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Increased Intracellular Ca^{2+} Induces Ca^{2+} Influx in Human T Lymphocytes

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One current hypothesis for the initiation of Ca^{2+} entry into nonelectrically excitable cells proposes that Ca^{2+} entry is linked to the state of filling of intracellular Ca^{2+} stores. In the human T lymphocyte cell line Jurkat, stimulation of the antigen receptor leads to release of Ca^{2+} from internal stores and influx of extracellular Ca^{2+}. Similarly, treatment of Jurkat cells with the tumor promoter thapsigargin induced release of Ca^{2+} from internal stores and also resulted in influx of extracellular Ca^{2+}. Initiation of Ca^{2+} entry by thapsigargin was blocked by chelation of Ca^{2+} released from the internal storage pool. The Ca^{2+} entry pathway also could be initiated by an increase in the intracellular concentration of Ca^{2+} after photolysis of the Ca^{2+}-cage, nitr-5. Thus, three separate treatments that caused an increase in the intracellular concentration of Ca^{2+} initiated Ca^{2+} influx in Jurkat cells. In all cases, Ca^{2+}-initiated Ca^{2+} influx was blocked by treatment with any of three phenothiazines or W-7, suggesting that it is mediated by calmodulin. These data suggest that release of Ca^{2+} from internal stores is not linked capacitatively to Ca^{2+} entry but that initiation is linked instead by Ca^{2+} itself, perhaps via calmodulin.

INTRODUCTION

The signal transduction cascade leading to receptor-mediated release of Ca^{2+} from internal storage pools after the generation of inositol 1,4,5-trisphosphate ($InsP_3$)[1] is well described (Berridge and Irvine, 1989). However, in T lymphocytes, as in many cell types, the regulatory mechanism for receptor-mediated influx of Ca^{2+} is not known. One proposal for the regulation of influx is the capacitative model (Putney, 1986). This model suggests that initiation of Ca^{2+} entry is controlled by the state of filling of intracellular Ca^{2+} stores. The model is derived from experiments showing that emptying of the Ca^{2+} stores into the cytosol in a variety of cell types is linked to activation of Ca^{2+} entry (Merritt and Rink, 1987; Pandol *et al.*, 1987; Takemura *et al.*, 1989; Kass *et al.*, 1990). The early version of the capacitative model of Ca^{2+} influx postulated a direct physical link between the Ca^{2+} entry pathway and intracellular Ca^{2+} stores (Putney, 1986). However, further experimentation suggested that a direct link need not exist and that Ca^{2+} enters the cytosol before sequestration in intracellular stores (Takemura *et al.*, 1989; Kwan and Putney, 1990). In the absence of a direct link, this current version requires an unknown second messenger to signal the filling state of the stores to the Ca^{2+} entry pathway.

Stimulation of the T lymphocyte antigen receptor, either through contact with an antigenically relevant target cell (Gray *et al.*, 1987) or with monoclonal antibody directed at the CD3 component of the antigen receptor (Weiss *et al.*, 1984; Haverstick *et al.*, 1991), results in a two-component increase in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). The rise in $[Ca^{2+}]_i$ has been shown to be due to an initial release of Ca^{2+} from intracellular stores followed by a sustained influx of extracellular Ca^{2+} (Weiss *et al.*, 1984; Nisbet-Brown *et al.*, 1985; Gray *et al.*, 1987). Although it is possible to pharmacologically manipulate the changes in $[Ca^{2+}]_i$ such that influx can occur without release and vice versa (Haverstick *et al.*, 1991), these two events, along with activation of protein kinase C, previously have been shown to be necessary for cytolytic T lymphocyte function (Haverstick *et al.*, 1991).

The capacitative model assumes that the state of filling of the internal stores directly initiates Ca^{2+} entry (Putney, 1986). Thus, presumably, filled stores would inactivate Ca^{2+} entry. The internal release component

[1] Abbreviations used: $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $InsP_3$, inositol 1,4,5-trisphosphate; BAPTA, bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate.

constitutes <20% of the total rise in $[Ca^{2+}]_i$ after antigen receptor stimulation of T lymphocytes, with the remaining increase due to Ca^{2+} entry (Haverstick *et al.*, 1991). Two possibilities could account for the fact that the magnitude of influx is in apparent excess of the amount needed to refill intracellular stores. Refilling of the stores may be a slow process with concomitant Ca^{2+} leak out of the cytosol. Alternatively, although the trigger for influx may be depletion of stores, filled stores may not immediately feedback to shut down influx. However, it is also possible that the state of filling of the internal stores is not directly sensed by the Ca^{2+} entry pathway.

To examine the initiation of the Ca^{2+} entry pathway, we have used three separate methods to increase $[Ca^{2+}]_i$ in Jurkat cells. Two of these methods, one receptor-dependent and the other receptor-independent, resulted in release of Ca^{2+} from internal stores, whereas the third directly elevated Ca^{2+} by photolysis of a Ca^{2+} cage. The total increase in $[Ca^{2+}]_i$ resulting from all three treatments was in large part due to the influx of extracellular Ca^{2+}. Treatment with calmodulin inhibitors blocked influx under all three conditions, suggesting the involvement of calmodulin in the pathway by which elevated $[Ca^{2+}]_i$ induces Ca^{2+} influx.

MATERIALS AND METHODS

Reagents

The human T-lymphocyte cell line, Jurkat, was maintained in RPMI-1640 (BioWhittaker, Walkersville, MD) supplemented with 5% fetal bovine serum. Monoclonal antibody directed at the CD3 component of the T-cell antigen receptor (Manger *et al.*, 1987) was grown and purified using published techniques (Andrew and Titus, 1991). W-7, DEDA, H-89, and KT5720 were all purchased from CalBiochem (La Jolla, CA). All other reagents were purchased from Sigma Chemical (St. Louis, MO).

Determination of $[Ca^{2+}]_i$

Jurkat cells were washed, suspended in culture medium, and incubated for 1 h at 37°C in the presence of the acetoxymethyl ester of the Ca^{2+}-indicator dye indo-1 (indo-1/AM; Molecular Probes, Eugene, OR) at 1 μM. Cells were washed three times in buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 140 mM NaCl, 0.1% glucose, 1% fetal bovine serum) and suspended at a final concentration of 1×10^6/ml in buffer A. The $[Ca^{2+}]_i$ was determined from the fluorescence ratio (398:480 nm) with excitation at 340 nm in an SLM 8000 spectrofluorometer (SLM/Aminco, Urbana, IL) in the T-format as previously described (Gray *et al.*, 1987). Calibration was conducted as published previously (Gray *et al.*, 1987) using parameters developed for earlier generations of Ca^{2+}-indicator dyes (Grynkiewiz *et al.*, 1985; Scanlon *et al.*, 1987).

Mn^{2+} Quench of Indo-1 Fluorescence

Measurement of Ca^{2+} entry by quench of indo-1 fluorescence intensity was carried out using previously published procedures (Gray *et al.*, 1987). For the data of Figures 6B and 10B, cells were incubated as above, washed in buffer A, and resuspended in modified buffer A (buffer A without 1 mM $CaCl_2$ and with 1 mM $MnCl_2$). For these figures, the data are plotted as relative fluorescence intensity to demonstrate the quench of indo-1 fluorescence emission at 398 nm. Monitoring of fluorescence emission at this wavelength is a sensitive indicator of Ca^{2+} entry (Gray *et al.*, 1987; Mason *et al.*, 1991).

