.). Cells loaded at 37°C in the absence of chloroquine often displayed an eccentrically located, highly fluorescent spot indicating dye compartmentation. The spot's fluorescence was brighter when excited at 380 nm than at 350 nm and was largely insensitive to changes in cytoplasmic [Ca²⁺], thus causing underestimates of cytoplasmic [Ca²⁺]. Loading at reduced temperature in the presence of chloroquine greatly reduced the accumulation of fura-2 in this compartment (Malgaroli, Milani, Meldolesi, and Pozzan, 1987) but had no discernable effect on the oscillatory behavior of the cells. After loading, cells were washed, resuspended in chloroquine-free culture medium, and maintained in the dark. Before each experiment, cells were allowed to adhere to poly-D-lysine-coated glass coverslips for 10 min. Video microscopic measurement of [Ca²⁺]ᵢ was conducted essentially as described previously (Lewis and Cahalan, 1989) using a Nikon Diaphot or Zeiss Axiovert inverted microscope. Briefly, excitation at 350 ± 5 nm and 380 ± 6 nm was supplied by a 75-watt xenon arc lamp (attenuated 10–100-fold) and interference filters (Omega Optical, Brattleboro, VT) in a computer-controlled filter wheel (Lambda 10, Sutter Instruments, Novato, CA). Excitation light was deflected by a 400-nm dichroic mirror through a 40× oil-immersion objective (Nikon Fluor, NA 1.3 or Zeiss Achrostigmat, NA 1.3), and emitted fluorescence at λ > 480 nm was collected with an intensified CCD camera (Hamamatsu Corp., Bridgewater, NJ). For each wavelength, 4–8 video frames per time point were digitized, averaged, and background-subtracted, and the 350/380 ratio images (R) were computed and stored digitally using a VideoProbe image processor (ETM Systems, Irvine, CA). [Ca²⁺]ᵢ was estimated offline for individual cells using the equation [Ca²⁺]ᵢ = K* (R–R_{min})/(R_{max}–R). R_{min}, R_{max}, and K* were derived from an in situ calibration of fura-2 in Jurkat T cells using an intracellular dialysis method described previously (Almers and Neher, 1985; Lewis and Cahalan, 1989). More routine calibrations of fura-2 in free solution (Grynkiewicz, Poenie, and Tsien, 1985) were used with the appropriate correction factors to provide values of R_{min}, R_{max}, and K*. This procedure allowed us to perform frequent calibrations to verify the accuracy of the system while taking into account the changes in fura-2 properties caused by the intracellular environment.

Spectral Analysis of [Ca²⁺]ᵢ Oscillations

Data were analyzed using the program Igor Pro (Wavemetrics Inc., Lake Oswego, OR). We used the power spectral density (PSD) function of the [Ca²⁺]ᵢ measurements to analyze both the frequency and magnitude of oscillations in single cells and cell populations. The data from each cell were processed by excluding the initial 400-s segment and subtracting a least-squares fit of a third-order polynomial function from the remainder to remove low frequency components resulting from slow [Ca²⁺]ᵢ changes unrelated to the oscillations. The PSD function was computed using an FFT algorithm, summing the squares of the resulting complex values, and normalizing by the number of data points.

The PSD function was used to estimate the oscillatory frequency distribution of cell populations and the number of cells oscillating at different agonist concentrations. Oscillating cells were detected automatically as those with maximum power at frequencies ≥ 3 mHz and peak power ≥ 150 nM²/Hz. This criterion effectively eliminates all cells that have fewer than three oscillatory cycles or peak-to-peak amplitudes smaller than 200 nM.

Rapid Perfusion Experiments

Rapid solution changes were achieved using a chamber consisting of a rectangular Teflon well sandwiched between two glass coverslips, with influx and efflux openings at either end. The

chamber had a volume of ~ 10 μl, and 90% exchange was achieved in < 1 s using a perfusion rate of ~ 5 ml/min. For these experiments, ratio images were collected every 1.5 s using four-frame averages for each wavelength. The time interval between the 350 and 380-nm images (466 ms maximum) affected estimates of $d[Ca^{2+}]_i/dt$ by $< 1\%$.

Ca^{2+} influx across the plasma membrane was estimated from the change in slope ($\Delta d[Ca^{2+}]_i/dt$) caused by rapid extracellular perfusion with Ca^{2+}-free Ringer's containing 1 mM EGTA. The slope before removal of Ca^{2+} was measured using a linear regression fit to four data points (6 s) immediately before the solution change. The slope following EGTA perfusion was determined from the most negative value of $d[Ca^{2+}]_i/dt$ within 5 s of the solution change. The minimum slope typically occurred within 3 s. The Ca^{2+} content of the stores was estimated in the same experiment by treating cells with 2 μM ionomycin + 1 mM EGTA 10 s after removing Ca_o^{2+}. The slopes were determined using the local minima and maxima of $d[Ca^{2+}]_i/dt$ in a 30-s window surrounding the addition of ionomycin. Relative store content was estimated by subtracting the declining slope after perfusion with EGTA from the rising slope produced by the addition of ionomycin.

Oscillation phase at the time of Ca^{2+} removal was determined using the following procedure. The period τ of the oscillations was estimated from the time between the minima of two consecutive $[Ca^{2+}]_i$ troughs immediately before the solution change. The time from the last minimum to the removal of Ca_o^{2+} (t) was measured, and the phase ϕ was calculated using the equation $\phi = t/\tau \times 360°$. Both Ca^{2+} influx and store content were measured as a function of phase and averaged into 18° bins.

The average shape of a $[Ca^{2+}]_i$ oscillation was calculated by averaging a single cycle (from trough to trough) from all the cells. Because the oscillation period varies somewhat among cells, each cycle was first normalized to a standard length by interpolation between data points. The average $[Ca^{2+}]_i$ was then plotted as a function of phase from 0 to 360°.

RESULTS

Low Doses of Thapsigargin Trigger [Ca²⁺]ᵢ Oscillations

Previous studies using T-cell suspensions have shown that TG elicits a sustained $[Ca^{2+}]_i$ rise whose amplitude is dependent on TG concentration (Gouy et al., 1990; Mason et al., 1991a,b). We examined this effect in single human T cells using concentrations of TG ranging from 1 to 1,000 nM. The responses of single cells and the average responses of > 250 cells is shown for three experiments in Fig. 1. At low (1 nM) and high (≥ 100 nM) concentrations, the population average resembles the effect of TG on single cells. In response to 1 nM TG, $[Ca^{2+}]_i$ rose with a sigmoidal time course from the resting level of 61 ± 39 nM to a plateau value of 138 ± 52 nM (mean ± SD, $n = 258$), whereas 100 nM TG elevated $[Ca^{2+}]_i$ from a baseline of 53 ± 39 nM to a plateau of 1,179 ± 396 nM ($n = 384$).

In contrast, concentrations of TG between 10 and 40 nM triggered pronounced $[Ca^{2+}]_i$ oscillations. These oscillations were asynchronous and therefore were not apparent in the population average (Fig. 1 B). The oscillation amplitude was variable among cells, typically increasing during the initial 1,500 s and then remaining constant or declining slowly as shown in Fig. 1 B. During prolonged exposure to 10 nM TG (duration > 50 min), the oscillations often gave way to a sustained $[Ca^{2+}]_i$ plateau somewhat higher than the peak of the oscillations. This effect is presumably due to the cumulative effect of irreversible inhibition of $ER-Ca^{2+}$ ATPases by TG (Thastrup, Cullen, Drobak, Hanley, and Dawson, 1990). Thapsigargin triggered

