Journal of Cellular Biochemistry 58:344–359 (1995)

# T Cell Receptor-Mediated $Ca^{2+}$ Signaling: Release and Influx Are Independent Events Linked to Different $Ca^{2+}$ Entry Pathways in the Plasma Membrane

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**Abstract** In this study, we showed that cross-linking CD3 molecules on the T cell surface resulted in $Ca^{2+}$ release from the intracellular stores followed by a sustained $Ca^{2+}$ influx. Inhibition of release with TMB-8 did not block the influx. However, inhibition of phospholipase C activity suppressed both $Ca^{2+}$ release and influx. Once activated, the influx pathway remained open in the absence of further hydrolysis of PIP2. Thapsigargin, a microsomal $Ca^{2+}$-ATPase inhibitor, stimulated $Ca^{2+}$ entry into the cells by a mechanism other than emptying $Ca^{2+}$ stores. In addition, $Ca^{2+}$ entry into the $Ca^{2+}$-depleted cells was stimulated by low basal level of cytosolic $Ca^{2+}$, not by the emptying of intracellular $Ca^{2+}$ stores. Both the $Ca^{2+}$ release and influx were dependent on high and low concentrations of extracellular $Ca^{2+}$. At low concentrations, $Mn^{2+}$ entered the cell through the $Ca^{2+}$ influx pathway and quenched the sustained phase of fluorescence; whereas, at higher $Mn^{2+}$ concentration both the transient and the sustained phases of fluorescence were quenched. Moreover, $Ca^{2+}$ release was inhibited by low concentrations of $Ni^{2+}$, $La^{3+}$, and EGTA, while $Ca^{2+}$ influx was inhibited by high concentrations. Thus, in T cells $Ca^{2+}$ influx occurs independently of IP3-dependent $Ca^{2+}$ release. However, some other PIP2 hydrolysis-dependent event was involved in prolonged activation of $Ca^{2+}$ influx. Extracellular $Ca^{2+}$ influenced $Ca^{2+}$ release and influx through the action of two plasma membrane $Ca^{2+}$ entry pathways with different pharmacological and biochemical properties. 

**Key words:** $Ca^{2+}$ signaling, release, refilling, thapsigargin, T cells, $Ca^{2+}$ influx

An increase in free intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]i$) is essential for many cellular functions, such as secretion, contraction, proliferation, and differentiation. In non-excitable cells, an increase of $[Ca^{2+}]i$ results from the activation of phosphoinositide signaling pathway by hormones, growth factors, and immune mediators. Occupancy of the receptors by agonists activates a membrane-bound phosphoinositide-specific phospholipase C (PLC), which in turn hydrolyzes phosphatidylinositol bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [for review see Berridge, 1987]. IP3 stimulates transient release of $Ca^{2+}$ from IP3-sensitive $Ca^{2+}$ stores, by binding to a tetrameric receptor which itself is a $Ca^{2+}$ channel [for review see Berridge, 1993]. Release of $Ca^{2+}$ from the IP3-sensitive stores is followed by a sustained influx of $Ca^{2+}$ into the cytoplasm from the extracellular medium. These results in a biphasic increase of $[Ca^{2+}]i$, a transient phase followed by a sustained phase, in a population of cells. However, with a single cell, the $Ca^{2+}$ signal appears as oscillating spikes, and $Ca^{2+}$ influx becomes necessary to sustain this $Ca^{2+}$ oscillation [Berridge, 1993]. Recently, it has also been demonstrated that IP3 stimulates oscillatory release of $Ca^{2+}$ from the intracellular stores [Ferris et al., 1992; Oldershaw et al., 1991; Parker and Ivora, 1990]. The sustained phase of increased $[Ca^{2+}]i$ in the cell population is believed to result from the asynchronous sustained $[Ca^{2+}]i$ oscillation by single cells [Lewis and Cahalan, 1989].

Although the mechanisms of $Ca^{2+}$ mobilization by IP3 is relatively well understood, controversy remains regarding the mechanism of $Ca^{2+}$ influx into the cytosol from the extracellular medium. One hypothesis suggests that IP3 or one of its metabolites, such as IP4, directly opens a $Ca^{2+}$ channel in the membrane [for

Received October 6, 1994; accepted November 10, 1994.

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review see Irvine, 1992]. That was based on the findings that: (1) intracellular administration of a metabolically stable IP3 analogue or IP4 activated $Ca^{2+}$ influx [Guse et al., 1992; Hansen et al., 1991; Striggow and Bohnensack, 1994]; (2) application of IP3 or IP4 to the inner surface of plasma membrane activated a $Ca^{2+}$ current [Kuno and Gardner, 1987; Luckhoff and Clapham, 1992]; (3) IP3 and IP4 have binding sites in the plasma membrane of several cell types [Irvine, 1992; Kalinoski et al., 1992; Khan et al., 1992a,b]; and (4) IP3 synergizes with IP4 in the stimulation of $Ca^{2+}$ influx [DeLisle et al., 1992; Irvine, 1992]. Another hypothesis, capacitative $Ca^{2+}$ entry, proposed that emptying intracellular $Ca^{2+}$ stores by IP3 triggers $Ca^{2+}$ influx into the cytosol [Putney, 1990]. To test this hypothesis, three principal experimental approaches were taken to empty the intracellular $Ca^{2+}$ stores [Irvine, 1992]: (1) stimulation of cells in $Ca^{2+}$-free medium with agonists which generate IP3; (2) inhibition of the $Ca^{2+}$-ATPase of intracellular $Ca^{2+}$ stores by specific inhibitors, such as thapsigargin (Tg); and (3) prolonged incubation of cells in $Ca^{2+}$-free medium. Addition of $Ca^{2+}$ to the cells following one of those treatments, resulted in $Ca^{2+}$ influx. These results were interpreted to suggest that emptying of the $Ca^{2+}$ stores caused $Ca^{2+}$ influx. However, it is not clear how the empty $Ca^{2+}$ stores stimulate $Ca^{2+}$ influx. Recently, it has been shown that those three methods of emptying the $Ca^{2+}$ stores generated a phosphate-containing diffusable messenger, which stimulated $Ca^{2+}$ influx [Parekh et al., 1993; Randriamampita and Tsien, 1993]. It has also been shown that thapsigargin-induced $Ca^{2+}$ entry was dependent on GTP hydrolysis [Bird and Putney, 1993], which indicates a role for GTP in capacitative $Ca^{2+}$ entry. In contrast, it was shown that inhibition of IP3-mediated $Ca^{2+}$ release by heparin did not affect $Ca^{2+}$ influx, whereas an anti-PIP2 antibody inhibited both the release and influx [Huang et al., 1991]. These findings contradicted the capacitative hypothesis. Furthermore, based on several studies, it appeared that both potential mechanisms of capacitative and second messenger-dependent $Ca^{2+}$ entry may function in the same cell type, such as in rat hepatocytes or human T cells [Hansen et al., 1991; Kuno and Gardner, 1987; Llopis et al., 1992; Zweifach and Lewis, 1993]. Thus, the mechanism regulating $Ca^{2+}$ entry into different cell types remains an enigma.

The objective of this study was to assess the regulatory mechanisms of $Ca^{2+}$ release from IP3-sensitive stores and $Ca^{2+}$ entry into the cell. For that, T cells, which have clonally specific antigen receptor (TCR) in close physical association with an invariant protein complex CD3 [Abraham et al., 1992], were used. Several monoclonal antibodies to the TCR or CD3 mimic an antigen by activating T lymphocytes. Binding of an antigen or one of those monoclonal antibodies to the TCR-CD3 complex initiates the hydrolysis of PIP2 and the subsequent biphasic rise of $[Ca^{2+}]i$ [Premack and Gardner, 1992]. In this study, we have shown that $Ca^{2+}$ influx into murine T cells was not controlled by IP3-mediated $Ca^{2+}$ release from intracellular stores, but by some other consequence of PIP2 hydrolysis. The $Ca^{2+}$ influx pathway remained active for an extended period of time in the absence of further PIP2 hydrolysis. Our results also indicated that $Ca^{2+}$ release and influx were linked to two different $Ca^{2+}$ entry pathways in the plasma membrane. We have provided experimental evidence to show that Tg and the cellular depletion of intracellular $Ca^{2+}$ activated a $Ca^{2+}$ influx pathway by mechanisms other than the emptying of intracellular $Ca^{2+}$ stores.

## MATERIALS AND METHODS

### Reagents

Fluorescein isothiocyanate (FITC) conjugated and unconjugated monoclonal antibody to the epsilon subunit of mouse CD3 complex, anti-CD3 antibody (αCD3), were purchased from Boehringer Mannheim (Indianapolis, IN). Acetomethyl ester of Fura-2 (Fura-2, AM) was obtained from Molecular Probes (Eugene, OR). Anti-hamster IgG antibody (αIgG) was from Cappel (Durham, NC). Hank's balanced salt solution (HBSS) and Eagle's minimum essential medium (EMEM) were purchased from JRH Sciences (Lenexa, KS). U73122 (1-(6-((17β-3-methoxyestra-1,3,5 (10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and U74343 (1-(6-((17β-3-methoxyestra-1,3,5 (10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione) were obtained from BIOMOL (Plymouth Meeting, PA). TMB-8 (8-(diethylamino)octyl-3,4,5-trimethoxy benzoate-hydrochloride) was purchased from Calbiochem (San Diego, CA). All other chemicals were from Sigma (St. Louis, MO).