Increasing $[Ca^{2+}]_i$ via the Ca^{2+} Cage, nitr-5

Cells were incubated simultaneously with 1 μM indo-1/AM for determination of $[Ca^{2+}]_i$ and 0.5 μM of the acetoxymethyl ester derivative of the calcium chelator nitr-5 (nitr-5/AM; CalBiochem) for 1 h at 37°C under the conditions outlined above. Cells were washed three times in buffer A and suspended at a final concentration of 1×10^6/ml. After collecting a baseline reading, photolysis of nitr-5 was accomplished by flashing the cuvette four times within 1 min using a xenon flash lamp apparatus (model JML-87; Gert Rapp, Dossenheim, Germany).

Chelation of Intracellular Free Ca^{2+} by BAPTA

To chelate intracellular free Ca^{2+}, Jurkat cells were simultaneously incubated with 1 μM indo-1/AM and 10 μM BAPTA/AM under the conditions outlined above. The presence of BAPTA had no effect on the spectral characteristics of indo-1 (see RESULTS).

Statistical Analyses

Measurement of the percent inhibition of changes in $[Ca^{2+}]_i$ under the various conditions were made with GraphPAD software (GraphPAD, San Diego, CA) using the computer calculated measure of the area under the curve during the 120-s period immediately after the addition of stimuli. This area, as a measure of the total releasable pool, was normalized to the area obtained in the absence of the inhibitor and expressed as a percent. In all cases, the comparison was made from samples prepared in parallel to minimize day to day variability. IC_{50} values for the data presented in Table 3 were determined using the same software package.

RESULTS

Both Receptor-Independent and Receptor-Dependent Release of Ca^{2+} from Intracellular Stores Initiate Ca^{2+} Entry

Thapsigargin, by inhibiting the endoplasmic reticulum Ca uptake pump (Thastrup *et al.*, 1990), depletes $InsP_3$-sensitive intracellular stores of Ca^{2+} without generating $InsP_3$ (Takemura *et al.*, 1989; Gouy *et al.*, 1990), presumably by allowing uncompensated Ca^{2+} leak (Thastrup *et al.*, 1990). In buffer containing Ca^{2+}, a variety of cell types show prolonged influx of extracellular Ca^{2+} resulting from release of Ca^{2+} from internal stores after treatment with thapsigargin (Takemura *et al.*, 1989; Gouy *et al.*, 1990; Mason *et al.*, 1991; Menniti *et al.*, 1991; Sarkadi *et al.*, 1991). Such receptor-independent Ca^{2+} influx can also be seen in Jurkat cells. Treatment with 1 μM thapsigargin resulted in a sustained increase in $[Ca^{2+}]_i$ (Figure 1A, solid trace). This change in $[Ca^{2+}]_i$ was due to both release of Ca^{2+} from internal stores and influx of extracellular Ca^{2+} because a portion of the increase was inhibited by Ni^{2+} (Figure 1A, dotted trace) and Ni^{2+} is known to block Ca^{2+} entry through blockage of Ca^{2+} channels (Hagiwara and Takahashi, 1967; Sage and Rink, 1987). For the data shown in Figure 1A, there was an 83% reduction in the increase in $[Ca^{2+}]_i$ in the presence of Ni^{2+}. This value is representative of several

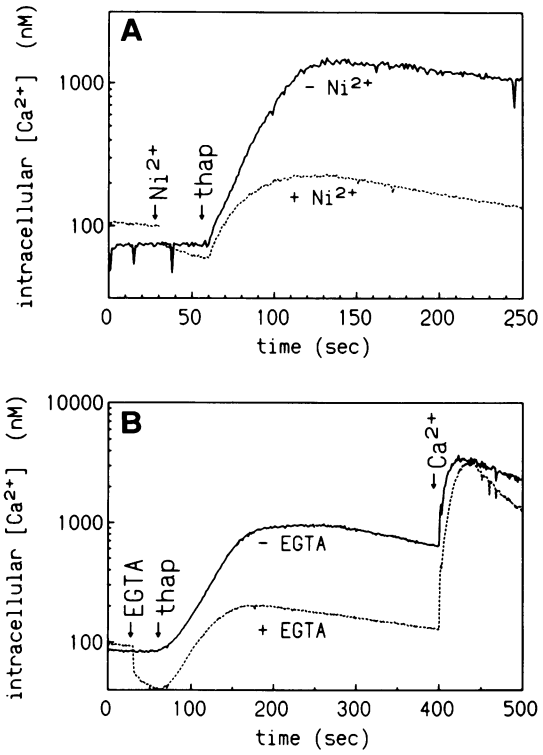

Figure 1. Receptor-independent increases in $[Ca^{2+}]_i$. (A) Changes in $[Ca^{2+}]_i$ were monitored after the addition of 1 μM thapsigargin (thap) at 60 s in the absence (solid trace) or presence (dotted trace) of 10 mM $NiCl_2$ (Ni^{2+}), added at 30 s. (B) Changes in $[Ca^{2+}]_i$ were monitored after the addition of 1 μM thapsigargin (thap) in the absence (solid trace) or presence (dotted trace) of chelation of extracellular Ca^{2+} (EGTA). At 400 s (Ca^{2+}), the extracellular buffer in both experiments was brought to 5 mM free extracellular Ca^{2+}. All traces are the means of three determinations.

experiments as outlined in Table 1. Similar results were seen following chelation of extracellular Ca^{2+} (Sarkadi *et al.*, 1991) (Figure 1B).

Initiation of the Ca^{2+} entry pathway by thapsigargin did not require the presence of extracellular Ca^{2+}. Chelation of extracellular Ca^{2+} by ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Figure 1B, dotted trace) reduced by 78% the extent of the increase in $[Ca^{2+}]_i$ as compared with that seen when extracellular Ca^{2+} was present (Figure 1B, solid trace). However, addition of free Ca^{2+} to a final concentration of 5 mM at a later time caused $[Ca^{2+}]_i$ to increase to comparable levels in both cases (Figure 1B). These data indicate that the Ca^{2+} entry pathway had been opened by thapsigargin treatment even in the absence of extracellular Ca^{2+}. Similar results in other cell types have been taken as evidence in support of the capacitative model (Takemura *et al.*, 1989). However, in a general sense, these results are consistent with any model in which increased $[Ca^{2+}]_i$ activates a pathway that, in turn, initiates Ca^{2+} entry. The mechanism by which the pathway is inactivated remains a matter of speculation, although the data suggest that elevated $[Ca^{2+}]_i$ is not required for continued influx of extracellular Ca^{2+}.

Elevated $[Ca^{2+}]_i$ is also seen in Jurkat cells after stimulation of the antigen receptor. Subsequent to receptor stimulation, there is generation of $InsP_3$ and release of Ca^{2+} from $InsP_3$-sensitive intracellular Ca^{2+} stores (Imboden and Stobo, 1985). Similar to thapsigargin treatment, receptor-induced release of Ca^{2+} from intracellular stores was followed by influx of extracellular Ca^{2+}. Compared with the rise in the absence of extracellular Ni^{2+} (Figure 2A, solid trace), there was an 81% reduction in the extent of the increase in $[Ca^{2+}]_i$ in its presence (Figure 2A, dotted trace). This is similar to the reduction in the effect of thapsigargin by EGTA (78%, Figure 1B) or Ni^{2+} (83%, Figure 1A). These data are representative of several experiments (Table 1).