similar $[Ca^{2+}]_i$ oscillations in the human leukemic T-cell line Jurkat and in murine splenic T cells, both at 22°C and at 37°C, indicating that this phenomenon is not restricted to a single experimental preparation or to non-physiologic temperatures (data not shown).
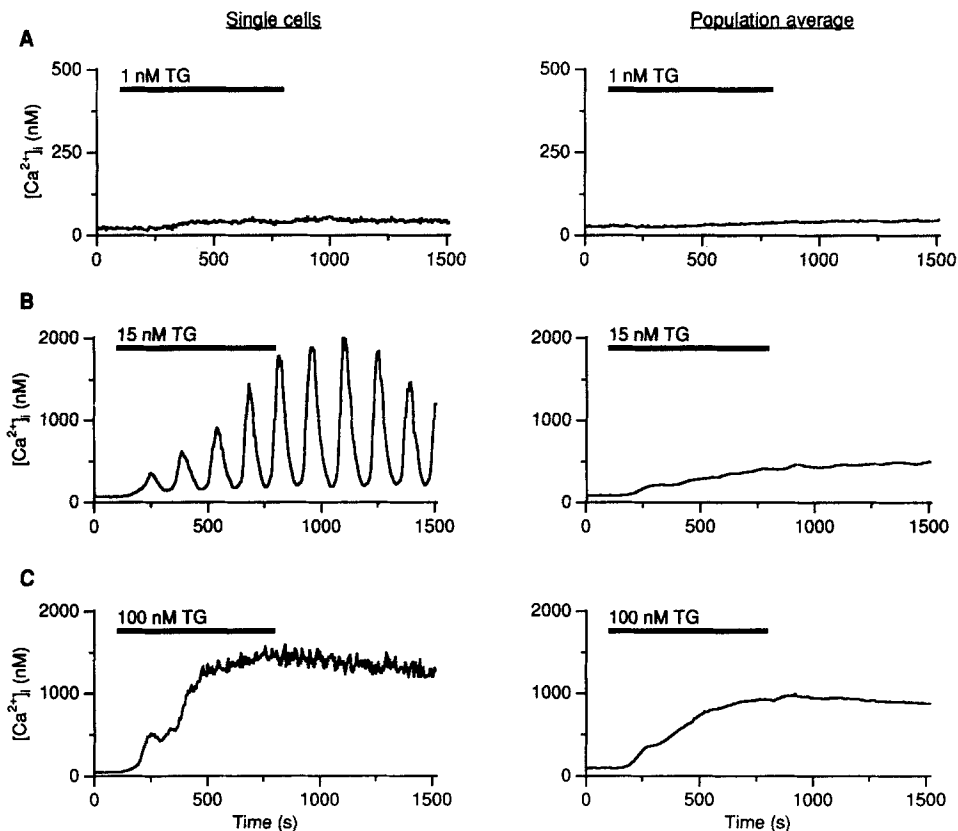

FIGURE 1. TG triggers $[Ca^{2+}]_i$ oscillations in a concentration-dependent manner. Single-cell responses to concentrations of 1 (A), 15 (B), and 100 nM (C) TG are shown on the left. Average responses of 200–300 cells are shown on the right. TG was applied during the time indicated by the bar. Note the vertical scale is amplified fourfold in A. The single-cell responses to 1 and 100 nM TG resemble the respective population averages; however, pronounced $[Ca^{2+}]_i$ oscillations are evoked by 15 nM TG in individual T cells. The oscillations are hidden in the average due to asynchrony in the cell population.

We investigated the frequency characteristics of TG-induced oscillations using spectral analysis (see Materials and Methods). The power spectral density function (PSD) produces a characteristic signature for each cell that encompasses the shape, amplitude, and periodicity of the oscillations. It can therefore be used as a relatively unbiased indicator to compare oscillations in different preparations and under different conditions. The PSD function also provides a consistent method for quantifying the proportion of oscillating cells in a population. Thus, it can be used to

group cells into oscillating and non-oscillating subsets using arbitrary yet absolutely defined criteria.

Power spectra for three T cells are shown in Fig. 2 *A*. Oscillations were prominent in one of the cells, and the corresponding spectrum has a sharp peak at 7 mHz
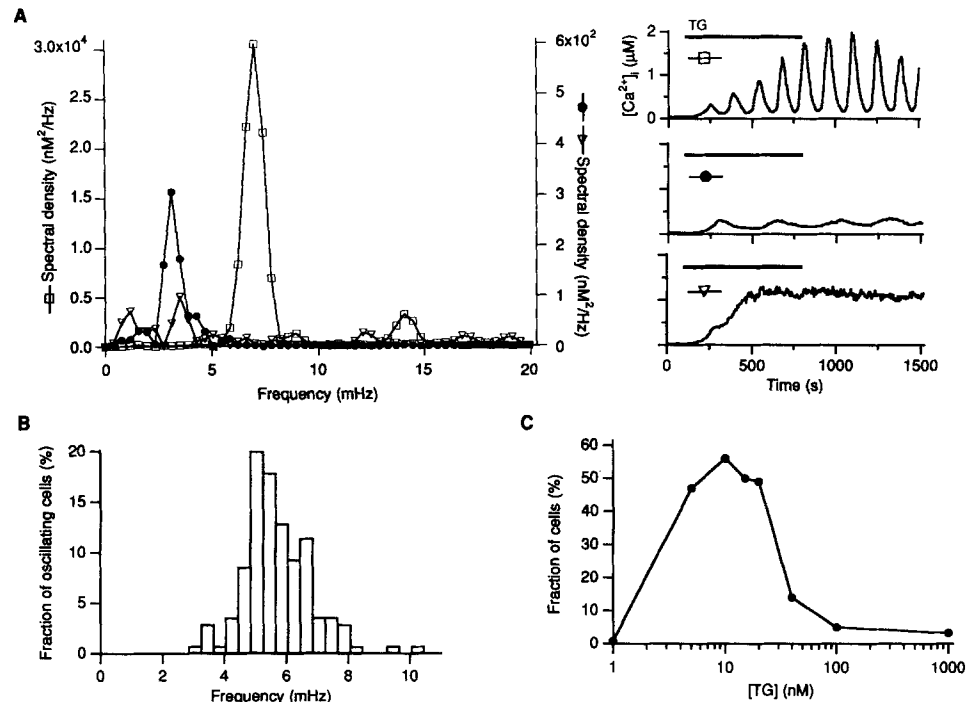

FIGURE 2.   Power spectra of TG-induced $[Ca^{2+}]_i$ oscillations. (*A*) Superimposed PSD functions for three cells (*left*) derived from $[Ca^{2+}]_i$ responses illustrated to the right. TG was applied during the bar. One cell shows robust oscillations at 7 mHz, with a small PSD peak at the first harmonic frequency due to oscillation asymmetry (□; 15 nM TG). A second cell oscillates with power at approximately two times the threshold criterion at a frequency of 3.1 mHz (●; 15 nM TG). A third cell does not oscillate and its spectrum is flat (∇; 100 nM TG). Note the PSD scale for the latter two cells (●, ∇) is amplified 50-fold. (*B*) Distribution of oscillation frequencies for T cells stimulated with 15 nM TG. For each cell, the oscillation frequency was determined from the frequency at maximum power. The results are expressed as the fraction of cells within the oscillating population having a particular frequency. (*C*) Dose-dependence of oscillations induced by TG. The graph shows the fraction of the total cell population showing oscillations in response to varying concentrations of TG. The criteria for determining oscillatory activity are described in the Materials and Methods.

(corresponding to a period of 143 s), while the spectrum for a nonoscillating cell is essentially flat. A third cell with small oscillations is shown for which the PSD peak barely exceeded the threshold we selected as an indicator of oscillatory behavior (150 nM^2/Hz; see Materials and Methods). The spectra of oscillating cells often show a

small peak at the first harmonic frequency, a consequence of the asymmetric shape of the oscillations. The rising phase of each oscillation is generally more rapid than the falling phase, and the $[Ca^{2+}]_i$ peaks are sharper than the troughs.

While the oscillations in each cell were tuned to a narrow frequency range, there was substantial frequency variation across the population. Oscillation frequency was determined for each cell from the location of the PSD function maximum. The median frequency for 250 cells stimulated with 10 nM TG was 5.3 mHz (period = 189 s), with the frequency distribution shown in Fig. 2 B. Under the conditions of these experiments (800-s TG treatment, 2 mM extracellular Ca^{2+}), oscillations were most prevalent at TG concentrations between 5 and 25 nM (Fig. 2 C). This dose dependence is a function of both the duration of TG exposure and the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), as an increase of either parameter augmented the proportion of cells that oscillated after stimulation with 1–5 nM TG (data not shown).

Oscillations are Caused by Other Agents that Deplete Intracellular Ca^{2+} Stores

The unexpected finding that TG can trigger $[Ca^{2+}]_i$ oscillations in T cells prompted us to investigate whether this effect was common to other agents that deplete internal Ca^{2+} stores. We examined the response of T cells to increasing doses of cyclopiazonic acid (CPA) and tert-butylhydroquinone (tBHQ), two ER-Ca^{2+}-ATPase inhibitors that are structurally unrelated to TG. Previous studies have shown that these agents also stimulate Ca^{2+} influx in T-cell populations (Kass, Duddy, Moore, and Orrenius, 1989; Llopis, Chow, Kass, Gahm and Orrenius, 1991; Mason et al., 1991a). We found that within a narrow concentration range, both CPA and tBHQ induce $[Ca^{2+}]_i$ oscillations that closely resemble those produced by TG. CPA and tBHQ are most effective at concentrations of 5–20 and 80–200 μM, respectively (Fig. 3). The concentrations of TG, CPA, and tBHQ that induce oscillations correlate well with their relative potencies of ER Ca^{2+}-ATPase inhibition (Mason et al., 1991a). Oscillations induced by CPA resembled those evoked by TG in terms of the distribution of frequencies (Fig. 3 C) and the median frequency (4.4 mHz; period = 227 s). tBHQ was less effective than TG or CPA. Under optimal conditions, tBHQ induced oscillations in only 20% of the cells, and these generally had a smaller amplitude than those triggered by the other agents. This result may be due to the reported inhibition of the depletion-activated Ca^{2+} influx pathway by tBHQ (Mason et al., 1991a; Foskett and Wong, 1992).