### Purification of T Cells

Splenic T cells were obtained from C57BL/6N mice (Charles River, Raleigh, NC) that were housed in specific pathogen free conditions. Single cell suspensions were prepared in EMEM; the red blood cells (RBC) were lysed with tris-ammonium chloride hypotonic solution and the RBC-free spleen cells were resuspended in EMEM containing 10% calf serum (EMEM-CS). To 100 mm tissue culture plates, $5 \times 10^7$ cells were added and incubated for 1 h at 37°C in a humidified incubator. The nonadherent cells were removed, resuspended in EMEM-CS, and about $1 \times 10^8$ cells were loaded on a nylon wool column, which had been prewarmed and equilibrated with EMEM-CS. After 1 h incubation at 37°C, nonadherent cells were gently eluted from the column with EMEM-CS. This T cell preparation was 85–90% pure, as determined by flow cytometric analysis of the cells staining with αCD3-FITC.

### Cell Stimulation and Measurement of $[Ca^{2+}]i$ and $Mn^{2+}$ Entry

T cells at $5 \times 10^6$ cells · $ml^{-1}$ were incubated on a shaker with 5 μM Fura-2, AM in HEPES buffered HBSS for 45 min at 25°C. Cells were then washed and resuspended at $2 \times 10^6$ cells $ml^{-1}$ in HBSS-HEPES. The Fura-2 loaded cells were stimulated by cross-linking the surface CD3 molecules with a combination of αCD3 and αIgG antibodies. Cells were first incubated with αCD3 antibody for 60 sec and then measurement of Fura 2 fluorescence initiated. After 20 sec, 10 μg $ml^{-1}$ αIgG was added to the cells. All the incubations with the antibodies were done at 37°C with constant stirring. Fluorescence was measured with a spectrophotometer (Hitachi, F2000) at 340 nm (calcium-bound Fura-2) and 380 nm (free Fura 2) for excitation and 510 nm for emission. The $[Ca^{2+}]i$ was calculated from the ratio (R) of fluorescence from 340 and 380 nm excitation wave lengths, using the equation $Kd \times (R - Rmin/Rmax - R) \times (Fmin\lambda 2/Fmax\lambda 2)$, where Kd = 285 nM for the apparent dissociation constant for $Ca^{2+}$ and Fura-2 at physiological conditions [Groden et al., 1991]. Fmax and Fmin, the fluorescence at the saturating and zero concentration of external $Ca^{2+}$ respectively, were obtained by the sequential addition of 0.02% digitonin and 10 mM EGTA (pH 8.5). $Mn^{2+}$ entry was measured by monitoring the quenching of intracellular Fura-2 fluorescence by extracellular $Mn^{2+}$ at both the excitation wave lengths.

### Quantitation of Total Intracellular $Ca^{2+}$ Content

Fura-2 loaded cells were suspended in $Ca^{2+}$-free HBSS and fluorescence measurement initiated. Digitonin was added after 50 and 100 sec to ensure that complete release of Fura-2 and cellular $Ca^{2+}$ occurred. After 200 sec, 1.3 mM $Ca^{2+}$ was added to obtain the Fmax value as well as to assure that Fura-2, but not the $Ca^{2+}$, was at a saturating concentration in the solution after digitonin lysis of the cells. The Fmin value was obtained by adding EGTA after the $Ca^{2+}$ addition. The $Ca^{2+}$ concentration in the solution was calculated with the same equation used to calculate $[Ca^{2+}]i$. The calculated $Ca^{2+}$ concentration in the solution was converted to the absolute amount of $Ca^{2+}$ in the cells.

## RESULTS

### Cross-Linking of CD3 Molecules Induced a Biphasic Rise of $[Ca^{2+}]i$ in T Lymphocytes

The $Ca^{2+}$ signaling process in murine T lymphocytes was first studied to establish a baseline for this model system. The αCD3 antibody was used as a stimulator. It is well documented that binding of the antibody to the CD3 molecules alone is not enough, but cross-linking of the whole complex is essential to deliver a full activation signal to the T cells [Abraham et al., 1992; Chakrabarti and Engleman, 1991]. Thus, the T cells were stimulated with a combination of αCD3 and αIgG, as described. The saturating concentration of αIgG (10 μg · $ml^{-1}$) used alone did not cause any change in $[Ca^{2+}]i$. The highest concentration of αCD3 (1 μg · $ml^{-1}$) alone induced a very little rise of $[Ca^{2+}]i$, from a basal level of 55 nM to 140 nM after 6 min, whereas, the background increase was from a basal level of 55 to 90 nM (Fig. 1). However, cross-linking of CD3 molecules with a combination of αCD3 and αIgG resulted in a biphasic rise of $[Ca^{2+}]i$, which was dependent on the dose of αCD3 (Fig. 1). The transient rise peaked at about 120 sec and then gradually declined to a steady-state level by about 200 sec. This steady-state level was sustained for as long as 20 min (data not shown). Because of a nonspecific increase in basal $[Ca^{2+}]i$, it was not possible to monitor the sustained $[Ca^{2+}]i$ beyond 20 min. These results showed that cross-linking of the TCR-CD3 complexes is critical to initiate a full T cell response,

with respect to $Ca^{2+}$ mobilization and influx. Unless specifically mentioned, in all subsequent experiments T cells were stimulated by cross-linking TCR-CD3 complexes with 10 $\mu g \cdot ml^{-1}$ αIgG and 1 $\mu g \cdot ml^{-1}$ αCD3. For simplicity, stimulation in this way will be referred to as "αCD3 stimulation" of T cells.

### Transient Phase of Increased $[Ca^{2+}]i$ Was Due to the $Ca^{2+}$ Release From the Intracellular Stores and Was Dependent on the Extracellular $Ca^{2+}$

In order to determine which component of the increased $[Ca^{2+}]i$ was due to $Ca^{2+}$ influx into the cytosol, the T cells were stimulated in the absence of extracellular $Ca^{2+}$. Under this condition, αCD3 stimulation of T cells should cause only the $Ca^{2+}$ release from the intracellular stores, but not the influx from the outside. The sustained phase of the increase $[Ca^{2+}]i$ was reduced to the background level (from 220 to 90 nM), indicating that this phase was due to $Ca^{2+}$ influx from outside into the cytosol (Fig. 2A). Interestingly, there was about 80% reduction of the transient phase of increased $[Ca^{2+}]i$, from 400 nM in $Ca^{2+}$-containing medium to 120 nM in $Ca^{2+}$-free medium (Fig. 2A). It is possible that

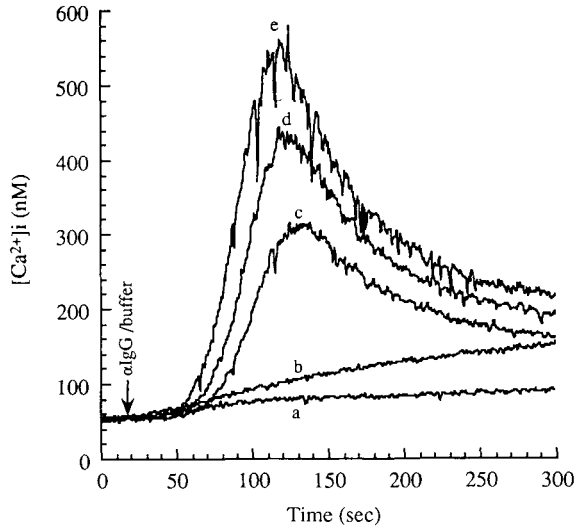



**Fig. 1.** Stimulation of a biphasic rise of $[Ca^{2+}]i$ in T cells after cross-linking of CD3 molecules. T cells were loaded with Fura-2. In a plastic cuvette, $2 \times 10^6$ cells $ml^{-1}$ were incubated with αCD3 antibody for 60 sec. Then fluorescence measurement was initiated and 20 sec later 10 $\mu g \cdot ml^{-1}$ αIgG was added to the cells. All the incubations were done at 37°C with constant stirring. The excitation wavelengths were 340 and 380 nm, and emission wavelength was 510 nm. Traces a, αIgG alone; b, 1 $\mu g \cdot ml^{-1}$ αCD3 alone; c, αIgG + 0.25 μg αCD3; d, αIgG + 0.5 μg αCD3; e, αIgG + 1 μg αCD3.

a major proportion of $Ca^{2+}$ influx occurs at the same time as the transient $Ca^{2+}$ release, giving rise to a relatively higher peak of the transient phase in the presence, and a smaller peak in the absence, of extracellular $Ca^{2+}$. Alternatively, $Ca^{2+}$ influx occurred after the $Ca^{2+}$ release; however, like influx, release was also dependent on extracellular $Ca^{2+}$. To delineate between these two possibilities, $Ba^{2+}$ was substituted for $Ca^{2+}$ in the extracellular medium. $Ba^{2+}$ causes a spectral shift similar to $Ca^{2+}$ after binding to Fura-2, can substitute $Ca^{2+}$ in many $Ca^{2+}$-dependent processes, can readily pass through the plasma membrane $Ca^{2+}$ channel and IP3-gated $Ca^{2+}$ channel, and can enter the $Ca^{2+}$ stores from cytosol [Ozaki et al., 1992; Premack et al., 1994]. However, the ability of this cation to pass through a $Ca^{2+}$ transporter, such as a carrier, exchanger, or pump in the cell membrane, is extremely limited [Ozaki et al., 1992; Premack et al., 1994]. As a result, increased Fura-2 fluorescence induced by the entry of divalent cation is sustained for a relatively longer period of time with $Ba^{2+}$ than with $Ca^{2+}$. In the present experiment, Fura-2 loaded T cells were stimulated with αCD3 and αIgG in the presence or absence of 1.3 mM $Ba^{2+}$ or $Ca^{2+}$, and the increase of the 340:380 ratio measured. In the presence of $Ca^{2+}$ both the transient and the sustained phases of increased fluorescence ratio were observed (Fig. 2B). However, in the presence of $Ba^{2+}$ only the sustained phase was seen, and the transient phase was no different than in $Ca^{2+}$-free medium. The integrity of the increased 340:380 ratio due to $Ba^{2+}$ entry was maintained after 120 sec, when the transient increase of the ratio, both in the presence and absence of extracellular $Ca^{2+}$, entered a declining phase (Fig. 2B). If influx started at the same time as release, an increase of the transient phase of 340:380 ratio should have occurred due to $Ba^{2+}$ entry. These results showed that the transient increase of $[Ca^{2+}]i$ was due to the $Ca^{2+}$ release from the intracellular stores and was dependent on extracellular $Ca^{2+}$. The sustained phase was due to the $Ca^{2+}$ influx, which began after $Ca^{2+}$ release.