The effects of Ni^{2+} described thus far are not due to a change in the spectral characteristics of indo-1. As shown in Figure 2B, the emission scan of indo-1 was virtually identical in the presence or absence of Ni^{2+}. There was a slight (2 nm) red shift in the presence of Ni^{2+}, but because the slit widths of the excitation and emission filters were set at 4 nm, this difference is unlikely to be a valid one. Regardless of this slight shift, the R_{max} and R_{min} in the absence and presence of Ni^{2+} were 4.64/4.59 and 0.81/0.83, respectively. Conse-

Table 1. Comparison of the increase in $[Ca^{2+}]_i$ in the presence and absence of influx of extracellular Ca^{2+}

Conditions	Range of inhibition (%) (n)
Thapsigargin[a]	
Plus Ni^{2+}	79–84 (5)
Plus EGTA	78–85 (6)
OKT3[b]	
Plus Ni^{2+}	78–83 (5)
Plus EGTA	79–82 (6)
Plus Ni^{2+} (nitr-5)[c]	77–82 (4)
OKT3, Ni^{2+}, thapsigargin[d]	77–84 (4)
OKT3, Ni^{2+}, thapsigargin[e]	77–83 (4)

[a] Percent inhibition of the increase in $[Ca^{2+}]_i$ after treatment of Jurkat cells with 100 μM thapsigargin with prior addition of 10 mM $NiCl_2$ or 5 mM EGTA compared with treatment with thapsigargin alone.

[b] Percent inhibition of the increase in $[Ca^{2+}]_i$ after treatment of Jurkat cells with 1 μg/ml anti-CD3 antibody OKT3 with prior addition of 10 mM $NiCl_2$ or 5 mM EGTA compared with antibody treatment alone.

[c] Percent inhibition of the increase in $[Ca^{2+}]_i$ after treatment of Jurkat cells with 1 μg/ml OKT3 with prior addition of 10 mM $NiCl_2$ and photolysis of nitr-5.

[d] Percent inhibition of the increase in $[Ca^{2+}]_i$ after treatment of Jurkat cells with 1 μg/ml OKT3, followed by 10 mM $NiCl_2$, and then 100 μM thapsigargin, compared with treatment with thapsigargin alone.

[e] Percent inhibition of the increase in $[Ca^{2+}]_i$ after treatment of Jurkat cells with 1 μg/ml OKT3, followed by 10 mM $NiCl_2$, and then 100 μM thapsigargin, compared with treatment with antibody alone.

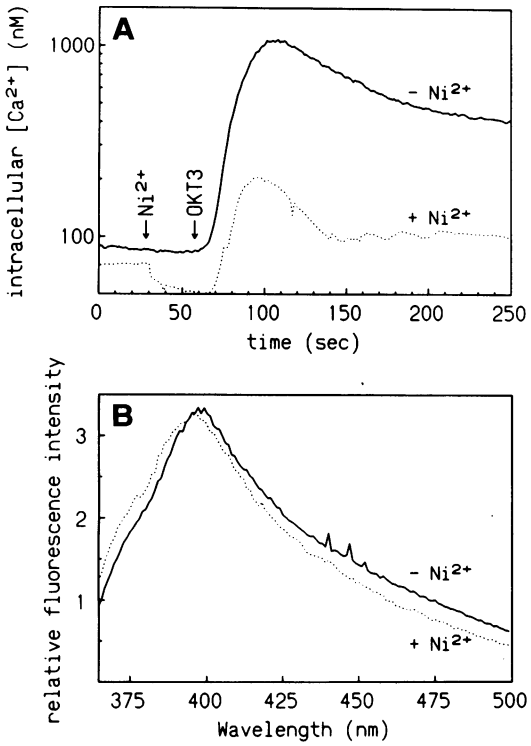

Figure 2. Receptor-dependent changes in $[Ca^{2+}]_i$. (A) Changes in $[Ca^{2+}]_i$ were monitored after stimulation of Jurkat cells with 1 μg/ml of anti-CD3 monoclonal antibody OKT3 (OKT3) at 30 s as outlined in MATERIALS AND METHODS in the absence (solid trace) or presence (dotted trace) of prior addition of Ni^{2+} at 10 mM (Ni^{2+}, 30 s). All traces are the means of three determinations. (B) Emission scan of indo-1 free acid in 1 mM Ca^{2+} in the absence (solid trace) or presence (dotted trace) of 10 mM $NiCl_2$. Excitation was at 340 nm, and emission was determined from 360 nm to 500 nm. The traces are representative of >10 determinations.

quently, β' used for calculation of $[Ca^{2+}]_i$ (Grynkiewiz *et al.*, 1985; Scanlon *et al.*, 1987) was also unchanged by the presence of Ni^{2+}.

Sustained Influx of Extracellular Ca^{2+} is not Dependent on Empty Intracellular Stores

As shown in Figure 3 (dotted trace), the receptor-induced rise in $[Ca^{2+}]_i$ lasted ≥300 s. During the sustained portion of this receptor-dependent increase, $[Ca^{2+}]_i$ could be reduced rapidly to near basal levels by addition of Ni^{2+} to the extracellular buffer (Figure 3, solid trace). Because Ni^{2+} is known to affect Ca^{2+} entry (Hagiwara and Takahashi, 1967; Haverstick *et al.*, 1991), these data suggest that the sustained portion of the increase in $[Ca^{2+}]_i$ was due entirely to influx.

Although this experiment suggested that release of Ca^{2+} from internal stores did not contribute to the sustained portion of elevated $[Ca^{2+}]_i$, it did not provide any information as to the status of the internal Ca^{2+} stores. To address this question, a second stimulation was applied after the Ni^{2+}-induced return of $[Ca^{2+}]_i$ to the basal level. The addition of thapsigargin (Figure 3, solid trace) led to a second increase in $[Ca^{2+}]_i$. Because Ni^{2+} was still present to block influx, the second increase was due only to internal release. The second increase in $[Ca^{2+}]_i$ was 79% less than the change in $[Ca^{2+}]_i$ after receptor stimulation and 82% less than the change induced by treatment of Jurkat cells with thapsigargin without prior receptor stimulation. These values are very similar to those shown in Figures 1 and 2 and are representative of multiple experiments (Table 1). Because the amount of Ca^{2+} released from the intracellular Ca^{2+} stores was virtually identical under all conditions, these data suggest that the internal stores were refilled at the time of the second receptor-independent stimulation. As shown in Figure 3, the elevation of $[Ca^{2+}]_i$ at the time of thapsigargin treatment was mediated solely by Ca^{2+} entry. Taken together, these data indicate that refilling of intracellular Ca^{2+} stores occurs soon after initiation of Ca^{2+} influx and that depleted intracellular stores do not directly regulate the sustained portion of the increase in $[Ca^{2+}]_i$.