The ability of TG, CPA, and tBHQ to elicit $[Ca^{2+}]_i$ oscillations in T cells suggests that either inhibition of ER Ca^{2+}-ATPases or depletion of internal stores is part of the underlying mechanism. To distinguish between these two possibilities, we examined the effect of ionomycin on resting T cells. Ionomycin, an electroneutral Ca^{2+} ionophore, partitions into both plasma and organellar membranes and conducts Ca^{2+} down its concentration gradient. In this way, it liberates Ca^{2+} from intracellular stores and activates I_{CRAC} without affecting ER Ca^{2+}-ATPases (Hoth and Penner, 1992). At low concentrations of ionomycin (≤ 100 nM), the bulk of Ca^{2+} influx appears to result from activation of I_{CRAC} rather than from direct transport by ionomycin itself. In support of this idea, depolarization with 165 mM K^+ rapidly eliminated the $[Ca^{2+}]_i$ rise induced by 5–50 nM ionomycin (data not shown). This

effect is too large to be explained by the moderate voltage sensitivity reported for ionomycin-mediated transport (Fasolato and Pozzan, 1989) but is consistent with the strong reduction of Ca^{2+} influx through depletion-activated Ca^{2+} channels under depolarized conditions (e.g., see Fig. 6 C). Fig. 4 A illustrates the effect of increasing concentrations of ionomycin on a single T cell. At concentrations of 5–40 nM, ionomycin elicited $[Ca^{2+}]_i$ oscillations in 28% of the cells ($n = 292$) with a median frequency of 9.0 mHz (period = 111 s; $n = 48$). These oscillations resemble those induced by Ca^{2+}-ATPase inhibitors in several respects. First, they are evoked only
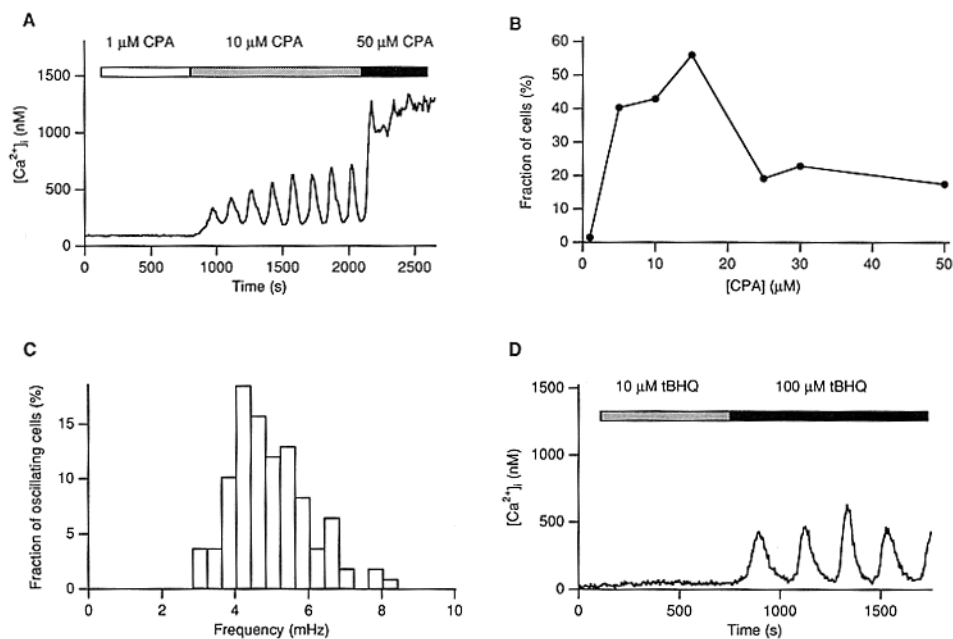

FIGURE 3. $[Ca^{2+}]_i$ oscillations are evoked by CPA and tBHQ. (A) CPA-induced $[Ca^{2+}]_i$ oscillations in a single T cell. The cell was exposed to 1, 10, and 50 μM CPA during the times indicated. (B) Dose dependence of oscillations induced by CPA. Oscillating cells were scored as in Fig. 2 C during a 1,000-s exposure to CPA. (C) Distribution of oscillation frequencies among cells stimulated with CPA. As for TG, most cells oscillate at a frequency of ~5 mHz. (D) Oscillations triggered by 100 μM tBHQ in a single cell. While similar in frequency, these oscillations were generally smaller and less common than those elicited by TG or CPA.

within a narrowly defined concentration range. Second, their shape is similar, with a rising phase that is faster than the declining phase. Finally, they have the same absolute dependence on a Ca^{2+} influx pathway that is inhibited by Ni^{2+} or depolarization, as described below for TG-induced oscillations. Despite different modes of operation, Ca^{2+}-pump inhibitors and ionomycin share an ability to release Ca^{2+} from internal stores. Therefore, the similarities of $[Ca^{2+}]_i$ oscillations produced by these different compounds suggests that Ca^{2+} release from internal stores, rather than Ca^{2+}-ATPase inhibition per se, is an essential part of the oscillation mechanism.

Oscillations triggered by Ca^{2+}-pump inhibition and by ionomycin differ significantly with respect to their frequencies. Ionomycin-induced $[Ca^{2+}]_i$ oscillations are nearly twice as rapid as those triggered by TG, CPA, or tBHQ (Fig. 4 *B*). Interestingly, the frequency observed with ionomycin is quite similar to that seen in T cells stimulated with PHA or anti-TCR antibodies, mitogens that generate intracellular IP_3 (Lewis and Cahalan, 1989; Donnadieu et al., 1992a; Hess et al., 1993). Thus, the oscillation frequency depends on the means by which Ca^{2+} is lost from intracellular stores, whether by increasing the rate of release (ionomycin, IP_3) or by inhibiting reuptake (Ca^{2+}-ATPase inhibitors).
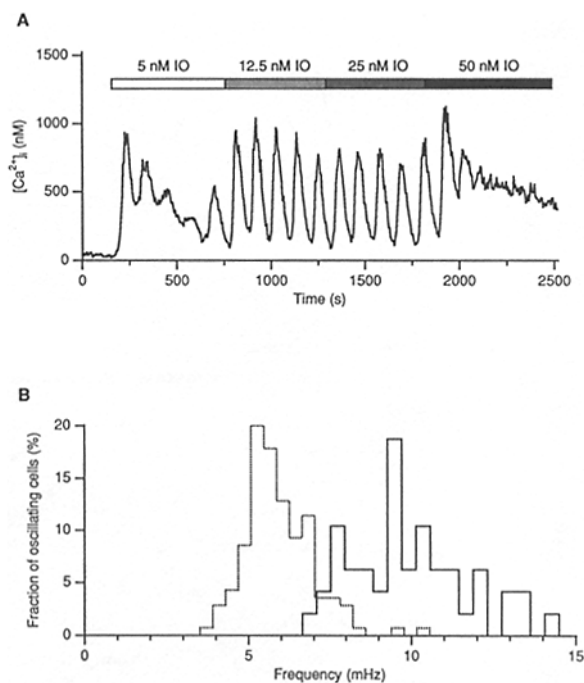

FIGURE 4. $[Ca^{2+}]_i$ oscillations triggered by ionomycin. (*A*) $[Ca^{2+}]_i$ response of a single T cell stimulated with increasing concentrations of ionomycin (IO) applied during the bars. Sustained oscillations are evoked only within a narrow concentration window. (*B*) Comparison of oscillation frequencies for cells stimulated with ionomycin and TG. On average, ionomycin-induced oscillations (*solid line*) are roughly twice as rapid as those elicited by TG (*dashed line*).