### Inhibition of $Ca^{2+}$ Release Did Not Affect $Ca^{2+}$ Influx Into the Cytosol

The basis of the capacitative hypothesis is that emptying of intracellular $Ca^{2+}$ stores by IP3 triggers a $Ca^{2+}$ influx into the cytosol [Putney, 1990]. That hypothesis was tested by examining the effect of TMB-8 on αCD3-stimulated

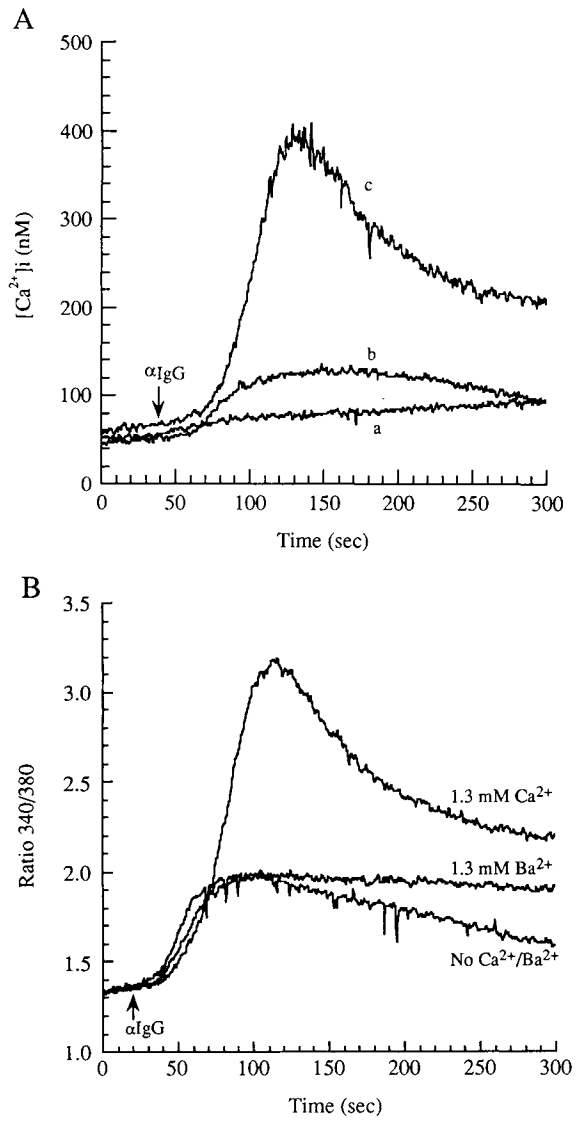



**Fig. 2.** aCD3 stimulation of $[Ca^{2+}]i$ increase and $Ba^{2+}$ entry in T cells in $Ca^{2+}$-free medium. **A:** Fura-2 loaded T cells were suspended in $Ca^{2+}$-free HBSS (trace b) or in HBSS containing 1.3 mM $Ca^{2+}$ (traces a and c), stimulated with αCD3 and αIgG and $[Ca^{2+}]i$ measured. Trace a, unstimulated control; traces b–c, αCD3 stimulation. **B:** Fura-2 loaded T cells were suspended in $Ca^{2+}$-free HBSS or in HBSS containing 1.3 mM $Ca^{2+}$ or $Ba^{2+}$, stimulated with αCD3 and αIgG, and changes in the fluorescence ratio from 340 and 380 nm excitation wavelengths were recorded.

$Ca^{2+}$ release and influx in T cells. TMB-8 has been shown to specifically suppress $Ca^{2+}$ release without affecting $Ca^{2+}$ influx in various cell types [Donowitz et al., 1986]. Preincubation of cells for 5 min with TMB-8 resulted in the inhibition of αCD3-stimulated $Ca^{2+}$ release from the intracellular stores (Fig. 3). Complete inhibition was observed with 200 μM TMB-8. Not only was the amplitude reduced, but also the latency period of release was prolonged. In contrast, neither the amplitude nor the latency of the $Ca^{2+}$ influx into cytosol was affected by any dose of TMB-8 (Fig. 3). TMB-8 also inhibited the $Ca^{2+}$ release, observed in $Ca^{2+}$-free medium, with complete inhibition at 200 μM (data not shown). This indicated that TMB-8 can completely inhibit, not just slowing down, the $Ca^{2+}$ release process. Taken together, these results suggest that neither the $Ca^{2+}$ release process nor the empty state of $Ca^{2+}$ stores were essential for triggering $Ca^{2+}$ entry into the cytosol from the extracellular medium.

## Hydrolysis of PIP2 Was Required for $Ca^{2+}$ Influx

Because IP3 stimulated release of $Ca^{2+}$ was not involved in $Ca^{2+}$ influx, we explored whether any product of PIP2 hydrolysis played a role. For this purpose, we used U73122, which has been shown to inhibit PLC activity in human neutrophils and platelets [Bleasedale et al., 1990; Smith et al., 1990]. For a control, U73343, an inactive structural analogue of U73122 [Bleasedale et al., 1990; Smith et al., 1990], was used. Treatment of the T cells with U73122, 5 min prior to the αCD3 stimulation, resulted in a dose dependent inhibition of the amplitude of both $Ca^{2+}$ release and influx (Fig. 4). The greatest inhibition was observed with 200 nM U73122;

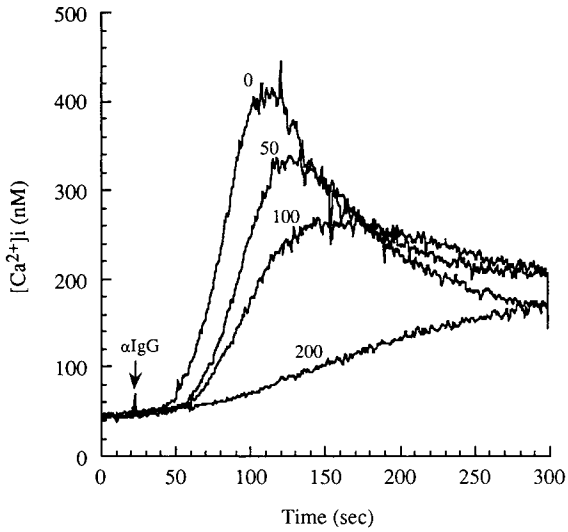



**Fig. 3.** Effect of TMB-8 on αCD3-stimulated biphasic rise of $[Ca^{2+}]i$. Fura-2 loaded T cells were pretreated for 5 min with various concentrations of TMB-8 and then stimulated with 1 μg αCD3 and 10 μg αIgG. The numbers on the $[Ca^{2+}]i$ traces represent the TMB-8 concentrations in μM.

whereas 250 nM of U73343 affected neither the release nor the influx. U73122 also slightly increased the latency period of $Ca^{2+}$ release, whereas latency of influx was not affected by this inhibitor. These results indicated that another cellular event resulted from PIP2 hydrolysis, other than IP3-stimulated $Ca^{2+}$ release, triggered $Ca^{2+}$ influx.

### Hydrolysis of PIP2 Resulted in the Prolonged Opening of $Ca^{2+}$ Influx Channel

The addition of $Ca^{2+}$ to the cells, stimulated for extended periods of time in $Ca^{2+}$-free medium, resulted in $Ca^{2+}$ influx; this finding was interpreted to suggest that the emptying of the $Ca^{2+}$ stores triggered subsequent $Ca^{2+}$ entry [see review in Irvine, 1992]. However, it was equally possible that $Ca^{2+}$ influx pathway was activated independent of $Ca^{2+}$ release, but remained open for an extended period of time, in the absence of any $Ca^{2+}$ influx. To delineate between these two possibilities, we sought to determine how long $Ca^{2+}$ release and $Ca^{2+}$ influx mechanisms remained active after the initiation and the termination of the phosphoinositide signaling pathway. For that, T cells were stimulated with αCD3 and αIgG for various time periods in the absence of extracellular $Ca^{2+}$. Measurements of $[Ca^{2+}]i$ were then initiated