Ca^{2+} Influx can be Initiated by a Rise in $[Ca^{2+}]_i$ in the Absence of Release of Ca^{2+} from Internal Stores

Although the data thus far are generally consistent with the capacitative model for regulation of Ca^{2+} influx (Takemura *et al.*, 1989; Kwan and Putney, 1990), there remains an alternative explanation. It is possible that the Ca^{2+} released from the internal stores initiates a pathway that remains active after repletion of the stores. To address this possibility, the effects of an increase in $[Ca^{2+}]_i$ in the absence of release of Ca^{2+} from internal stores on Ca^{2+} entry was examined. The photolabile

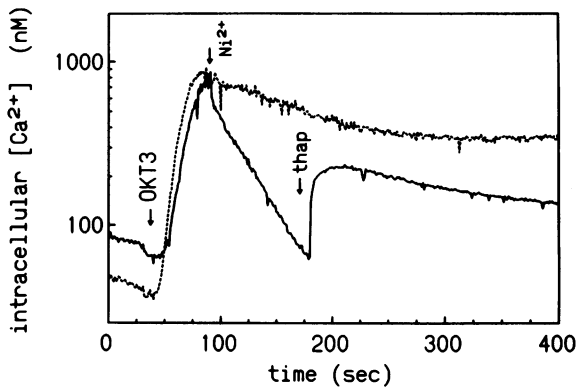

Figure 3. Rapid refilling of intracellular Ca^{2+} stores after antigen receptor stimulation. Changes in $[Ca^{2+}]_i$ were monitored after stimulation of Jurkat cells with 1 μg/ml of anti-CD3 monoclonal antibody OKT3 (OKT3) at 30 s as outlined in MATERIALS AND METHODS (solid and dotted trace). Ni^{2+} indicates the addition of 10 mM $NiCl_2$ at 90 s (solid trace). At 180 s, these cells were further stimulated with 1 μM thapsigargin (thap). All traces are the means of three determinations.

calcium chelator nitr-5 releases Ca^{2+} after illumination at 360 nm due to a change in K_d from ~150 nM to 6 μM (Tsien and Zucker, 1986). Jurkat cells were incubated with indo-1/AM and nitr-5/AM, washed, and examined in a spectrofluorometer (Figure 4A) to determine the resting $[Ca^{2+}]_i$. The cell suspension was then exposed to high-intensity light for 1 min (Figure 4A, fl). During exposure to the flash lamp, there was a rise in $[Ca^{2+}]_i$ of about 225 nM in the presence of extracellular Ca^{2+} (Figure 4A, solid trace). This increase was likely due, at least in part, to the release of Ca^{2+} from nitr-5 because it was not seen in cells incubated with indo-1/AM alone and exposed to the lamp (Figure 4B, dashed trace) nor in cells that had been incubated with nitr-5/AM but were not exposed to the flash lamp. In addition to Ca^{2+} released from nitr-5, a portion of the increase in $[Ca^{2+}]_i$ seen after exposure to the flash lamp apparatus was due to influx of extracellular Ca^{2+}, as outlined below.

Cells incubated with both indo-1/AM and nitr-5/AM and suspended in buffer containing Ni^{2+} showed a rise in $[Ca^{2+}]_i$ after exposure to the flash lamp of smaller magnitude (65 nM) than cells suspended in buffer without Ni^{2+} (225 nM). Additionally, for cells in buffer containing Ni^{2+}, there was no further rise after the release of Ca^{2+} from nitr-5 (Figure 4A, dotted trace). However, cells suspended in buffer containing Ca^{2+} but not Ni^{2+} showed a continued rise in $[Ca^{2+}]_i$ after photolysis (Figure 4A, solid trace). Because this second component of the increase in $[Ca^{2+}]_i$ was blocked by extracellular Ni^{2+} (Figure 4A) or chelation of extracellular Ca^{2+} by EGTA, it was due to influx of extracellular Ca^{2+}. Taken together, these results indicate that increased $[Ca^{2+}]_i$, in the likely absence of release of Ca^{2+} from intracellular stores, can initiate Ca^{2+} entry by some mechanism.

It was possible that the changes in the Ca^{2+} signal were due to damage to the cells after exposure of the cell suspension to the flash lamp apparatus. This was unlikely because there was no change in $[Ca^{2+}]_i$ when cells were exposed to the flash lamp in the absence of nitr-5 (Figure 4B, dashed trace). Additionally, after the change in $[Ca^{2+}]_i$ in response to flash photolysis of nitr-5, it was possible to further increase $[Ca^{2+}]_i$ by antigen receptor stimulation (Figure 4B, solid and dotted trace). The receptor-induced increase seen in the presence of Ca^{2+} influx was similar in magnitude to that seen in cells that were not incubated with nitr-5/AM (Figure 2A). These results suggest that nitr-5 had not disrupted the functional connection between release of Ca^{2+} from internal stores and entry of extracellular Ca^{2+}. Similarly, in the presence of Ni^{2+}, the magnitude of the rise in $[Ca^{2+}]_i$ in response to receptor stimulation was nearly as great in the presence of nitr-5 as in its absence (Figure 2A, dotted trace). In both cases the maximum $[Ca^{2+}]_i$ reached was the same (~110 nM), and the percent reduction compared with antigen receptor stimulation in the absence of Ni^{2+} was similar (81 vs. 75%, in the absence and presence of photolysis, respectively). These data suggest that the increase in $[Ca^{2+}]_i$ seen during photolysis was not due to damage to the cell suspension.

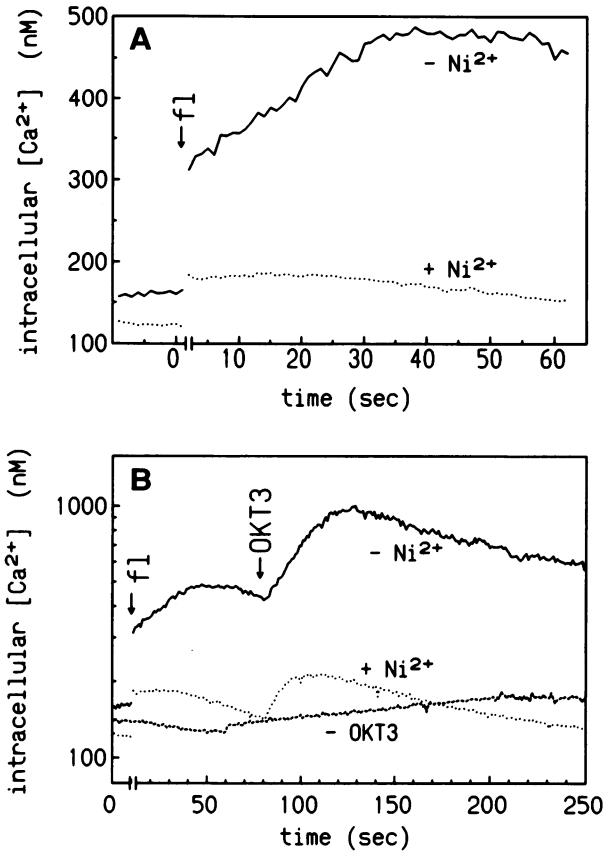

Figure 4. Effect of a rise in $[Ca^{2+}]_i$ on Ca^{2+} influx. (A) Cells were incubated with 1 μM indo-1/AM and 0.5 μM nitr-5/AM as outlined in MATERIALS AND METHODS. Prior addition of 10 mM $NiCl_2$ to the cell suspension shown by the dotted trace is indicated by $+Ni^{2+}$. fl indicates exposure to the flash lamp apparatus as outlined in MATERIALS AND METHODS. (B) Jurkat cells incubated with indo-1/AM and nitr-5/AM were exposed to the flash lamp in the absence ($-Ni^{2+}$) or presence ($+Ni^{2+}$) of 10 mM $NiCl_2$ as in A and then treated with 1 μg/ml of OKT3 (OKT3). Cells shown by the dashed trace (−OKT3) were incubated with indo-1/AM in the absence of nitr-5/AM and exposed to the flash lamp apparatus with no further treatment to show that there was no effect of the flash lamp on the spectral characteristics of indo-1. Traces are the means of three determinations.