[Ca²⁺]ᵢ Oscillations Depend on Partial Emptying of Intracellular Ca²⁺ Stores

The narrow concentration ranges in which TG, CPA, tBHQ, and ionomycin induce oscillations suggest that the relative rates of ER Ca^{2+} release and reuptake are critical. We therefore determined the concentration dependence of TG- and ionomycin-induced Ca^{2+} efflux from internal stores. In the absence of external Ca^{2+}, inhibition of ER Ca^{2+}-ATPases by TG creates a net Ca^{2+} leak from the ER and an increase in $[Ca^{2+}]_i$ that slowly declines as Ca^{2+} is pumped out of the cell (Fig. 5*A*). Using the maximal rate of the TG-induced $[Ca^{2+}]_i$ rise as a qualitative measure of pump inhibition, it appears that TG concentrations that elicit oscillations (i.e., 5–25 nM) cause submaximal inhibition of the ER Ca^{2+} pumps. Likewise, levels of ionomycin that induce oscillations (i.e., 10–25 nM) also release ER Ca^{2+} at a

submaximal rate (Fig. 5 B), though roughly twice as rapidly as does TG. These results suggest that during the generation of oscillations in physiological $[Ca^{2+}]_o$, TG and ionomycin only partially deplete internal stores.

Partial emptying of the Ca^{2+} stores is expected to elicit a low rate of Ca^{2+} influx by stimulating only a fraction of the cell's depletion-activated Ca^{2+} channels. The question arises, is partial depletion itself essential for oscillations to occur, or is it only
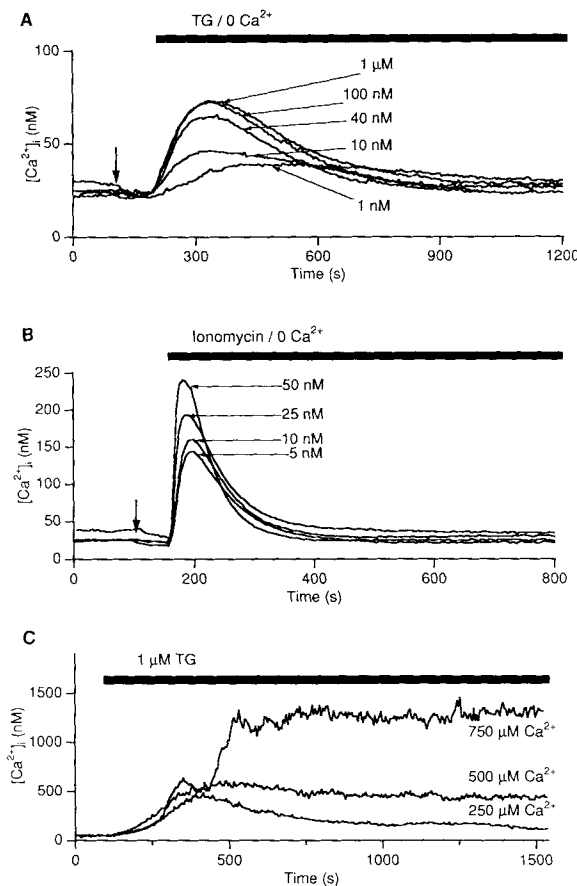
FIGURE 5. Oscillations depend on a submaximal rate of ER Ca^{2+} release. (A) Intracellular Ca^{2+} release by TG. Cells were exposed to nominally Ca^{2+}-free Ringer's at the arrow followed by 1–1,000 nM TG + 0 Ca^{2+} during the bar. The average response of >200 cells is shown for each concentration. $[Ca^{2+}]_i$ rises due to net leakage from the ER, then falls due to Ca^{2+} pumping across the plasma membrane. At concentrations that elicit oscillations in the presence of Ca_o^{2+} (see Fig. 2 C), TG releases ER Ca^{2+} at a submaximal rate. (B) Intracellular Ca^{2+} release by ionomycin. Cells were stimulated with 5–50 nM ionomycin in Ca^{2+}-free Ringer's as in A. Doses of ionomycin that cause oscillations (10–25 nM) release ER Ca^{2+} at a fraction of the maximal rate. (C) Complete inhibition of ER Ca^{2+} pumps prevents oscillations even in reduced $[Ca^{2+}]_o$. The three cells shown were stimulated with 1 μM TG in the presence of 250,

500, or 750 μM Ca_o^{2+}. These conditions bracket the range of $[Ca^{2+}]_i$ increases typical of oscillating cells (stimulated with 15 nM TG, 2 mM Ca_o^{2+}; see Fig. 1), yet oscillations do not occur.

required to evoke a critical rate of Ca^{2+} entry across the plasma membrane? To distinguish between these two possibilities, we stimulated cells with a saturating amount of TG (1 μM) in the presence of reduced $[Ca^{2+}]_o$. In this way, TG-sensitive stores were maintained in a depleted state whereas Ca^{2+} influx occurred at a rate characteristic of oscillating cells. Fig. 5 C illustrates the typical result of these experiments. In nine experiments, >800 cells treated with 1 μM TG failed to

oscillate when exposed to $[Ca^{2+}]_o$ from 0.1 to 1 mM. Thus, partial depletion of Ca^{2+} stores is required for the generation of $[Ca^{2+}]_i$ oscillations, suggesting that the store content plays a critical role in the underlying mechanism.

Calcium Influx is Required to Sustain $[Ca^{2+}]_i$ Oscillations

Removal of Ca^{2+}_o, treatment with 5 mM Ni^{2+}, or substitution of external K^+ for Na^+ rapidly eliminated TG-induced oscillations in all cells studied (Fig. 6). These three
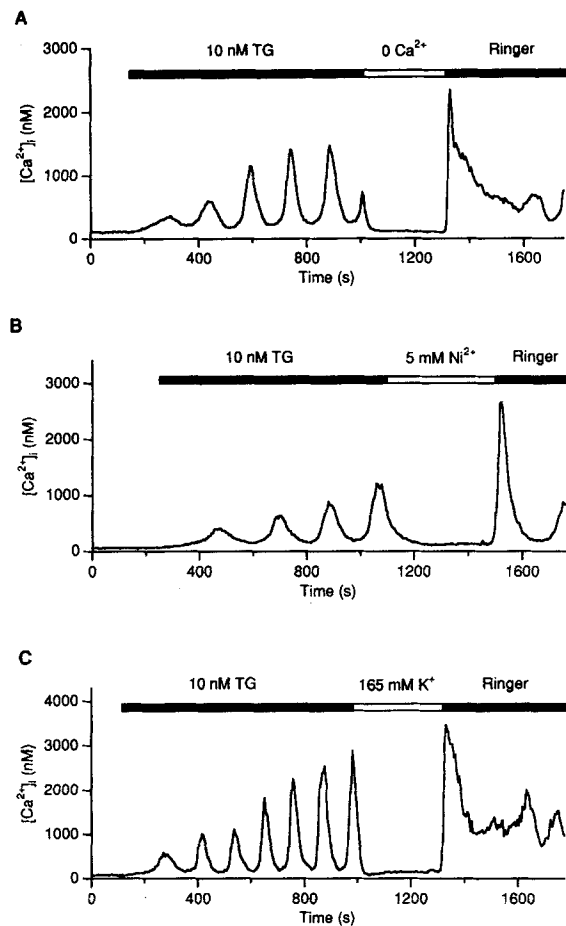

FIGURE 6. $[Ca^{2+}]_i$ oscillations depend critically on Ca^{2+} influx. In all experiments, cells were stimulated with 10 nM TG in Ringer's solution. Responses of three single cells are shown. (*A*) Effect of Ca^{2+}_o removal on TG-induced oscillations. Perfusion with Ca^{2+}-free Ringer's (without TG) rapidly and reversibly eliminates $[Ca^{2+}]_i$ oscillations. Control perfusion with normal Ringer's lacking TG had no effect (data not shown). (*B*) Inhibition of $[Ca^{2+}]_i$ oscillations by 5 mM Ni^{2+}. (*C*) Depolarization of cells with K^+ Ringer's immediately blocks the oscillations.

conditions are known to inhibit Ca^{2+} entry through depletion-activated Ca^{2+} channels (Lewis and Cahalan, 1989; Hoth and Penner, 1992; Zweifach and Lewis, 1993). 5 mM Ni^{2+} blocks I_{CRAC} directly. The effect of depolarization by high $[K^+]_o$ is largely explained by a reduction in the driving force for Ca^{2+} entry, as the gating of I_{CRAC} is not directly voltage dependent. The large $[Ca^{2+}]_i$ overshoot seen upon restoration of Ca^{2+} influx in each experiment is consistent with the feedback inhibition of I_{CRAC} by intracellular Ca^{2+} (Lewis and Cahalan, 1989; Hoth and Penner, 1993). The results of

these experiments indicate an essential role for Ca^{2+} influx in generating oscillations. Analogous results were reported for oscillations evoked by PHA or anti-TCR antibodies (Lewis and Cahalan, 1989; Donnadieu et al., 1992a; Hess et al., 1993). This similarity provides additional evidence that TCR- and depletion-triggered oscillations share a common mechanism.