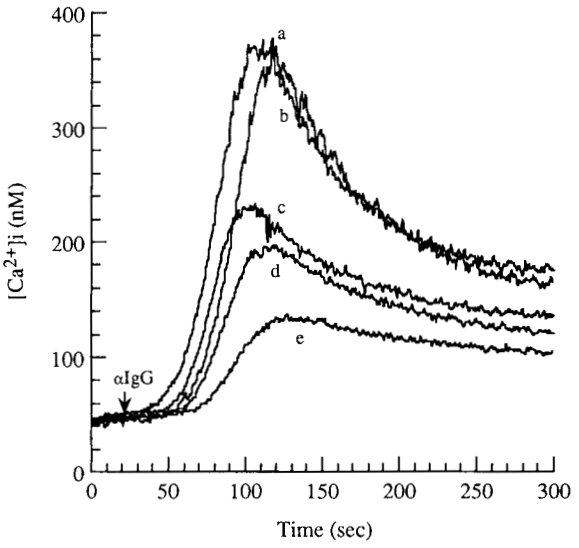
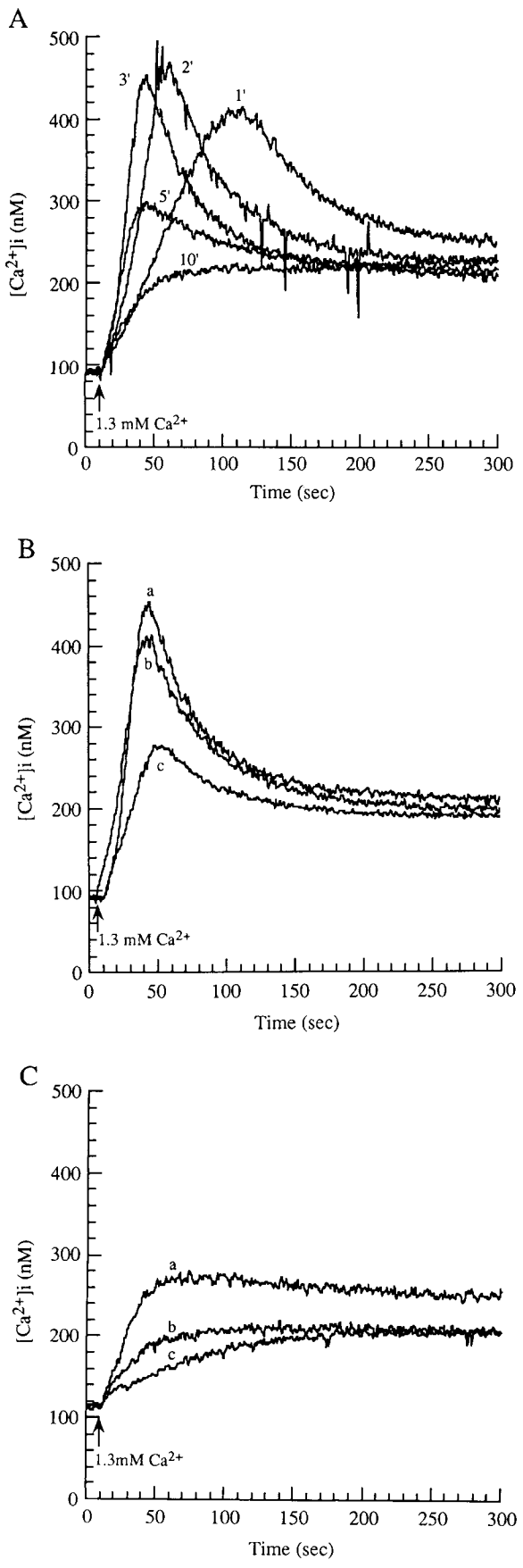



**Fig. 4.** Inhibition of αCD3-stimulated increase of $[Ca^{2+}]i$ by the PLC inhibitor, U73122. T cells were stimulated with 1 μg αCD3 and 10 μg αIgG, and $[Ca^{2+}]i$ was determined. Some samples were treated with PLC inhibitor for 5 min, before stimulation. Traces a, control; b, 250 nM U73343; c, 100 nM U73122; d, 150 nM U73122; e, 200 nM U73122.

and 1.3 mM $Ca^{2+}$ was added to the cells. Addition of $Ca^{2+}$ to these cells resulted in both $Ca^{2+}$ release and influx (Fig. 5A). The $Ca^{2+}$ release was maximal in the cells stimulated for 2–3 min; levels then declined gradually, such that cells stimulated for 10 min showed no $Ca^{2+}$ release but only $Ca^{2+}$ influx. In contrast, maximal $Ca^{2+}$ influx was observed after 1 min of stimulation and remained steady even after 10 min of stimulation. Moreover, the addition of a PLC inhibitor for the last minute of a 3 min stimulation, did not suppress $Ca^{2+}$ release influx (Fig. 5B). However, addition of the same inhibitor for the last 2 min of a 3 min stimulation, suppressed the $Ca^{2+}$ release substantially without affecting $Ca^{2+}$ influx. These results demonstrated that PIP2 hydrolysis and IP3 accumulation were maximal after 2 min of stimulation and remained at a constant level for up to 3 min of stimulation. After that time, PIP2 hydrolysis and IP3 levels gradually decreased with concomitant reduction in $Ca^{2+}$ release. In contrast, once activated $Ca^{2+}$ influx in T cells remained steady for an extended period of time even in the absence of PIP2 hydrolysis and IP3 production. The $Ca^{2+}$ influx into the cells stimulated for 10 min was not due to the emptying of $Ca^{2+}$ stores during stimulation, because this was not suppressed by the treatment of the cells with 100 μM TMB-8 for 5 min before stimulation (Fig. 5C). Further, addition of PLC inhibitor for the last 7 min of a 10 min stimulation did not suppress $Ca^{2+}$ influx (Fig. 5C). These results showed that $Ca^{2+}$ influx was activated within 60 sec of stimulation. Once activated, influx was independent of PIP2 hydrolysis and remained active for a relatively long period of time. These results also further showed that emptying of the intracellular $Ca^{2+}$ stores was not essential for the agonist induced $Ca^{2+}$ influx.

### Thapsigargin Stimulated $Ca^{2+}$ Entry Independent of Emptying the $Ca^{2+}$ Stores

Microsomal $Ca^{2+}$-ATPase inhibitor, such as Tg, was shown to induce emptying of the $Ca^{2+}$ stores and $Ca^{2+}$ influx in many cell types [Thomas and Hanley, 1994]. From this finding, investigators have proposed that emptying of the intracellular $Ca^{2+}$ stores stimulated $Ca^{2+}$ influx. That interpretation, however, is inconsistent with our present findings. Therefore, we investigated whether Tg stimulates $Ca^{2+}$ entry by a mechanism other than emptying $Ca^{2+}$ stores. For this, cells were stimulated with various con-




centrations of Tg in the presence or absence of 1.3 mM $Ca^{2+}$ and the pattern of $[Ca^{2+}]i$ changes under these two conditions were compared. Our results showed that Tg induced a modest release of $Ca^{2+}$ from the intracellular stores over a period of several minutes (Fig. 6A). The $Ca^{2+}$ release remained constant over a dose range of 1 to 1,000 nM Tg. In contrast, $Ca^{2+}$ influx increased in direct relation to the levels of Tg over the same dose range (Fig. 6B). If release was the determining factor for influx, then influx should have also remained constant over the dose range of 1 to 1,000 nM Tg. Moreover, the amount of $Ca^{2+}$ released by Tg stimulation was much less than the amount released by αCD3 stimulation. Yet, Tg stimulated a much larger amount of $Ca^{2+}$ influx than αCD3. If $Ca^{2+}$ influx was stimulated by $Ca^{2+}$ release, then the amount of Tg-stimulated $Ca^{2+}$ influx should have been less than or at the most equal to the αCD3-stimulated $Ca^{2+}$ influx. Collectively, these results demonstrated that Tg can stimulate $Ca^{2+}$ entry into the cells by a mechanism other than emptying the $Ca^{2+}$ stores.

### Decrease of Basal $[Ca^{2+}]i$ Stimulated $Ca^{2+}$ Influx in T Cells

Addition of $Ca^{2+}$ to cells depleted of intracellular $Ca^{2+}$ by incubation in $Ca^{2+}$-free medium, resulted in a sustained $Ca^{2+}$ influx [Mason et al., 1991; Montero et al., 1991; Randriamampita and Tsien, 1993]. That influx, however, did not occur when the cells were reloaded with $Ca^{2+}$ after depletion. These results were interpreted in the light of the capacitative $Ca^{2+}$ entry mechanism. In the present study, we incubated the T cells in $Ca^{2+}$-free HBSS at 37°C for 60 min and then 1.3 mM $Ca^{2+}$ was added in one aliquot. T cells were incubated for an additional 15 min, loaded with Fura-2, resuspended in $Ca^{2+}$-free HBSS, and $[Ca^{2+}]i$ measured. Incubation of cells in the $Ca^{2+}$-free medium resulted in a reduction

**Fig. 5.** Kinetics of $Ca^{2+}$ signaling in T cells. In all the samples, T cells were first incubated with 1 μg αCD3 for 60 sec in $Ca^{2+}$-free HBSS at 37°C. **A:** αIgG was then added to the cells and incubated for the various times indicated, before the initiation of fluorescence measurement. 1.3 mM $Ca^{2+}$ was added to the cells 10 sec after the beginning of fluorescence measurements. **B:** T cells were incubated with αIgG for 3 min. During that time period, cells received only 10 μl DMSO (trace a), or 200 nM U73122 at 1 (trace b) or 2 min (trace c). **C:** T cells were incubated with αIgG for 10 min. Samples received 10 μl DMSO (trace a) or 200 nM U73122 (trace b) at 3 min, during a 10 min incubation with αIgG. Some samples were treated with TMB-8 for 5 min before 10 min stimulation (trace c).