There were two additional possibilities to explain the data shown in Figure 4. First, it is possible that, due to the Ca^{2+} buffering capacity of cytosolic nitr-5, Ca^{2+} was actively removed from the intracellular stores. In this case, the influx component could be due to nitr-5-depleted intracellular stores. However, when cells that had been incubated with nitr-5/AM were examined over 500 s, there was no change in $[Ca^{2+}]_i$. Thus, unphotolyzed nitr-5 with a very high Ca^{2+} affinity did not initiate Ca^{2+} entry, whereas photolyzed nitr-5 with a much lower Ca^{2+} affinity did initiate this pathway.

It is also possible that some portion of the increase in $[Ca^{2+}]_i$ seen after photolysis of nitr-5 was due to Ca^{2+}-induced Ca^{2+} release from internal stores (Randriamampita *et al.*, 1991). To address this possibility, the thapsigargin-sensitive internal Ca^{2+} stores were examined in cells incubated with indo-1/AM alone or in combination with nitr-5/AM. When Ca^{2+} influx was blocked by the addition of Ni^{2+}, there was no difference between the change in $[Ca^{2+}]_i$ after thapsigargin treatment of cells incubated with indo-1/AM alone (Figure 5, dotted trace) and incubated in the presence of both indo-1/AM and nitr-5/AM (Figure 5, solid trace). These data indicate, as suggested above, that the presence of unphotolyzed cytosolic nitr-5 did not reduce intracellular Ca^{2+} stores. Additionally, prior photolysis of nitr-5 did not alter the magnitude of the thapsigargin response (Figure 5, dashed trace). Therefore, it is unlikely that the Ca^{2+} released from nitr-5 was inducing Ca^{2+} release from intracellular stores. Taken together, the data presented in Figures 4 and 5 suggest that a rise in $[Ca^{2+}]_i$, independent of the status of the intracellular Ca^{2+} stores, initiates Ca^{2+} entry.

Chelation of Intracellular Free Ca^{2+} Eliminates the Thapsigargin-Induced Initiation of Ca^{2+} Entry

The data thus far indicate that Ca^{2+} itself is the mediator of Ca^{2+} entry. If this were the case, then chelation of intracellular free Ca^{2+} should block initiation of Ca^{2+} entry. To chelate intracellular free Ca^{2+}, Jurkat cells were incubated with the acetoxymethyl ester of BAPTA. As shown in Figure 6A, when Jurkat cells were incubated with both indo-1/AM and BAPTA/AM, the normally occurring increase in $[Ca^{2+}]_i$ after thapsigargin treatment

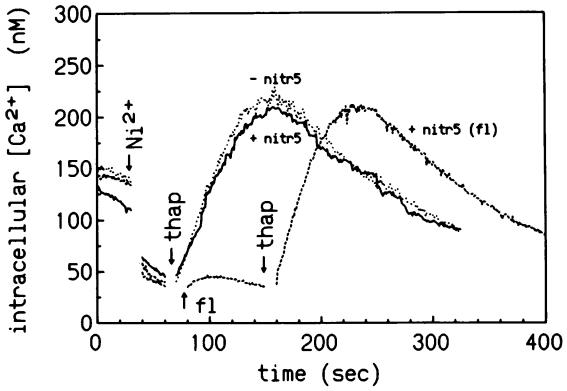

Figure 5. Effect of nitr-5 on thapsigargin-induced release of Ca^{2+} from intracellular stores. Changes in $[Ca^{2+}]_i$ were monitored as outlined in MATERIALS AND METHODS. Under all conditions, influx of extracellular Ca^{2+} was blocked by addition of 10 mM $NiCl_2$ at 30 s (Ni^{2+}). The response to 1 μM thapsigargin (thap) was monitored in cells incubated with indo-1/AM alone (−nitr5, dotted trace) or with indo-1/AM and nitr-5/AM in the absence (+nitr5, solid trace) and presence (+nitr5 (fl), dotted trace) of photolysis of nitr-5. Traces are the means of three determinations.

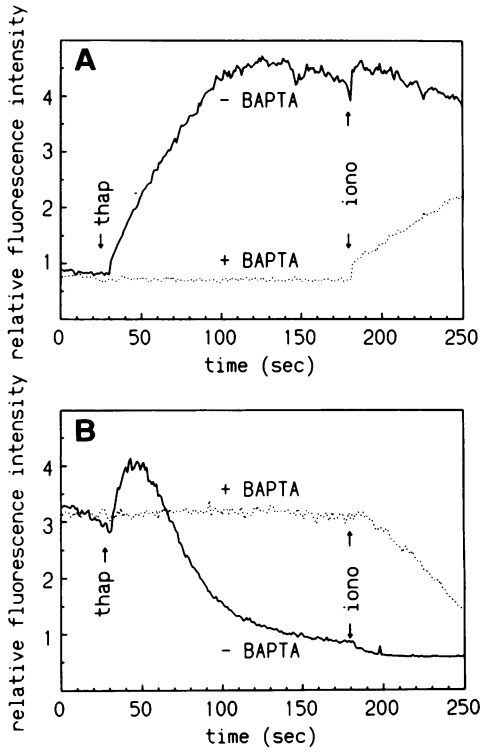

Figure 6. Effect of chelation of intracellular Ca^{2+} on Ca^{2+} entry. Changes in $[Ca^{2+}]_i$ were determined as a change in the fluorescence emission of indo-1 at 398 nm with excitation at 340 nm (Gray *et al.*, 1987; Mason *et al.*, 1991). (A) Changes in $[Ca^{2+}]_i$ after treatment with 1 μM thapsigargin (thap). Jurkat cells were incubated with indo-1/AM alone (solid trace) or with indo-1/AM and BAPTA/AM (dotted trace) and suspended in Ca^{2+}-replete buffer as outlined in MATERIALS AND METHODS. At 180 s, 5 μM ionomycin (iono) was added to the cuvette. (B) Cells incubated with indo-1/AM alone (solid trace) or with indo-1/AM and BAPTA/AM and resuspended in buffer containing 1 mM $MnCl_2$ in place of 1 mM $CaCl_2$ as outlined in MATERIALS AND METHODS. Treatment was as in A. All traces are representative of at least three experiments.

was completely inhibited. These data demonstrate that introduction of a Ca^{2+} chelator, which might deplete intracellular Ca^{2+} stores, did not induce Ca^{2+} entry. This is consistent with the effects of nitr-5 (Figures 4 and 5). Additionally, because chelation of free intracellular Ca^{2+} blocked Ca^{2+} entry, the data suggest that changes in $[Ca^{2+}]_i$ directly initiate Ca^{2+} entry.

This was further shown by taking advantage of the capacity of Mn^{2+} to quench indo-1 fluorescence. Mn^{2+} can substitute for Ca^{2+} in various Ca^{2+} entry pathways (Gray *et al.*, 1987), as opposed to Ni^{2+} which blocks Ca^{2+} entry. Quench of indo-1 fluorescence emission at 398 nm by Mn^{2+} indicates initiation of the Ca^{2+} entry pathway (Gray *et al.*, 1987). When indo-1 fluorescence was monitored in the absence of intracellular BAPTA and the presence of extracellular Mn^{2+} (Figure 6B, solid trace), the thapsigargin-induced changes in $[Ca^{2+}]_i$ were seen as an increase in the fluorescence emission due to

release of Ca^{2+} from internal stores followed by a decrease in the emission due to Ca^{2+} entry as reflected by quench of the emission signal by Mn^{2+} entry. However, when intracellular free Ca^{2+} was chelated by BAPTA, there was no change in the fluorescence emission of indo-1 induced by thapsigargin treatment (Figure 6B, dotted trace). These results suggest that chelation of intracellular free Ca^{2+}, released from the internal storage pools, inhibits the Ca^{2+} entry pathway and provide further evidence that initiation of Ca^{2+} entry is in response to an increase in $[Ca^{2+}]_i$.