Ca^{2+}-induced Ca^{2+} Release Does Not Contribute Significantly to $[Ca^{2+}]_i$ Oscillations

The extreme sensitivity of the oscillations to inhibition of Ca^{2+} entry implies that they are not the direct result of repetitive Ca^{2+} release from intracellular stores. However,
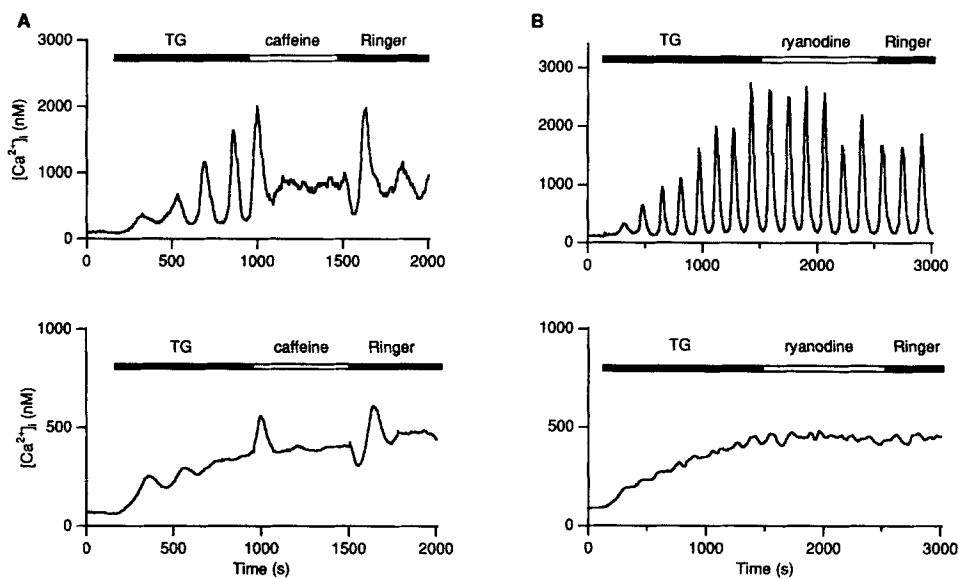

FIGURE 7.   Effects of caffeine and ryanodine on $[Ca^{2+}]_i$ oscillations. The upper graphs represent single cells while the lower graphs show population averages from > 200 cells in each experiment. (A) Effect of caffeine on TG-stimulated T cells. 10 mM caffeine was applied during the period indicated after stimulation with 10 nM TG. Under these conditions, caffeine elicits a transient $[Ca^{2+}]_i$ rise and eliminates the oscillations in single cells. (B) Ryanodine lacks a significant effect on $[Ca^{2+}]_i$ oscillations. Cells stimulated with 15 nM TG were exposed to 100 μM ryanodine during the period indicated.

a contribution of Ca^{2+}-induced Ca^{2+} release (CICR) cannot be excluded on this basis, particularly if CICR is highly dependent on Ca^{2+} influx. This possibility was initially tested using caffeine, which in many cells releases Ca^{2+} from the CICR store by increasing the Ca^{2+} sensitivity of the ryanodine receptor Ca^{2+} channel (Fabiato, 1985; Rousseau, LaDine, Liu, and Meissner, 1988; Wakui, Osipchuk, and Petersen, 1990; Friel and Tsien, 1992). Treatment of resting T cells with 10 mM caffeine did not release intracellular Ca^{2+} from stores (not shown). In cells pretreated with 15 nM TG, caffeine induced a $[Ca^{2+}]_i$ transient that was most apparent in the population average (Fig. 7 A). After this transient rise, oscillations were effectively suppressed in

96% of the cells ($n = 343$). Removal of caffeine triggered a biphasic $[Ca^{2+}]_i$ transient in the average response, apparently due to synchronization of one to two subsequent oscillations in the cell population. Approximately 25% of cells stimulated with 2 nM TG (subthreshold for eliciting oscillations) began to oscillate after caffeine application; these oscillations were small, never exceeding a peak amplitude of 300 nM (data not shown). These effects of caffeine were independent of any inhibitory action on cyclic nucleotide phosphodiesterases, because they could not be mimicked by isobutylmethylxanthine (100 μM), a more specific phosphodiesterase inhibitor.

Despite the actions of caffeine described above, two lines of evidence argue strongly against a significant role for CICR in the oscillation mechanism. First, ryanodine lacks a clear effect. Ryanodine inhibits the function of the ryanodine receptor in a use-dependent manner, either by locking the channel in a subconductance state or by blocking it (Smith, Imagawa, Ma, Fill, Campbell, and Coronado, 1988). When applied to oscillating cells, ryanodine (1–100 μM) had no effect over periods of 300–1,200 s in 89% of 862 cells studied (Fig. 7 B). Furthermore, the absence of CICR was shown directly by measuring changes in $[Ca^{2+}]_i$ following Ca_o^{2+} removal at different phases of the oscillation cycle. As illustrated in Fig. 8 A, at no time during the rising part of an oscillation cycle does $[Ca^{2+}]_i$ continue to increase after Ca_o^{2+} is removed, consistent with the extreme dependence on Ca^{2+} entry shown in Fig. 6. After removal of Ca_o^{2+}, $[Ca^{2+}]_i$ changes at a rate $(d[Ca^{2+}]_i/dt_{-Ca})$ determined by Ca^{2+} fluxes into and out of the cytoplasmic compartment. $d[Ca^{2+}]_i/dt_{-Ca}$ is expected to vary with $[Ca^{2+}]_i$ due to the Ca^{2+}-dependence of Ca^{2+}-ATPases (Donnadieu et al., 1992a). In addition, any finite contribution of CICR to the oscillation rising phase will oppose Ca^{2+} efflux and therefore make $d[Ca^{2+}]_i/dt_{-Ca}$ more positive during the rising phase relative to the declining phase of the cycle. Fig. 8 B shows the rate of Ca^{2+} efflux from the cytosol as a function of $[Ca^{2+}]_i$ for the rising and falling phases of the oscillations. Linear regression fits to both data sets are roughly identical, indicating that the net $[Ca^{2+}]_i$ removal rate varies with $[Ca^{2+}]_i$ but not with phase. These results, together with the ryanodine data, provide strong evidence against a role for CICR in generating $[Ca^{2+}]_i$ oscillations in T cells.

Changes in ER Ca^{2+} Content and Ca^{2+} Influx Are Linked to $[Ca^{2+}]_i$ Oscillations

The results presented above suggest that $[Ca^{2+}]_i$ oscillations in T cells result from fluctuating Ca^{2+} influx coupled to periodic changes in the content of intracellular stores. We tested this hypothesis by estimating the rate of Ca^{2+} influx and the content of Ca^{2+} stores during TG-stimulated oscillations. The rate of Ca^{2+} entry was estimated by rapidly removing Ca_o^{2+} with 1 mM EGTA (Fig. 9 A). The rate of $[Ca^{2+}]_i$ change immediately before EGTA addition $(d[Ca^{2+}]_i/dt_{+Ca})$ includes contributions from Ca^{2+} influx and pumping across the plasma membrane as well as release and reuptake by the stores. By selectively eliminating the influx component, EGTA produces a change in slope $(\Delta d[Ca^{2+}]_i/dt_{-Ca} = d[Ca^{2+}]_i/dt_{-Ca} - d[Ca^{2+}]_i/dt_{+Ca})$ that reflects the ongoing rate of Ca^{2+} entry. Because oscillations are asynchronous among cells in the population, Ca_o^{2+} removal occurs at a different phase in the cycle for each cell. Fig. 9 B shows Ca^{2+} influx measured from $\Delta d[Ca^{2+}]_i/dt_{-Ca}$ in 195 cells, superimposed on an averaged oscillation from the same population of cells and