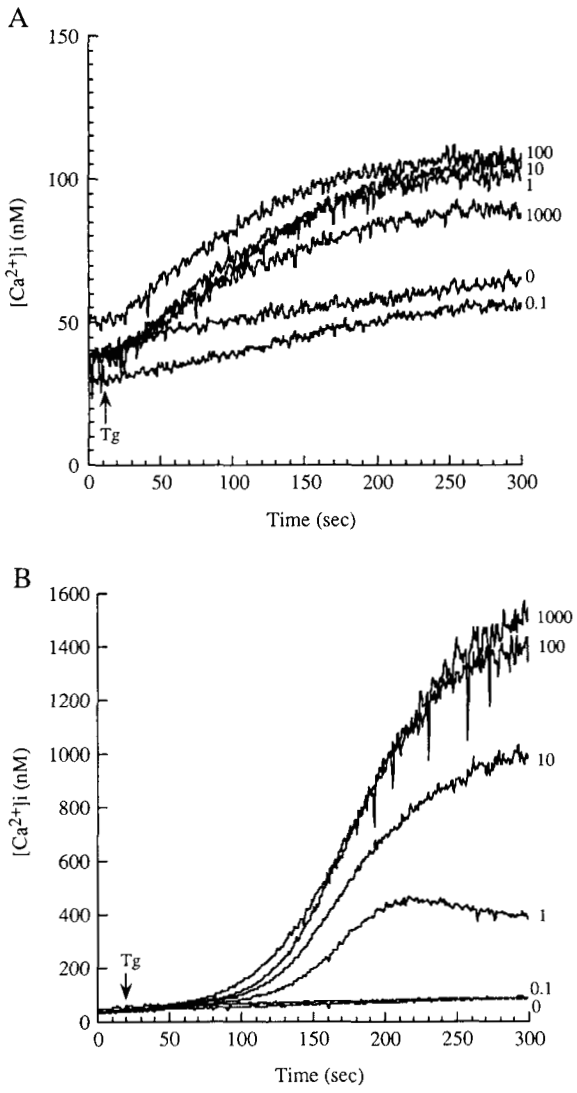



**Fig. 6.** Effect of thapsigargin on $[Ca^{2+}]i$ in T cells. Fura-2 loaded T cells were suspended in HBSS containing 0 mM (**A**) or 1.3 mM (**B**) $Ca^{2+}$, and fluorescence measurement was initiated. After 20 sec, thapsigargin was added to the cells. The numbers on the traces represent the thapsigargin concentrations in nM.

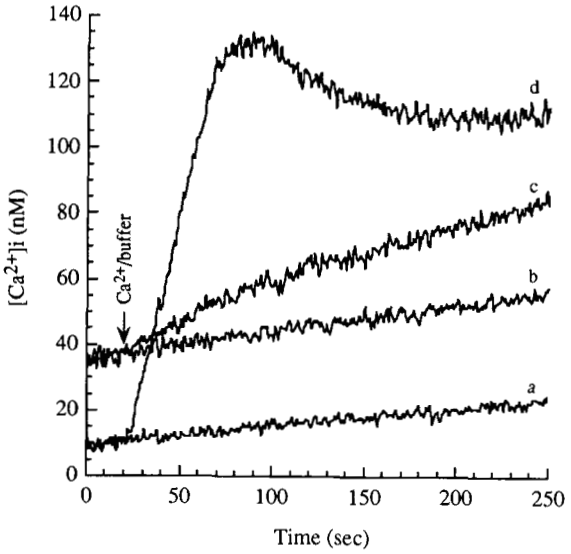



**Fig. 7.** Calcium influx into T cells depleted of intracellular $Ca^{2+}$. T cells were depleted of intracellular $Ca^{2+}$ by incubating in $Ca^{2+}$-free medium for 60 min, incubated for an additional 15 min with 0 or 1.3 mM $Ca^{2+}$ and then loaded with Fura-2 AM. The cells were resuspended in $Ca^{2+}$-free medium and $[Ca^{2+}]i$ measurement was initiated. After 10 sec, 0 mM (traces a and b) or 1.3 mM $Ca^{2+}$ (traces c and d) was added to the cells. Traces a and d, $Ca^{2+}$-depleted cells; b and c, $Ca^{2+}$-reloaded cells.

of basal $[Ca^{2+}]i$ to about 9 nM. Addition of $Ca^{2+}$ to those cells caused a sustained increase of $[Ca^{2+}]i$ to about 130 nM (Fig. 7). However, in the $Ca^{2+}$-reloaded cells, basal $[Ca^{2+}]i$ increased to about 40 nM and the addition of $Ca^{2+}$ to those cells caused a slow increase of $[Ca^{2+}]i$ (Fig. 7). These results showed that depleting T cells of intracellular $Ca^{2+}$ activated a plasma membrane $Ca^{2+}$ influx pathway.

$Ca^{2+}$ entry into the $Ca^{2+}$ depleted cells may be stimulated either by the emptying of intracellular $Ca^{2+}$ stores, as suggested by the capacitative hypothesis, or by the reduction in basal $[Ca^{2+}]i$. To delineate between these two possibilities, we sought to determine the total $Ca^{2+}$ content in the $Ca^{2+}$ depleted and $Ca^{2+}$ reloaded cells. Since, the calcium concentration in the intracellular stores is much higher (mM level) than that of the cytosol (nM level) [Carafoli, 1987], emptying of the intracellular stores should result in a significant difference in total $Ca^{2+}$ content between $Ca^{2+}$ depleted and reloaded cells. The values were, however, not significantly different ($895 \pm 91$ vs. $975 \pm 84$ pmol/$10^6$ cells). This demonstrated that intracellular $Ca^{2+}$ stores were not significantly depleted even after prolonged incubation of the cells in $Ca^{2+}$-free medium. Based on these results we suggest that $Ca^{2+}$ entry into the $Ca^{2+}$ depleted cells was perhaps stimulated by the reduction in the basal $[Ca^{2+}]i$, but not by the emptying of the intracellular $Ca^{2+}$ stores. Small amount of $Ca^{2+}$ entry into the $Ca^{2+}$-reloaded cells suggests that incubation of cells in $Ca^{2+}$-free medium may also induce some nonspecific $Ca^{2+}$ entry.

### Extracellular $Ca^{2+}$ Modulation of the Transient $Ca^{2+}$ Release From the Intracellular Store

Attenuation in $Ca^{2+}$-free medium and complete suppression by TMB-8 of the transient phase of increased $[Ca^{2+}]i$ suggested that extracellular $Ca^{2+}$ may have a regulatory effect on

$Ca^{2+}$ release. To analyze this, the effect of various concentrations of extracellular $Ca^{2+}$ ($[Ca^{2+}]o$) on the αCD3 stimulated rise in $[Ca^{2+}]i$ was investigated. Stimulation of T cells in the absence of $[Ca^{2+}]o$ resulted in a slight increase of the transient phase of $[Ca^{2+}]i$; addition of $Ca^{2+}$ to these cells resulted in the gradual increase in $[Ca^{2+}]i$ (Fig. 8A). The sustained increase in $[Ca^{2+}]i$ was prominent even at low $[Ca^{2+}]o$ and was maximal at 0.5 mM $[Ca^{2+}]o$. In contrast, the transient phase of increased $[Ca^{2+}]i$ was prominent at 0.5 mM and reached a maximum with 1.3 mM $[Ca^{2+}]o$. Addition of 1.3 mM $Ba^{2+}$ caused only the sustained but not the transient increase of 340;380 ratio, indicating that the transient phase was not due to the higher initial driving force of the cation entry. Furthermore, low levels of EGTA (< 1 mM) suppressed only $Ca^{2+}$ release, whereas high levels of EGTA (2 mM or above) suppressed both the $Ca^{2+}$ release and influx (data not shown). The transient and the sustained phases of increased $[Ca^{2+}]i$, following the addition of extracellular calcium, were associated with $Ca^{2+}$ release from the intracellular store and $Ca^{2+}$ influx into the cytosol, respectively. This was demonstrated by about 80% suppression of the transient phase but not the sustained phase of increased $[Ca^{2+}]i$ by TMB-8 (Fig. 8B). These results showed that both $Ca^{2+}$ influx into the cytosol and $Ca^{2+}$ release from the intracellular stores were differentially dependent on extracellular $Ca^{2+}$.

### Characterization of $Ca^{2+}$ Entry Pathways in T Cells by αCD3 Stimulated $Mn^{2+}$ Entry

We further analyzed the kinetics of $Ca^{2+}$ influx, by determining the temporal pattern and rate of the Fura-2 fluorescence quenching by $Mn^{2+}$. That ion serves as a surrogate for $Ca^{2+}$ and can cross the $Ca^{2+}$ channels [Foder et al., 1989; Hallam and Rink, 1985]. Accordingly, cells were stimulated in the presence of various concentrations of extracellular $Mn^{2+}$ and 1.3 mM$[Ca^{2+}]o$, and total Fura-2 fluorescence at 340 nm, instead of conventional 360 nm (isosbestic point for Fura-2) excitation wave length, was determined. This should allow the precise determination of the temporal pattern of $Ca^{2+}/Mn^{2+}$ entry with respect to $Ca^{2+}$ release. If $Mn^{2+}$ begins to enter the cell before or at the same time as $Ca^{2+}$ release, quenching of both the transient and sustained phase of fluorescence should occur. If $Mn^{2+}$ entry begins after the $Ca^{2+}$ release, then only the sustained phase of fluorescence