The results shown in Figure 6 contradict those obtained by another laboratory (Mason *et al.*, 1991). Differences in methodology make direct comparison of our results with those described earlier (Mason *et al.*, 1991) difficult. Figure 6 shows that under the conditions used in the current study, there was complete elimination of thapsigargin-induced increases in $[Ca^{2+}]_i$ due to release from internal stores. Because of differences in experimental design, it is unclear if the methods used in the previous study resulted in a similarly complete elimination of changes in $[Ca^{2+}]_i$ due to release.

The effects of BAPTA shown in Figure 6 were not due to alterations in the spectral characteristics of indo-1 or to chelation of Mn^{2+} by BAPTA. As shown in Figure 7A, the emission intensity of indo-1 was reduced after the addition of Mn^{2+}, as would be expected from quench of indo-1 fluorescence by Mn^{2+}. Subsequent addition of BAPTA did not further alter the emission profile of the solution, suggesting that BAPTA was not acting as a Mn^{2+} chelator. As seen in Figure 7B, addition of BAPTA did not effect the emission intensity of indo-1 in the absence of added Ca^{2+}. With the subsequent addition of Mn^{2+}, however, there was quench of indo-1 emission. Taken together, the data of Figures 6 and 7 demonstrate that chelation of intracellular free Ca^{2+} blocks initiation of Ca^{2+} entry.

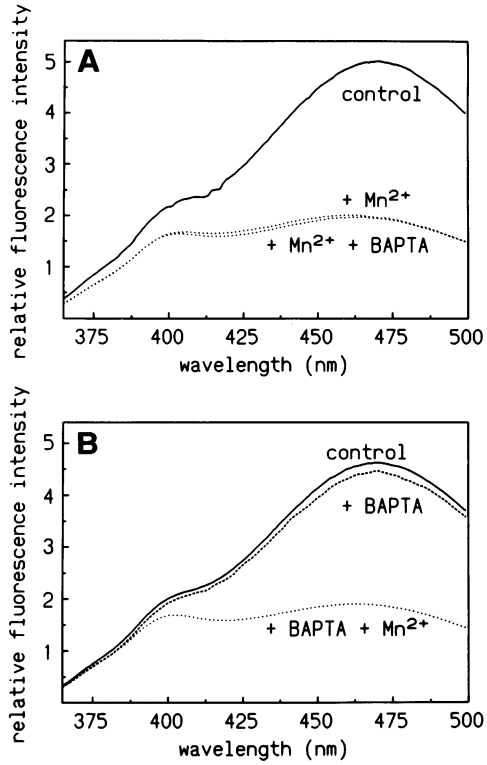

Figure 7. Effect of Mn^{2+} and BAPTA on the spectral characteristics of indo-1. (A) The fluorescence emission of indo-1 free acid in the absence of free Ca^{2+} (buffer A without $CaCl_2$) was monitored from 365 to 500 nm with excitation at 340 nm (solid trace). Mn^{2+} at a final concentration of 1 μM was then added and the solution rescanned (dotted trace). K_4BAPTA at 10 μM was then added and the solution scanned again (dashed trace). (B) The fluorescence emission of indo-1 free acid in the absence of free Ca^{2+} (buffer A without $CaCl_2$) was monitored from 365 to 500 nm with excitation at 340 nm (solid trace). K_4BAPTA at 10 μM was added and the solution scanned again (dotted trace). Mn^{2+} at a final concentration of 1 μM was added and the solution rescanned (dashed trace). Traces are representative of five determinations.

Ca^{2+} Entry can be Inhibited by Antagonists of Calmodulin

To determine the pathway responsible for the influx of Ca^{2+} in response to either a direct increase in $[Ca^{2+}]_i$ or one mediated by release of Ca^{2+} from internal stores, Jurkat cells were incubated with inhibitors of various protein kinases before stimulation with thapsigargin. As shown in Figure 8A (+calph), incubation with the relatively specific protein kinase C inhibitor calphostin C (Haverstick *et al.*, 1992) was without an effect on thapsigargin-induced increases in $[Ca^{2+}]_i$ (Figure 8A, control). Similar results were seen with another inhibitor of protein kinase C, staurosporine, as well as the protein kinase A inhibitors H-89 and KT5720 (Table 2). However, incubation with 10 μM trifluoperazine (Figure 8A, +tfp) blocked a major portion of the rise in $[Ca^{2+}]_i$ seen after treatment with thapsigargin. In addition to lowering the maximum $[Ca^{2+}]_i$ reached, in the presence of trifluoperazine the $[Ca^{2+}]_i$ returned to baseline rapidly. These results are similar to the response to thapsigargin in the absence of influx (Figure 1A). Addition of trifluoperazine similarly inhibited the increase in $[Ca^{2+}]_i$ seen after receptor stimulation (Figure 8B).

The phenothiazines, including trifluoperazine, inhibit the function of calmodulin by inhibiting the ability of activated calmodulin to bind to its molecular target (Hidaka and Ishikawa, 1992). As shown in Table 3, three compounds in this class of drugs inhibited the increase in $[Ca^{2+}]_i$ seen after thapsigargin treatment. In the experiment outlined in Table 3, the IC_{50} for inhibition of changes in $[Ca^{2+}]_i$ was 3.3 μM for trifluoperazine, 25 μM for chlorpromazine, and 63 μM for promethazine. These values are virtually identical to those previously published for inhibition of the function of activated calmodulin (Nemerow and Cooper, 1984).

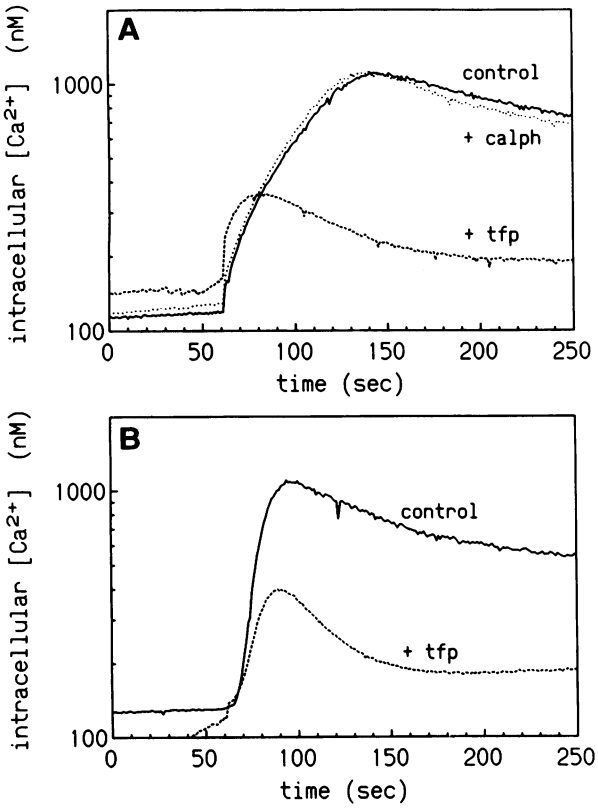

Figure 8. Effect of trifluoperazine on receptor-independent and receptor-dependent increases in $[Ca^{2+}]_i$. (A) Jurkat cells were incubated with 1 μM indo-1/AM, and changes in $[Ca^{2+}]_i$ were monitored as in MATERIALS AND METHODS. control, cells treated with 1 μM thapsigargin (thap) at 60 s; + calph, cells treated with 100 nM calphostin C at 30 s and then treated with thapsigargin at 60 s; + tfp, cells treated with 10 μM trifluoperazine (tfp) at 30 s and then treated with thapsigargin at 60 s. (B) Jurkat cells were incubated in the presence of 1 μM indo-1/AM and changes in $[Ca^{2+}]_i$ were monitored as in MATERIALS AND METHODS. control, cells treated with 1 μg/ml of OKT3 at 60s; + tfp, cells treated with 10 μM trifluoperazine (tfp) at 30 s and then treated with OKT3 at 60 s. All traces are the means of triplicate determinations.