normalized for phase (see Materials and Methods). Ca^{2+} influx increases during the rising part of the oscillation, peaking at the time when $[Ca^{2+}]_i$ is increasing most rapidly, and subsequently declines. This behavior further supports the conclusion that the oscillations are driven primarily by Ca^{2+} entry across the plasma membrane.
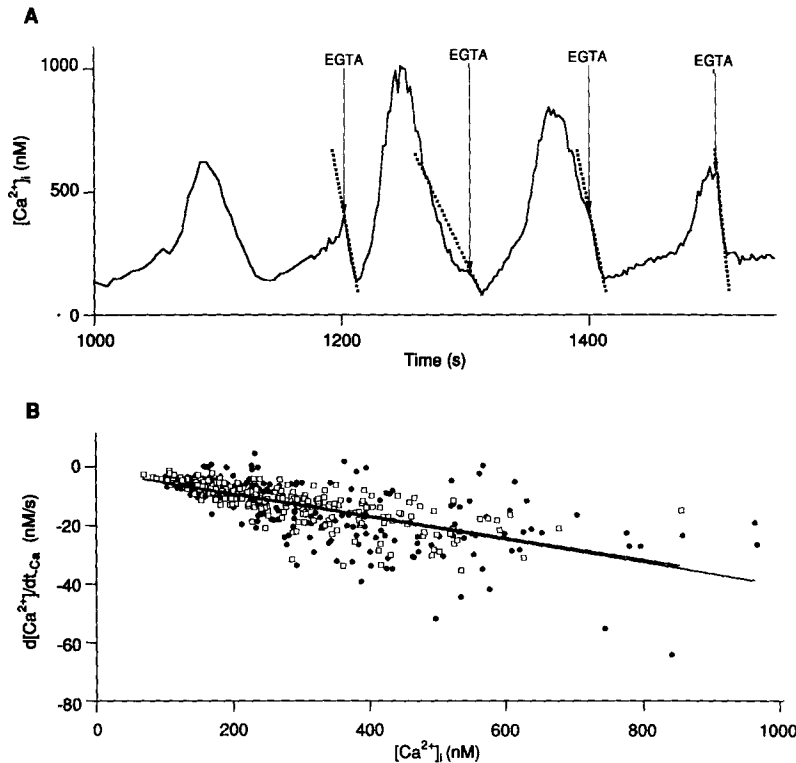
A

B

FIGURE 8.   CICR does not contribute significantly to $[Ca^{2+}]_i$ oscillations. (*A*) Ca^{2+}-free Ringer's solution + 1 mM EGTA was applied for 10 s to oscillating cells pretreated with 10 nM TG. $[Ca^{2+}]_i$ declines immediately upon removal of Ca_0^{2+} irrespective of the phase of the oscillation. Dashed lines illustrate the rate of $[Ca^{2+}]_i$ decrease immediately after EGTA addition. (*B*) The initial rate of $[Ca^{2+}]_i$ decrease in EGTA ($d[Ca^{2+}]_i/dt_{-Ca}$) is plotted against the immediately preceding $[Ca^{2+}]_i$ for cells in the rising phase (●) or falling phase (□) of the oscillation cycle. Data were obtained from the experiment shown in *A*. Linear regression fits of the form $d[Ca^{2+}]_i/dt_{-Ca} = m [Ca^{2+}]_i + b$ are superimposed on the two sets of data. For the rising phase, $m = -0.0376$ s⁻¹, $b = -1.66$ nM s⁻¹; for the falling phase, $m = -0.0384$ s⁻¹, $b = -1.86$ nM s⁻¹. $d[Ca^{2+}]_i/dt_{-Ca}$ has the same linear relationship to $[Ca^{2+}]_i$ during the rising and falling phases, indicating a lack of CICR during $[Ca^{2+}]_i$ oscillations.

Changes in the content of the Ca^{2+} stores were estimated from the rate of ionomycin-induced intracellular Ca^{2+} release. 10 s after treatment with EGTA, 2 μM ionomycin was added (in the continued presence of EGTA) as illustrated in Fig. 9 *A*. Ionomycin liberates Ca^{2+} from intracellular stores at a rate dependent on the

lumenal Ca^{2+} concentration, and this Ca^{2+} release is superimposed on the ongoing decline in $[Ca^{2+}]_i$ due to Ca^{2+}-ATPase activity in the plasma and ER membranes. Thus, the stores content can be estimated by measuring the change in $d[Ca^{2+}]_i/dt$ caused by ionomycin ($\Delta d[Ca^{2+}]_i/dt_{iono} = d[Ca^{2+}]_i/dt_{iono} - d[Ca^{2+}]_i/dt_{-Ca}$). Fig. 9 C shows the content of ionomycin-releasable stores as a function of the oscillation phase
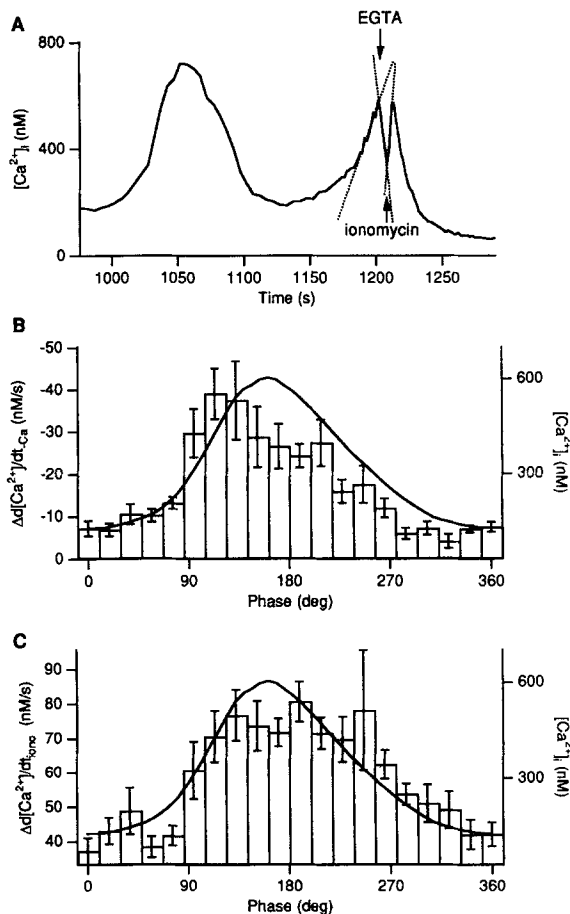

FIGURE 9. The content of Ca^{2+} stores and magnitude of Ca^{2+} influx fluctuate during $[Ca^{2+}]_i$ oscillations. (A) Measurement of Ca^{2+} influx and the content of Ca^{2+} stores. 1 mM EGTA was applied at the first arrow to oscillating cells pretreated with 10 nM TG, and the change in slope ($\Delta d[Ca^{2+}]_i/dt_{-Ca}$) was measured to estimate the ongoing rate of Ca^{2+} influx. After 10 s, 2 µM ionomycin + EGTA was added (*second arrow*), and the resulting change in slope ($\Delta d[Ca^{2+}]_i/dt_{iono}$) was determined as an estimate of the relative store content. (B) Ca^{2+} influx during the oscillation cycle. $\Delta d[Ca^{2+}]_i/dt_{-Ca}$ was measured for 195 cells in three experiments. Bars indicate mean values ± SEM. The averaged $[Ca^{2+}]_i$ oscillation, normalized for phase, is shown superimposed on the results using the vertical axis on the right. Ca^{2+} influx is maximal during the rising phase of the oscillation. (C) Content of Ca^{2+} stores during the oscillation cycle. Store content, as indicated by $\Delta d[Ca^{2+}]_i/dt_{iono}$, varies with $[Ca^{2+}]_i$. Data were obtained from the same experiment shown in B.

for the same cells as in Fig. 9 B. The average store content appears to fluctuate with roughly the same time course as $[Ca^{2+}]_i$, although a phase difference between the two signals cannot be excluded given the amount of cell-to-cell variation in the population. Together, the results of Fig. 9, B and C provide direct evidence for an oscillation

mechanism driven by signaling between Ca^{2+} stores and depletion-activated Ca^{2+} channels in the plasma membrane.

DISCUSSION

[Ca²⁺]ᵢ Oscillations in T Cells Are Not Explained by Existing Models

As discussed below, several characteristics of $[Ca^{2+}]_i$ oscillations in T cells are incompatible with mechanisms that have been proposed for other non-excitable cells. First, the oscillations can be generated independently of IP_3 by agents that partially deplete intracellular stores. Second, they are not produced or augmented by CICR. Finally, the oscillations are generated almost entirely by Ca^{2+} influx across the plasma membrane.