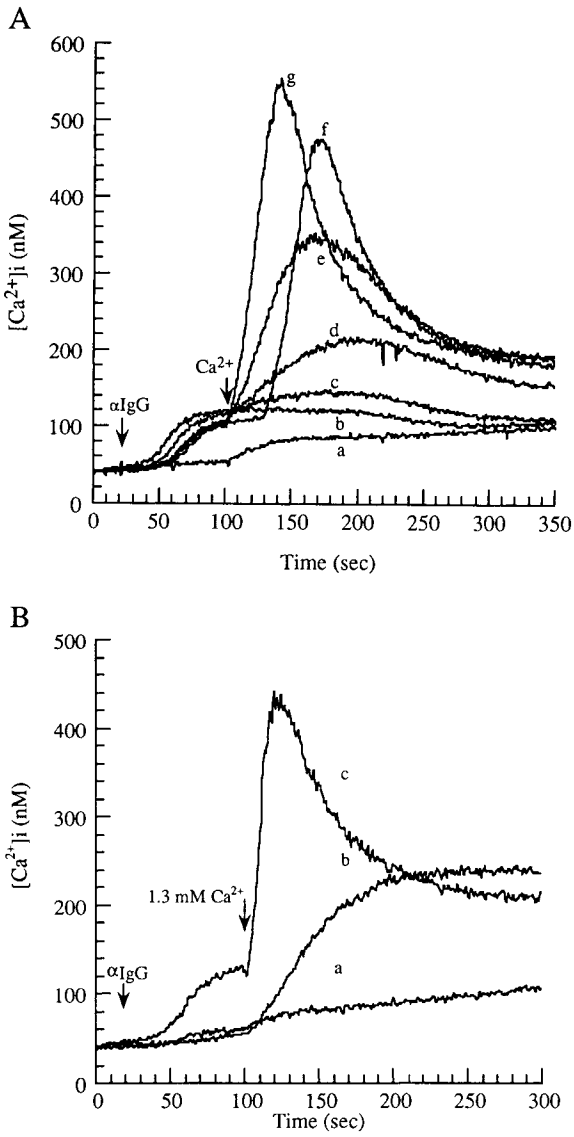



**Fig. 8.** Effect of extracellular $Ca^{2+}$ on the αCD3-stimulated biphasic rise of $[Ca^{2+}]i$. Fura-2 loaded cells were stimulated with 1 μg αCD3 and 10 μg αIgG in $Ca^{2+}$-free HBSS, and $[Ca^{2+}]i$ measured. **A:** Various doses of $Ca^{2+}$ were added to the cells at 100 sec, except in f where $Ca^{2+}$ was added at 130 sec. Trace a, 1.3 mM $Ca^{2+}$ was added to unstimulated cells. Traces b–g, $Ca^{2+}$ was added to stimulated cells: b, 0 mM; c, 0.125 mM; d, 0.25 mM; e, 0.5 mM; f, 0.8 mM; and g, 1.3 mM $Ca^{2+}$. **B:** Cells were treated with medium (trace a and c) or 200 μM TMB-8 (trace b), before αCD3 stimulation. Trace a, unstimulated control; traces b–c, αCD3-stimulation.

should be quenched. We observed a differential quenching of the transient and sustained phases of fluorescence at different concentration of $Mn^{2+}$. At relatively lower concentrations, entry of $Mn^{2+}$ into the stimulated cells resulted in the quenching of the sustained phase of fluorescence (Fig. 9), which was due to the $Ca^{2+}$ influx

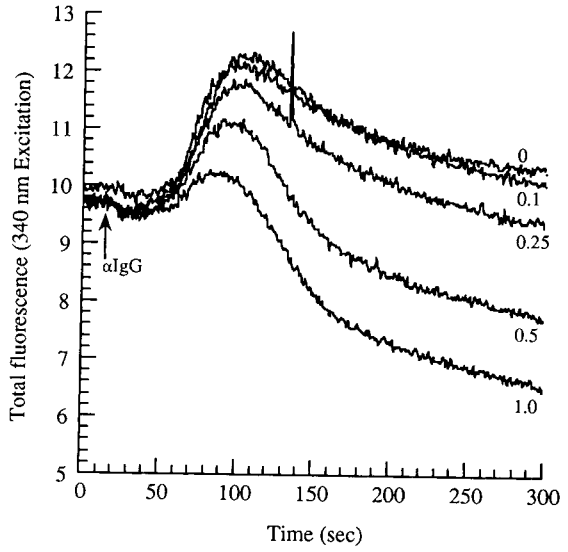



**Fig. 9.** αCD3 stimulation of $Mn^{2+}$ entry in T cells. Cells were stimulated with αIgG and 1 μg · $ml^{-1}$ αCD3 as described in Figure 1, except various concentrations of $Mn^{2+}$ were present in the buffer. The number of the traces represent the $Mn^{2+}$ concentrations in mM.

(Fig. 2). However, as the concentration of $Mn^{2+}$ was increased, the transient phase of fluorescence, which was due to $Ca^{2+}$ release (Fig. 2), was also reduced (Fig. 9). Without any stimulus, some quenching of the basal fluorescence was observed with the higher concentration of $Mn^{2+}$ tested. Accordingly, all the traces shown were corrected for this background quenching. Since, there was a distinct $Ba^{2+}$ permeable $Ca^{2+}$ influx pathway which opened after the $Ca^{2+}$ release, the stimulus-specific quenching of the transient phase of fluorescence suggests that there may be another poorly $Mn^{2+}$ permeable and $Ba^{2+}$ impermeable $Ca^{2+}$ entry pathway through which high concentrations of $Mn^{2+}$ can enter the cell at the same time as $Ca^{2+}$ release.

The relative kinetic behavior of the $Ca^{2+}$ entry pathways was obtained by plotting the quench ratio, the ratio of fluorescence in the absence of $Mn^{2+}$ to that in the presence of $Mn^{2+}$, against the $Mn^{2+}$ concentrations, throughout the stimulation period. Three distinct ranges of slopes were obtained (data not shown). Between 90 and 120 sec, the time period of transient $Ca^{2+}$ release, the slope was roughly $-0.19 \pm 0.01$ ($-0.15$ to $-0.22$); between 150 and 300 sec, the time period of sustained $Ca^{2+}$ influx, the slope was $-0.35 \pm 0.01$ ($-0.31$ to $-0.39$). Between 120 and 130 sec, roughly the interjunction time between transient and the sustained phases of increased $[Ca^{2+}]i$, the slope value was $-0.24$ ($-0.23$ to $-0.25$), about the average of the two slope values. These results further suggest that there are two $Ca^{2+}$ entry pathways in T cell plasma membrane, differentially permeable to $Mn^{2+}$.

### Modulation of $Ca^{2+}$ Signaling in T Cells by $La^{3+}$ and $Ni^{2+}$

The channel by which $Ca^{2+}$ influx occurs in nonexcitable cells has not been well characterized. However, it has been shown that the addition of a single dose of $Ni^{2+}$ or $La^{3+}$ several minutes after the addition of stimuli, inhibited the increase in $[Ca^{2+}]i$ [Chow and Jondal, 1990; Laskey et al., 1992; Lewis and Cahalan, 1989; Llopis et al., 1992; Zweifach and Lewis, 1993]. Based on those findings several investigators suggested that a $Ni^{2+}$- and $La^{3+}$-sensitive channel was involved in $Ca^{2+}$ influx. However, some reports showed that addition of $Ni^{2+}$ or $La^{3+}$ before the addition of stimuli inhibited both the $Ca^{2+}$ release and influx [Hallam et al., 1988; Haverstick and Gray, 1993; Mason et al., 1991]. Consequently we used those two cations to characterize the $Ca^{2+}$ influx pathway in T cells. For this, cells were incubated with various concentrations of $Ni^{2+}$ or $La^{3+}$ 5 min before the stimulation with αCD3. Low doses of $Ni^{2+}$ (0.5–2 mM) inhibited $Ca^{2+}$ release without affecting the $Ca^{2+}$ influx (Fig. 10A). However, at a dose above 2 mM, $Ni^{2+}$ inhibited both the $Ca^{2+}$ release and influx. Similarly, 20 μM $La^{3+}$ inhibited $Ca^{2+}$ release with a minor effect on $Ca^{2+}$ influx (Fig. 10B). Because of cell aggregation at concentrations greater than 20 μM $La^{3+}$, we were unable to test whether higher concentrations had any effect on $Ca^{2+}$ influx. These results may suggest that $Ca^{2+}$ release and influx are under the control of $Ca^{2+}$ entry pathways differentially sensitive to $Ni^{2+}$ and $La^{3+}$.

## DISCUSSION

In this study, we attempted to provide some insight into the $Ca^{2+}$ signaling mechanism in a non-excitable cell, the T cell, with an emphasis on the relationship between the $Ca^{2+}$ release from the intracellular stores and $Ca^{2+}$ entry into the cells. We provide evidence that $Ca^{2+}$ release and influx are independent events, linked to two different $Ca^{2+}$ entry pathways in the plasma membrane, one opening directly into the cytosol and the other linked to the $Ca^{2+}$ stores.

αCD3 stimulation of T cells resulted in a biphasic increase of $[Ca^{2+}]i$, a transient phase of higher

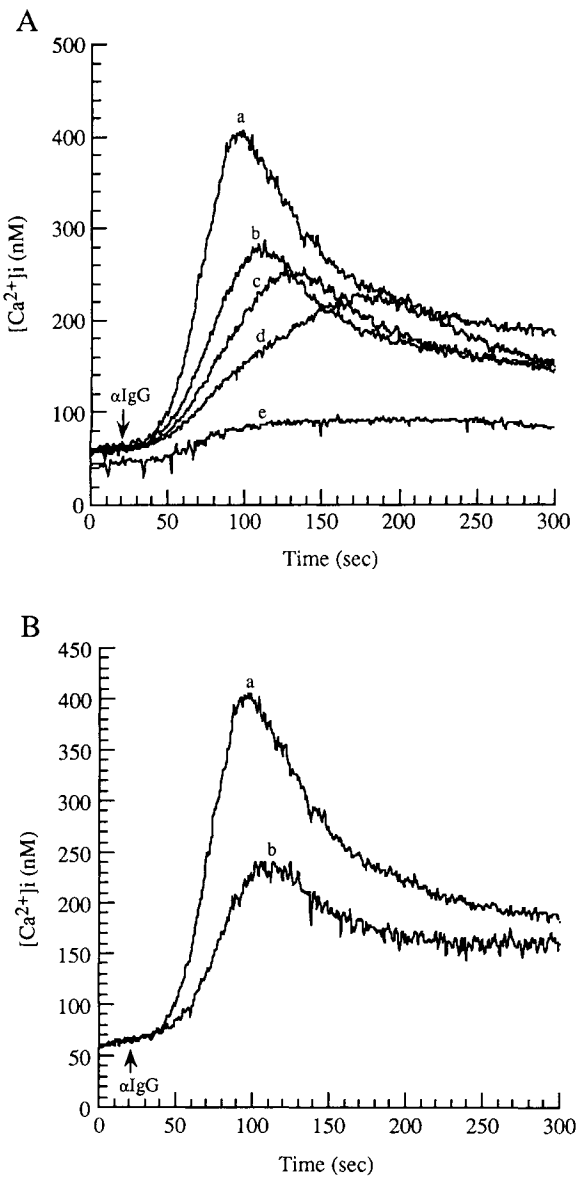



**Fig. 10.** Effect of $Ni^{2+}$ and $La^{3+}$ on the αCD3-stimulated rise of $[Ca^{2+}]i$. T cells were stimulated with 1 μg αCD3 and 10 μg αIgG, and $[Ca^{2+}]i$ was determined. **A:** $Ni^{2+}$ was added to the cells 5 min before stimulation; traces a, 0 mM $Ni^{2+}$; b, 0.5 mM $Ni^{2+}$; c, 1 mM $Ni^{2+}$; d, 2 mM $Ni^{2+}$; e, 5 mM $Ni^{2+}$. **B:** Cells were treated for 5 min with 0 (trace a) or 20 μM $La^{3+}$ (trace b) before stimulation.