In addition to inhibition of the function of activated calmodulin, the phenothiazines also have been reported to have effects on arachidonic acid metabolism. Thus, it was possible that the effects of trifluoperazine shown in Figure 8 were unrelated to calmodulin. However, as shown in Table 2, the more specific inhibitor of arachidonic acid metabolism, DEDA (Cohen *et al.*, 1984), was without an effect on receptor-independent increases in $[Ca^{2+}]_i$. Additionally, the calmodulin inhibitor W-7 (Hidaka *et al.*, 1981) did inhibit the increase in $[Ca^{2+}]_i$ seen in response to thapsigargin (Table 2). W-7 exerts its inhibitory actions in a manner entirely different from the phenothiazines. Rather than blocking the binding of activated calmodulin, W-7 and related compounds exert their actions by interfering with the activation of calmodulin by inhibiting Ca^{2+} binding to calmodulin

Table 2. Inhibition of thapsigargin-induced increases in $[Ca^{2+}]_i$

Conditions[a]	Change in $[Ca^{2+}]_i$ (nM)[b]
Control	913
Staurosporine, 100 nM	930
H-89, 100 nM	917
KT5720, 100 nM	925
DEDA, 100 nM	905
W-7, 100 μM	316

[a] Jurkat cells, incubated with indo-1/AM as outlined in MATERIALS AND METHODS, were stimulated with 1 μM thapsigargin 30 s after the addition of the indicated compounds. Control indicates no additions before thapsigargin.

[b] The change in $[Ca^{2+}]_i$ was calculated as the difference between the intracellular concentration of Ca^{2+} immediately before the addition of thapsigargin and the maximal $[Ca^{2+}]_i$ reached. In all cases, the maximal Ca^{2+} was obtained between 60 and 70 s after thapsigargin treatment.

(Hidaka and Ishikawa, 1992). Taken together, the data in Figure 8 and Tables 2 and 3 suggest that there is a component of the increase in $[Ca^{2+}]_i$ after release of Ca^{2+} from internal stores that is due to activation of a Ca^{2+}/calmodulin-dependent pathway.

It has been shown recently that certain inhibitors of calmodulin appear to directly block Ca^{2+} entry in a manner similar to Ni^{2+} (Li *et al.*, 1992). The data shown in Figure 9, however, indicate that neither trifluoperazine nor W-7 act in this way. When Ca^{2+} entry was initiated by either receptor engagement (Figure 9A) or

Table 3. Inhibition of thapsigargin-induced increases in $[Ca^{2+}]_i$ by phenothiazines[a]

Compound	Dose (μM)	Increased $[Ca^{2+}]_i$ (nM)	Percent inhibition[b]
None		1295	0
Trifluoperazine	10	300	77
	3	675	48
	1	945	27
	0.3	1220	6
Chlorpromazine	100	285	82
	30	890	31
	10	1125	13
	3	1270	2
Promethazine	100	350	73
	30	910	30
	10	1100	15

[a] Cells incubated with indo-1/AM were treated with the indicated dose of phenothiazines 30 s before stimulation with 1 μM thapsigargin. The increased $[Ca^{2+}]_i$ was calculated based on the difference between $[Ca^{2+}]_i$ immediately before addition of thapsigargin and the maximum value reached.

[b] Percent inhibition was calculated compared with the increase in $[Ca^{2+}]_i$ seen in the absence of phenothiazine treatment.

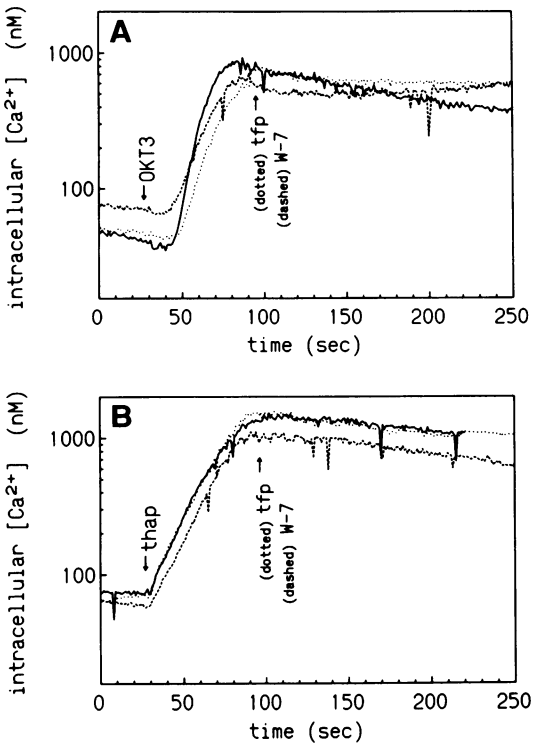

Figure 9. Effect of trifluoperazine and W-7 on receptor-dependent and -independent sustained influx of extracellular Ca^{2+}. (A) Changes in $[Ca^{2+}]_i$ were monitored after treatment of Jurkat cells with 1 μg/ml of anti-CD3 antibody OKT3 at 30 s (OKT3) with no further treatment (solid trace), with addition of 10 μM trifluoperazine (tfp, dotted trace) or with the addition of 100 μM W-7 (W-7, dashed trace) at 90 s. (B) Changes in $[Ca^{2+}]_i$ were monitored after treatment of Jurkat cells with 1 μM thapsigargin at 30 s (thap) with no further treatment (solid trace), with addition of 10 μM trifluoperazine (tfp, dotted trace) or with the addition of 100 μM W-7 (W-7, dashed trace) at 90 s.

thapsigargin (Figure 9B), subsequent addition of trifluoperazine or W-7 had no effect on the sustained portion of the increase in $[Ca^{2+}]_i$. This is in direct contrast to the effects of subsequent addition of Ni^{2+} on receptor-mediated (Figure 3) increases in $[Ca^{2+}]_i$. Thus, these compounds were not acting as direct blockers of the Ca^{2+} entry pathway.