T cell oscillations do not require IP₃. Intracellular IP_3 is an essential signaling element of many oscillation models. Ca^{2+} can exert positive and negative feedback by modulating the production of IP_3 (Meyer and Stryer, 1988; Cuthbertson and Chay, 1991; Harootunian, Kao, Paranjape, and Tsien, 1991) or IP_3's ability to release stored Ca^{2+} (Parker and Ivorra, 1990; Bezprozvanny, Watras and Ehrlich, 1991). TG fails to generate significant amounts of IP_3 in a variety of cells, including T cells (Takemura et al., 1989; Gouy et al., 1990; Putney, 1990; Foskett et al., 1991; Mason et al., 1991a). Thus, the ability of TG to evoke $[Ca^{2+}]_i$ oscillations in T cells demonstrates that IP_3-requiring models do not apply in this case. However, TCR stimulation is likely to trigger oscillations in T cells under physiological conditions through the ability of IP_3 to liberate Ca^{2+} from and thereby deplete intracellular stores (discussed below).

Partial depletion of Ca²⁺ stores is essential for triggering [Ca²⁺]ᵢ oscillations. The agents used in this study effectively induced oscillations only within narrow concentration ranges that caused partial inhibition of Ca^{2+} pumps (Fig. 5 A) or a submaximal rate of Ca^{2+} release (Fig. 5 B). Functional Ca^{2+}-ATPase activity in the ER was shown to be required for oscillations to occur; cells did not oscillate if the pumps were completely inhibited (1 μM TG), even when $[Ca^{2+}]_o$ was reduced to equalize the rate of influx to that of oscillating cells. The requirement for pump activity is consistent with the observed fluctuations in store content (Fig. 9 C) and implies that such fluctuations play an essential role in the oscillation mechanism.

Thapsigargin-triggered $[Ca^{2+}]_i$ oscillations have also been described in parotid cells (Foskett et al., 1991; Foskett and Wong, 1991; Foskett and Wong, 1992). The oscillations are similar to those of T cells with respect to their lack of IP_3 involvement, dependence on external Ca^{2+}, sensitivity to caffeine, and frequency. However, parotid-cell oscillations are sensitive to ryanodine and are triggered only by micromolar concentrations of TG that fully inhibit the ER Ca^{2+}-ATPases. Given the evidence that the IP_3-sensitive store is empty under these conditions, Foskett and colleagues have concluded that $[Ca^{2+}]_i$ oscillations in parotid cells are due to CICR from a TG-insensitive store, with the IP_3-sensitive store serving to activate a steady Ca^{2+} influx needed to refill the CICR store (Foskett and Wong, 1991). Thus, the underlying mechanism is distinct from that of the T cell but may share some elements such as capacitative Ca^{2+} entry. It is not known whether Ca^{2+} entry oscillates in parotid cells.

CICR does not cause T cell $[Ca^{2+}]_i$ oscillations. CICR has been studied in a variety of excitable and nonexcitable cells and is capable of generating $[Ca^{2+}]_i$ oscillations (Berridge and Galione, 1988; Wakui et al., 1990; Friel and Tsien, 1992). Two types of evidence argue against a role for CICR in the T cell oscillation mechanism. First, Ca_0^{2+} removal during the oscillations caused an immediate decline in $[Ca^{2+}]_i$ (Fig. 8 *A*), in contrast to the continued rise observed in cells in which CICR is the major contributor to the upstroke of each oscillation (Friel and Tsien, 1992). Even a small role for CICR in T cells is unlikely, as $[Ca^{2+}]_i$ declined at the same rate regardless of whether Ca_0^{2+} was removed during the rising or falling phase of the oscillation (Fig. 8 *B*). Second, ryanodine failed to have a significant effect in T cells (Fig. 7 *B*); ryanodine would be expected to inhibit CICR-dependent oscillations through its ability to block the CICR channel or deplete the CICR store (Malgaroli et al., 1990; Friel and Tsien, 1992).

In view of these data, it is surprising that caffeine causes a transient elevation of $[Ca^{2+}]_i$ in oscillating T cells followed by reversible inhibition of the oscillations (Fig. 7 *A*). It is not clear whether these effects are due to the opening of intracellular ryanodine receptors (perhaps having a low intrinsic Ca^{2+} sensitivity) or instead result from nonspecific actions of caffeine. The time course of the caffeine-induced spike is slow (rise time ≈ 50 s; Fig. 7 *A*) relative to that generally seen in other cells in which the Ca^{2+} transient reaches a peak within 1–10 s (Fabiato, 1985; Wakui et al., 1990; Friel and Tsien, 1992). In addition, caffeine inhibits I_{CRAC} ($\sim 25\%$ inhibition by 10 mM caffeine; A. Zweifach, personal communication) and IP_3 receptors (Parker and Ivorra, 1991). Thus, the effects of caffeine on $[Ca^{2+}]_i$ and oscillations cannot be taken as conclusive evidence for a functional CICR mechanism in T cells.

T cell oscillations depend absolutely on Ca^{2+} entry. In all previous models that have been proposed, $[Ca^{2+}]_i$ oscillations in nonexcitable cells result principally from periodic Ca^{2+} release and reuptake by intracellular stores. According to these models, Ca^{2+} influx from the external environment is needed only to trigger CICR or to offset the Ca^{2+} stores deficit that arises from competition between ER and plasma membrane Ca^{2+}-ATPases for cytosolic Ca^{2+}. In cells such as hepatocytes the contribution from influx is relatively small, as oscillations persist for many cycles following removal of extracellular Ca^{2+} (Woods et al., 1986; Woods, Cuthbertson, and Cobbold, 1987). The stores deficit and hence Ca^{2+} influx is more significant in RBL cells (Millard, Ryan, Webb, and Fewtrell, 1989) and endothelial cells (Jacob et al., 1988), in which only a few oscillations occur in the absence of Ca_0^{2+}. In contrast, $[Ca^{2+}]_i$ oscillations in T cells depend absolutely on Ca^{2+} entry. Conditions that reduce or eliminate influx through depletion-activated Ca^{2+} channels, such as Ca^{2+}-free Ringer's, 165 mM K^+, or 5 mM Ni^{2+}, immediately terminate ongoing oscillations (Fig. 6).

Cyclical Variation of Ca^{2+} Influx and the Content of Ca^{2+} Stores

The evidence presented above suggests a model in which $[Ca^{2+}]_i$ oscillations are generated by the interaction between intracellular Ca^{2+} stores and I_{CRAC} (described below). Two fundamental predictions of such a model are that the content of intracellular stores and the magnitude of Ca^{2+} influx should oscillate. EGTA and ionomycin were applied to assess these two parameters at random times during the

oscillation cycle (Fig. 9). One advantage of this approach is that it requires no assumptions regarding pharmacological specificity; the primary requirement is that the solution exchange and $[Ca^{2+}]_i$ measurements be done rapidly such that changes in $d[Ca^{2+}]_i/dt$ can be measured before $[Ca^{2+}]_i$ changes significantly (Friel and Tsien, 1992). One limitation is that $d[Ca^{2+}]_i/dt$ is influenced by the buffering capacity of fura-2, which is a function of $[Ca^{2+}]_i$ (Neher and Augustine, 1992). In addition, these cell population measurements are also influenced by cell-to-cell variability in store content and Ca^{2+} channel and pump expression. The limits of resolution imposed by such cell-to-cell variability prevent accurate measurement of the phase difference between cytosolic and ER $[Ca^{2+}]$. However, Ca^{2+} influx clearly reaches a peak before $[Ca^{2+}]_i$. This result agrees with a previous patch-clamp study in which I_{CRAC} was shown to oscillate in advance of $[Ca^{2+}]_i$ in PHA-stimulated Jurkat T cells (Lewis and Cahalan, 1989). Fluctuations in Ca^{2+} influx (inferred from Mn^{2+} entry) are also associated with $[Ca^{2+}]_i$ oscillations in carbachol-stimulated AR42J pancreatic acinar cells (Loessberg, Zhao, and Muallem, 1991). However, oscillations in these cells can occur in the absence of Ca_o^{2+}, indicating that Ca^{2+} influx plays a minor role compared to intracellular Ca^{2+} release.

TCR Stimulation and Direct Depletion of Stores Elicit Oscillations by a Similar Mechanism

$[Ca^{2+}]_i$ oscillations triggered through the TCR (Lewis and Cahalan, 1989; Donnadieu et al., 1992a) and through receptor-independent means of stores depletion share several features. First, both are sinusoidal-type oscillations, in that significant periods of quiescence do not occur between the $[Ca^{2+}]_i$ peaks (Fewtrell, 1993). Second, both types of oscillations vary substantially in size among cells and over time in single cells. Thus, they are not stereotyped, all-or-none events like the $[Ca^{2+}]_i$ oscillations in other cells such as hepatocytes (Woods et al., 1987). Third, both types of oscillations are eliminated by perfusion with Ca^{2+}-free media, high $[K^+]$, or Ni^{2+}, conditions that hinder Ca^{2+} influx via I_{CRAC} (Lewis and Cahalan, 1989). This result implies a common dependence on Ca^{2+} influx through depletion-activated Ca^{2+} channels. Finally, both oscillation types are linked to periodic fluctuations of Ca^{2+} influx that occur in advance of the $[Ca^{2+}]_i$ oscillations themselves (Lewis and Cahalan, 1989).