amplitude followed by a sustained phase of lower amplitude. To determine the relative contribution of $Ca^{2+}$ release and influx to this biphasic increase of $[Ca^{2+}]i$, T cells were stimulated in $Ca^{2+}$-free medium. The sustained phase of increased $[Ca^{2+}]i$ was reduced to the background level, indicating that this phase was due to $Ca^{2+}$ influx from outside into the cytosol. In addition, the transient phase was drastically reduced (~80% reduction) in $Ca^{2+}$-free medium. There could be two possibilities for these results. One possibility was that $Ca^{2+}$ influx began at the same time as $Ca^{2+}$ release, such that the small transient increase of $[Ca^{2+}]i$ in $Ca^{2+}$-free medium was solely due to $Ca^{2+}$ release, and the large transient increase of $[Ca^{2+}]i$ in $Ca^{2+}$-containing medium was due to both the release and influx. Alternatively, $Ca^{2+}$ release was dependent on the extracellular $Ca^{2+}$ and, therefore, it was reduced in $Ca^{2+}$-free medium. Our results showed that increase of 340:380 ratio, due to $Ba^{2+}$ entry, corresponded only to the sustained phase of increased $[Ca^{2+}]i$; the transient phase was the same as in $Ca^{2+}/Ba^{2+}$-free medium. If the transient phase of $[Ca^{2+}]i$ was due to the overlapping of $Ca^{2+}$ release with the influx, then $Ba^{2+}$ entry should have caused both the transient and sustained increase of 340:380 ratio. Besides, with 0.5 mM $[Ca^{2+}]o$, maximum sustained influx was observed; whereas, the transient phase was submaximal. If the transient increase of $[Ca^{2+}]i$ was due to both release and influx, then both the transient and the sustained phases of $[Ca^{2+}]i$ should have been observed with 0.5 mM $[Ca^{2+}]o$. These results showed that the transient phase of increased $[Ca^{2+}]i$ was due to $Ca^{2+}$ release and the sustained phase was due to influx, which began after the $Ca^{2+}$ release.

Next we sought to determine the regulatory mechanism of $Ca^{2+}$ influx. In the original capacitative model [Putney, 1986], $Ca^{2+}$ from the extracellular fluid entered into the cytosol via empty $Ca^{2+}$ stores, giving rise to the sustained phase of increased $[Ca^{2+}]i$. The model was further altered such that the empty state of the $Ca^{2+}$ stores triggered $Ca^{2+}$ entry directly into the cytosol, causing a sustained increase in $[Ca^{2+}]i$ [Putney, 1990]. In contrast, the second messenger hypothesis suggests that an inositol phosphate, such as IP3, IP4, or both, triggers $Ca^{2+}$ influx into the cytosol [Irvine, 1992]. The second messenger-mediated $Ca^{2+}$ entry hypothesis was mainly based on the findings that intracellular administration of inositol phosphates activated a $Ca^{2+}$ channel in the plasma membrane, in the absence of any $Ca^{2+}$ release. In this study we showed that complete inhibition of $Ca^{2+}$ release from the intracellular stores by TMB-8 did not affect $Ca^{2+}$ influx in T cells. However, the PLC inhibitor suppressed both the $Ca^{2+}$ release and

influx. These results showed that in T cells, $Ca^{2+}$ influx occurred independently of emptying of the $Ca^{2+}$ stores. However, some PIP2 hydrolysis-dependent cellular event, other than IP3-stimulated $Ca^{2+}$ release, was necessary for $Ca^{2+}$ influx into the cytosol.

Support for the capacitative model was based on the stimulation of $Ca^{2+}$ influx by emptying $Ca^{2+}$ stores. One approach to empty the $Ca^{2+}$ store was by stimulating the cells with agonist in $Ca^{2+}$-free medium. Subsequent addition of $Ca^{2+}$ to the cells resulted in $Ca^{2+}$ influx, even when the agonist was removed. However, we have shown that once activated, the $Ca^{2+}$ influx became independent of further PIP2 hydrolysis and remained active for an extended period of time. We have further shown that presence of TMB-8 during αCD3 stimulation in $Ca^{2+}$-free medium did not prevent subsequent $Ca^{2+}$ influx. Thus, a sustained activation of the influx pathway itself, not the emptying of $Ca^{2+}$ stores, caused $Ca^{2+}$ influx after removal of the agonist and the addition of $Ca^{2+}$. These results also corroborated our findings that $Ca^{2+}$ influx did not depend on $Ca^{2+}$ release. Another way of depleting the intracellular $Ca^{2+}$ stores was by preventing the reuptake of $Ca^{2+}$ into the stores by an inhibitor of the microsomal $Ca^{2+}$ pump, such as Tg. In the present study, Tg stimulated $Ca^{2+}$ entry in a dose dependent manner over a range of 1–1,000 nM, although the $Ca^{2+}$ release remained constant over that same dose range. Furthermore, the relative ratio of $Ca^{2+}$ release to influx was different with Tg than with αCD3 stimulation. This demonstrated that Tg stimulated $Ca^{2+}$ influx by a mechanism other than emptying the $Ca^{2+}$ stores. The third principal way of depleting the $Ca^{2+}$ stores was to incubate the cells for an extended period of time in $Ca^{2+}$ free medium. Our study with T cells also showed that depletion of intracellular $Ca^{2+}$ activated a $Ca^{2+}$ influx pathway, which was partially inhibited when the cells were reloaded with $Ca^{2+}$ after depletion. We observed, however, that this depletion process resulted in the reduction of basal $[Ca^{2+}]i$, but not the depletion of $Ca^{2+}$ stores. The reason is unclear at this time. It may be due to poor leakage of $Ca^{2+}$ from the intracellular stores, and that may explain why Tg-stimulated $Ca^{2+}$ release was very low in T cells. The basal $[Ca^{2+}]i$ was increased in cells reloaded with $Ca^{2+}$ after depletion. These results demonstrated that $Ca^{2+}$ influx into the $Ca^{2+}$ depleted cells was stimulated by lowered basal $[Ca^{2+}]i$, but not by emptying of the $Ca^{2+}$ stores. How lowered basal $[Ca^{2+}]i$ stimulates $Ca^{2+}$ influx is unclear. It may stimulate the synthesis of diffusable $Ca^{2+}$ influx factor as recently described [Parekh et al., 1993; Randriamampita and Tsien, 1993].

In this study we made a series of observations which led us to propose that two plasma membrane $Ca^{2+}$ entry pathways function in T cells, one which opens directly into the cytosol and the other into the $Ca^{2+}$ stores. First, αCD3 antibody stimulated a barely detectable amount of $[Ca^{2+}]i$ release in the absence of extracellular $Ca^{2+}$. However, addition of $Ca^{2+}$ after the stimulation resulted in a gradual increase of $[Ca^{2+}]i$. Maximum influx was observed at low $[Ca^{2+}]o$; a much higher level of $[Ca^{2+}]o$ was required for maximum $Ca^{2+}$ release. This biphasic nature of the increased $[Ca^{2+}]i$ may be due to the higher initial driving force for $Ca^{2+}$ entry followed by a gradual inhibition of the $Ca^{2+}$ channel until a new equilibrium is reached, at higher concentrations of extracellular $Ca^{2+}$. The above possibility was ruled out because: (1) TMB-8 inhibited only the transient phase of increased $Ca^{2+}$, (2) addition of 1.3 mM $Ba^{2+}$, instead of $Ca^{2+}$, to the cells resulted only in the sustained but not the transient increase of 340:380 ratio, and (3) addition of 1.3 mM $Ca^{2+}$ to the cells after 10 min of αCD3 stimulation resulted only in sustained but not transient increase of $[Ca^{2+}]i$. Furthermore, low levels of EGTA suppressed only $Ca^{2+}$ release, while the high levels suppressed both release and influx. These are consistent with many previous findings where $Ca^{2+}$ release can be significantly attenuated in the absence of extracellular $Ca^{2+}$ [Chow and Jondal, 1990; Chow et al., 1993; Gelfand et al., 1988; Hughes and Schachter, 1994; Li et al., 1994; Peters et al., 1992; Premack et al., 1994; Sargeant et al., 1993]. Second, a low concentration of $Mn^{2+}$ quenched only the sustained phase of fluorescence, indicating $Mn^{2+}$ entry through $Ca^{2+}$ channels into the cytosol. In contrast, high concentrations of $Mn^{2+}$ quenched both the transient and sustained phase of fluorescence. The slopes of the $Mn^{2+}$ concentration-dependent quench curve were different during the period of transient phase and the period of the sustained phase. Third, low doses of $Ni^{2+}$ and $La^{3+}$ suppressed the $Ca^{2+}$ release without affecting $Ca^{2+}$ influx. However, at high doses of $Ni^{2+}$ both the release and influx were suppressed. These results now show that there are