Interestingly, TCR-generated oscillations are more rapid than those triggered by inhibition of ER-Ca^{2+}-ATPases. Oscillations evoked by PHA or anti-TCR antibodies have a frequency of 8–12 mHz (Lewis and Cahalan, 1989; Donnadieu et al., 1992a; Hess et al., 1993), similar to that of ionomycin-induced oscillations, but roughly twice the frequency of those produced by TG, CPA, or tBHQ. These results suggest that oscillation frequency is determined in part by the rate at which Ca^{2+} redistributes across the ER membrane after a perturbation. Ca^{2+} redistributes slowly in the presence of Ca^{2+} pump inhibitors due to impaired Ca^{2+} uptake and slow Ca^{2+} release through leak channels; redistribution is enhanced by ionomycin or TCR ligands because of a higher (i.e., normal) Ca^{2+}-ATPase activity and an increased rate of Ca^{2+} efflux from the ER. This dependence of oscillation frequency on the mechanism of store depletion further strengthens the conclusion that TCR ligation elicits $[Ca^{2+}]_i$ oscillations through the IP_3-dependent depletion of internal stores.

A Depletion-regulated Ca^{2+} Influx Model for $[Ca^{2+}]_i$ Oscillations in T Cells

The results of this study support the depletion-regulated oscillation model summarized in Fig. 10. In this model, $[Ca^{2+}]_i$ oscillations arise primarily from pulsatile Ca^{2+} influx regulated by the content of intracellular Ca^{2+} stores. An important feature of the model is that the stores contribute little to changing $[Ca^{2+}]_i$ by themselves; instead, their main function is to control the amplitude and timing of Ca^{2+} entry. In a resting cell, the stores are full and $[Ca^{2+}]_i$ and Ca^{2+} influx are low. Addition of TG inhibits the ER pumps and unmasks a Ca^{2+} leak from the ER. The ensuing depletion activates I_{CRAC}, elevating $[Ca^{2+}]_i$ and thereby promoting store refilling. As the stores reload, I_{CRAC} is deactivated, allowing plasma-membrane Ca^{2+} pumps to reduce $[Ca^{2+}]_i$. The fall in $[Ca^{2+}]_i$ accelerates the leak of Ca^{2+} from the ER, causing
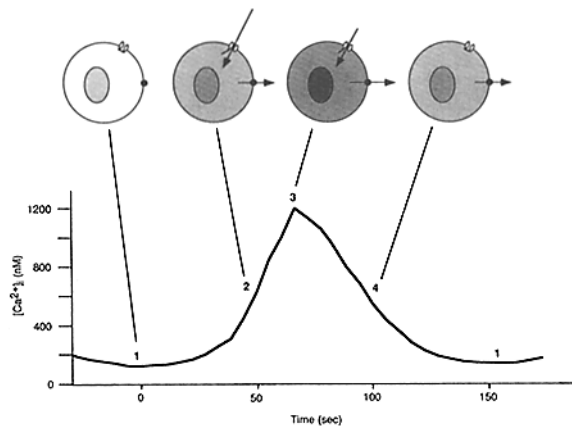

FIGURE 10. A mechanism for $[Ca^{2+}]_i$ oscillations induced by depletion of internal Ca^{2+} stores. The diagram shows qualitatively the predicted changes in cytosolic and store $[Ca^{2+}]$ (*shading*) and Ca^{2+} fluxes (*arrows*). Fluxes across the ER membrane are not shown explicitly because of uncertainty regarding the phase of store content with respect to cytosolic $[Ca^{2+}]$. (*1*) The stores are relatively depleted and Ca^{2+} channels are mostly closed during the period between oscillation peaks (Fig. 9, *B* and *C*). (*2*) The depleted stores cause Ca^{2+} channels in the plasma membrane to open after a delay, raising $[Ca^{2+}]_i$. As $[Ca^{2+}]_i$ increases the stores reload, initiating the deactivation of Ca^{2+} channels. (*3*) At the peak of the oscillation, the increased store content has reduced Ca^{2+} influx to a level that is balanced by pumping across the plasma membrane. (*4*) As the Ca^{2+} channels continue to close, $[Ca^{2+}]_i$ falls. The decline in $[Ca^{2+}]_i$ accelerates the leak of Ca^{2+} from the ER, causing renewed depletion and starting the next cycle.

depletion, reactivation of I_{CRAC}, and initiation of the next cycle. Agents that increase the Ca^{2+} permeability of the ER membrane (IP_3, ionomycin) will elicit a similar sequence of events but at a faster rate.

Negative feedback in the model is provided solely by reloading of the Ca^{2+} stores. Ca^{2+} may also inhibit I_{CRAC} channels directly (Hoth and Penner, 1992, 1993; Zweifach and Lewis, 1994), but this process is essentially complete within 100 ms and is therefore unlikely to be involved in generating oscillations lasting ~ 100 s. Direct feedback on Ca^{2+} channels has been proposed to explain TG-induced oscillations in a T-cell line (Donnadieu et al., 1992a). Such a model predicts that oscillations would depend only on the rate of Ca^{2+} influx, and that their frequency would be independent of the mechanism by which stores become depleted. Neither of these predictions holds; cells treated with high [TG] and reduced $[Ca^{2+}]_o$ do not oscillate

(Fig. 5 C), and oscillation frequency is higher with ionomycin than with Ca^{2+}-ATPase inhibitors (Fig. 4 B).

A critical feature of our model is that feedback occurs with an intrinsic delay, so that Ca^{2+} influx across the plasma membrane and Ca^{2+} release from internal stores oscillate out of phase with each other. Without such delays, release of Ca^{2+} from the stores would simply elevate Ca^{2+} entry until a new steady-state is reached between influx and [Ca^{2+}] in the cytoplasm and ER. In principle, delays could arise from a number of sources: the rate of store refilling, the rate of store leak, and the lag between ER depletion and activation of Ca^{2+} entry. Uncertainty in the estimates of store content and oscillation phase prevent us from determining the phase relationship between ER and cytosolic [Ca^{2+}]. However, significant delays may exist between the depletion of stores and activation of Ca^{2+} influx, and between subsequent repletion of stores and cessation of influx (Figs. 9, B and C). This observation may explain why Ca^{2+} influx is minimal when the stores appear most empty, a result that seems to contradict the notion that Ca^{2+} influx is activated by the depletion of the stores. In mast cells and T cells, the time constant for activation of I$_{CRAC}$ by intracellular IP$_3$ is ~20–30 s (Hoth and Penner, 1993; McDonald, Premack, and Gardner, 1993) and a similar rate of induction is seen following PHA stimulation (Zweifach and Lewis, 1993). Delayed activation of Ca^{2+} entry allows the stores to lose more Ca^{2+} before influx begins, thus enhancing the production or release of the depletion-triggered messenger.

The model presented here is the simplest one that can qualitatively account for the generation of [Ca^{2+}]$_i$ oscillations in T cells by partial depletion of Ca^{2+} stores. However, the model is certainly not a complete description, and a number of questions remain. How is the time course of I$_{CRAC}$ activation and deactivation determined by changes in Ca^{2+} store content? Recent evidence suggests that a diffusible factor is released from depleted stores to activate I$_{CRAC}$ (Randriamampita and Tsien, 1993), but its regulation in terms of synthesis, degradation, release, and reuptake are not well understood. Do additional sites of positive and negative feedback exist? Feedback by Ca^{2+} could occur at the level of the I$_{CRAC}$ channel, its activator, or through Ca^{2+}-activated K$^+$ channels, which affect Ca^{2+} entry by influencing the driving force. For example, membrane-potential oscillations like those observed in PHA-stimulated Jurkat T cells (Lewis, Grissmer, and Cahalan, 1991) may provide positive and negative feedback during the rising and falling phases of [Ca^{2+}]$_i$ oscillations. The T lymphocyte is the first example of a cytosolic [Ca^{2+}]$_i$ oscillator that arises from signalling between intracellular Ca^{2+} stores and depletion-activated Ca^{2+} channels in the plasma membrane. These results raise the possibility that a similar oscillation mechanism may operate in many of the other cells that possess depletion-regulated Ca^{2+} entry pathways.

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