two types of $Ca^{2+}$ entry pathways in T cell plasma membrane. One pathway was responsible for the sustained $Ca^{2+}$ influx into the cytosol (influx pathway). This pathway had a higher affinity for $Ca^{2+}$, was less sensitive to $Ni^{2+}$ and $La^{3+}$, and was highly permeable to $Mn^{2+}$. The second pathway was involved in the $Ca^{2+}$ entry into the cell simultaneously with $Ca^{2+}$ release. This pathway had a lower affinity for $Ca^{2+}$, was very sensitive to $Ni^{2+}$ and $La^{3+}$, was poorly permeable to $Mn^{2+}$. αCD3 stimulation of T cells in the presence of extracellular $Ba^{2+}$ resulted only in the sustained, but not the transient increase of cytosolic $Ba^{2+}$ concentration. This suggests that the influx pathway is permeable to $Ba^{2+}$ and is likely to be a $Ca^{2+}$ channel. Whereas, the second pathway is impermeable to $Ba^{2+}$ and is likely to be a $Ca^{2+}$ transporter rather than a channel.

It may also be argued that there was only one $Ca^{2+}$ entry pathway, through which $Ca^{2+}$ entry began at the same time as $Ca^{2+}$ release. However, initially the influx pathway was less active than during the sustained phase of $[Ca^{2+}]i$ increase. Therefore, it was more sensitive to $Ni^{2+}$ and $La^{3+}$, less permeable to $Mn^{2+}$, impermeable to $Ba^{2+}$ and required higher $[Ca^{2+}]o$, during the transient phase than the sustained phase of $[Ca^{2+}]i$ increase. If $Ca^{2+}$ influx began at the same time as $Ca^{2+}$ release, then 80% of the transient increase of $[Ca^{2+}]i$ was due to $Ca^{2+}$ influx, and that amount was 2–3-fold higher than the sustained $[Ca^{2+}]i$. The transient increase of $[Ca^{2+}]i$ was faster than the sustained one. Therefore, the $Ca^{2+}$ influx pathway must be more active during the transient than the sustained phase of $[Ca^{2+}]i$ increase. If that was true, the biochemical and the pharmacological behavior of the $Ca^{2+}$ entry pathway should have been exactly opposite to what was observed.

The attenuation in $Ca^{2+}$-free medium and the complete suppression by TMB-8 of the transient phase of increased $[Ca^{2+}]i$ suggest that the $Ca^{2+}$ entry through the second pathway and $Ca^{2+}$ release are tightly linked with each other. There could be four possible ways that they can be linked together. One possibility is that emptying of the $Ca^{2+}$ stores triggered the $Ca^{2+}$ entry through this second pathway, resulting in the higher amplitude of the transient phase of $[Ca^{2+}]i$ in the presence of extracellular $Ca^{2+}$. If it was true, $Ca^{2+}$ entry through the second pathway should have followed, instead of occurring simultaneously with, the $Ca^{2+}$ release, giving a phase differences between these two. In other words, three phases of increase of $[Ca^{2+}]i$ should have occurred; a small elevation due to release, as seen in $Ca^{2+}$-free medium, followed by a relatively larger increase of $[Ca^{2+}]i$ due to $Ca^{2+}$ entry through the second pathway, and then the sustained phase due to $Ca^{2+}$ influx through the influx pathway. Besides, addition of $Ca^{2+}$ to the cells, stimulated for 10 min, resulted only in the sustained influx. If the empty state of $Ca^{2+}$ store triggered $Ca^{2+}$ entry through the second pathway, then both the transient and the sustained increase of $[Ca^{2+}]i$ should have occurred. The second possibility is that the entry of $Ca^{2+}$ through the second pathway stimulated $Ca^{2+}$ release through the calcium-induced calcium release (CICR) mechanism [Berridge, 1993]. Thus, a small elevation of $[Ca^{2+}]i$ would trigger its own release from the intracellular stores. However, these possibilities can be ruled out based on our findings that: (1) addition of low $[Ca^{2+}]o$ (0.25 mM) after 80 sec of stimulation, resulted in the near maximal (90% of maximal) sustained $Ca^{2+}$ influx, without an appreciable change in the transient phase of $[Ca^{2+}]i$, and (2) addition of $Ca^{2+}$ to the cells, stimulated for 10 min, resulted in sustained $Ca^{2+}$ influx, but not the release. If CICR mechanism was true, then both the transient and the sustained phases of increased $[Ca^{2+}]i$ should have been seen in both of those situations. The third possibility is that $Ca^{2+}$ entry through the second pathway enhanced the IP3 action; increase of $Ca^{2+}$ concentration up to 300 nM augmented and beyond 300 nM suppressed the IP3-stimulated $Ca^{2+}$ release [Finch et al., 1991]. Our findings indicated that αCD3 stimulation of T cells caused IP3 accumulation sufficiently by 60 sec and maximally by 120 sec. However, addition of 0.25 mM $[Ca^{2+}]o$ to the cells after 80 sec of stimulation, resulted only in the sustained increase of $[Ca^{2+}]i$ to about 200 nM. If $Ca^{2+}$ augmented the IP3 action, then a significant amount of transient increase of $[Ca^{2+}]i$ should have occurred, after adding 0.25 mM $[Ca^{2+}]o$ at 80 sec of stimulation. Besides, the enhancing effect of $Ca^{2+}$ on IP3 action has become disputable, because several investigators failed to demonstrate this [Combettes and Champeil, 1994]. Finally, it is possible that the second $Ca^{2+}$ entry pathway was involved in the direct refilling of the $Ca^{2+}$ stores by extracellular $Ca^{2+}$ (refill pathway). The latter in turn regulated $Ca^{2+}$ release in the presence of IP3, such that extracellular $Ca^{2+}$ entered the partially empty $Ca^{2+}$ stores and was released into the

cytosol by IP3. This model accounts for the simultaneous occurrence of $Ca^{2+}$ release and $Ca^{2+}$ entry through the second pathway. In addition, the most crucial findings that could support this model were that refilling of the intracellular stores can occur without any increase in $[Ca^{2+}]i$ [Aub et al., 1982; Byron et al., 1992; Putney, 1977], and this can be prevented by $La^{3+}$ or $Ni^{2+}$ [Aub et al., 1982].

The model of refill pathway can also account for many findings in physiologically relevant ways. To coordinate between refilling of the $Ca^{2+}$ stores and IP3-stimulated $Ca^{2+}$ release, luminal $Ca^{2+}$ must have the opposite regulatory effects on the refill pathway as the IP3-operated $Ca^{2+}$ channel, IP3 receptor. Thus, we hypothesize that luminal $Ca^{2+}$ has a positive effect on the IP3-receptor channel and a negative one on the refill pathway. When $Ca^{2+}$ stores are full, the IP3 receptor is sensitive to IP3 and the refill pathway remains inactive. Consequently, after the IP3 stimulated $Ca^{2+}$ release, the IP3 receptor becomes insensitive to IP3 and the refill pathway is opened, allowing the entry of $Ca^{2+}$ into the empty store. The process of $Ca^{2+}$ release and refilling of the $Ca^{2+}$ store would continue as long as IP3 is present. This hypothesis is partly supported by the findings that partially depleted $Ca^{2+}$ stores are less sensitive to IP3 [Missiaen et al., 1992] and luminal $Ca^{2+}$ increases the affinity of IP3 for its receptor [Oldershaw and Taylor, 1993]. The physiological significance of dependence of IP3 stimulated $Ca^{2+}$ release on $[Ca^{2+}]o$ is not clear. One possibility is that this may participate in the oscillatory release of $Ca^{2+}$ from the intracellular stores. Oscillatory $Ca^{2+}$ release has been demonstrated in many cell types [Ferris et al., 1992; Oldershaw et al., 1991; Parker and Ivora, 1990]. However, the mechanism by which that occurs is not known. Based on our present findings, we believe that initial release of $Ca^{2+}$ by IP3 would empty the $Ca^{2+}$ store. This would allow extracellular $Ca^{2+}$ to enter the empty store and then be released into the cytosol by the action of IP3. Repetition of this process would result in an oscillatory $Ca^{2+}$ release. The asynchronous oscillation of $Ca^{2+}$ release in single cells should summate to a smooth transient rise of $[Ca^{2+}]i$ in a population of cells. In fact, in many single cell studies, a biphasic pattern of calcium oscillation was present, which includes a transient phase of higher amplitude followed by a sustained phase of moderate amplitude [Lewis and Cahalan, 1989; Li et al., 1994; Wacholtz and Lipsky, 1993; Zhang and Muallem, 1992; Zhao et al., 1990].

The proposed model accommodates not only all the present findings, but also the previous findings of which many appeared contradictory. A combined electrophysiological and fluorescence spectrophotometric study at the single cell level is necessary to further understand the nature of the $Ca^{2+}$ entry pathways and the $Ca^{2+}$ signaling mechanism in nonexcitable cells.

## ACKNOWLEDGMENTS

This work was supported by research grant CA 47050 from the National Institute of Health. We are sincerely grateful to Drs. Neil E. Hubbard, Richard Nuccitelli, Paul Thomas, and Gautam Bandyopadhyay for critical evaluation of the manuscript and to David Araya and Debora Lim for technical assistance.

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