The magnitude and duration of the increase in $[Ca^{2+}]_i$ induced by either thapsigargin or receptor stimulation in the presence of trifluoperazine (Figure 8) suggested release of Ca^{2+} from intracellular stores without Ca^{2+} influx (Figures 1 and 2). To address this possibility, the change in $[Ca^{2+}]_i$ in cells treated with trifluoperazine and then thapsigargin (Figure 10A, solid trace) was compared with the change in $[Ca^{2+}]_i$ in cells treated with EGTA to chelate extracellular Ca^{2+} and then exposed to thapsigargin (Figure 10A, dashed trace). In the presence of trifluoperazine, there was an 84% reduction in the magnitude of the increase in $[Ca^{2+}]_i$ seen after thapsigargin treatment alone. In the presence of EGTA, there was an 88% reduction in the magnitude of the increase compared with treatment of Jurkat cells with thapsigargin alone. These data suggest that trifluoperazine had no effect on thapsigargin-induced release of Ca^{2+} from intracellular stores but did inhibit Ca^{2+} influx. This was confirmed when cells were treated with both EGTA and trifluoperazine before addition of thapsigargin (Figure 10A, dotted trace). Under these conditions, there was a 91% reduction in the magnitude of the increase in $[Ca^{2+}]_i$, essentially identical to the inhibition seen with trifluoperazine or EGTA alone. Although these data are semiquantitative, they suggest that there were no differences in the increase in $[Ca^{2+}]_i$ seen among these three conditions. Taken together with the data shown in Figures 8 and 9, the data of Figure 10A suggest that a calmodulin-dependent pathway links the Ca^{2+} storage pool to initiation of Ca^{2+} entry.

To examine this question in more detail, Jurkat cells were suspended in buffer containing Mn^{2+} and treated with thapsigargin (Figure 10B, solid trace). Under these conditions, there was both release of Ca^{2+} from internal

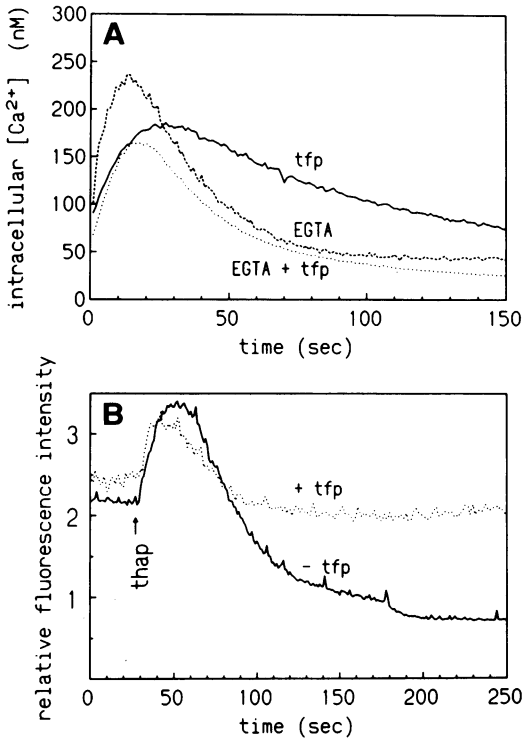

Figure 10. Effect of trifluoperazine on Ca^{2+} entry. (A) Changes in $[Ca^{2+}]_i$ induced by thapsigargin in the absence of influx and/or presence of trifluoperazine. Jurkat cells were incubated with indo-1/AM as outlined in MATERIALS AND METHODS. One minute before stimulation with 1 μM thapsigargin, cells were treated with 5 mM EGTA (EGTA, dashed trace), 10 μM trifluoperazine (tfp, solid trace) or both (EGTA + tfp, dotted trace). (B) Changes in $[Ca^{2+}]_i$ were monitored as in Figure 6. Jurkat cells, suspended in buffer containing Mn^{2+}, were treated with 1 μM thapsigargin at 30 s in the absence (solid trace) or presence (dotted trace) of 10 μM trifluoperazine added at zero time. Traces are representative of six determinations.

stores and initiation of Ca^{2+} entry as shown by an initial increase in the fluorescence emission of indo-1 followed by a reduction in the fluorescence emission, similar to the data shown in Figure 6B (solid trace). However, when Jurkat cells were suspended in buffer containing Mn^{2+} and treated with trifluoperazine before thapsigargin (Figure 10B, dotted trace), only release of Ca^{2+} from intracellular stores was seen. After an initial increase in fluorescence intensity, there was decrease of indo-1 fluorescence emission only to basal value. The lack of a further reduction in indo-1 fluorescence emission indicated that Mn^{2+} was not traversing the Ca^{2+} entry pathway. Similar results were seen when $[Ca^{2+}]_i$ was increased by antigen receptor stimulation. Thus, in the presence of trifluoperazine, Ca^{2+} entry was not initiated.

The results of Figure 10 indicate that a calmodulin-dependent pathway may link depletion of the Ca^{2+} storage pool to initiation of Ca^{2+} entry. To determine if the calmodulin-dependent initiation of Ca^{2+} entry could occur in the absence of depletion of intracellular Ca^{2+} stores, Jurkat cells were incubated with nitr-5/AM and treated with trifluoperazine (Figure 11). When nitr-5 was photolyzed, releasing free Ca^{2+}, the change in $[Ca^{2+}]_i$ was ~70 nM. This was similar in magnitude to that seen when cells were incubated with nitr-5/AM and resuspended in Ni^{2+} before photolysis (Figure 4A, dashed trace). The second component of the rise shown in Figure 4A (solid trace), due to influx of extracellular Ca^{2+}, was absent in the presence of trifluoperazine (Figure 11). Thus, initiation of Ca^{2+} entry in response to an elevation in $[Ca^{2+}]_i$ resulting from release of Ca^{2+} from nitr-5 was sensitive to inhibition by trifluoperazine. Taken together with the effect of trifluoperazine on receptor-dependent and -independent initiation of Ca^{2+} entry, these results suggest that subsequent to Ca^{2+} release from internal stores, there is a Ca^{2+}-dependent process, possibly mediated by calmodulin, that initiates Ca^{2+} influx.

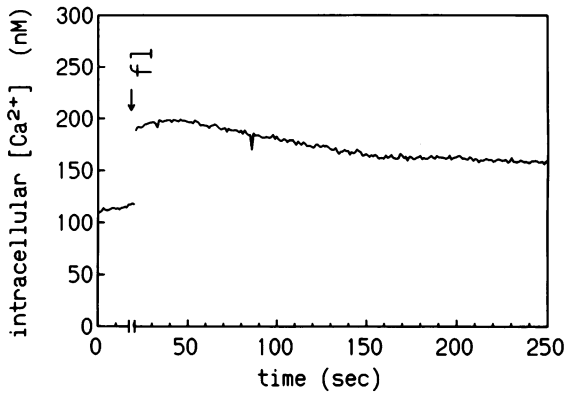

Figure 11. Effect of trifluoperazine on Ca^{2+} entry due to release of Ca^{2+} from nitr-5. Cells were incubated with 1 μM indo-1/AM and 0.5 μM nitr-5/AM and resuspended in Ca^{2+}-replete buffer as outlined in MATERIALS AND METHODS. One minute before exposure to the flash lamp apparatus (fl), the cell suspension was treated with 10 μM trifluoperazine. The trace is the mean of three determinations.

DISCUSSION

The capacitative model for the regulation of Ca^{2+} influx attempts to explain the fact that depletion of intracellular Ca^{2+} stores is associated with influx of extracellular Ca^{2+} (Putney, 1986; Menniti *et al.*, 1991). Although the early version of the capacitative model suggested a direct link between the intracellular Ca^{2+} depots and the Ca^{2+} entry pathway, revisions have suggested that the direct link need not exist (Kwan and Putney, 1990; Menniti *et al.*, 1991). Rather, the Ca^{2+} entry pathway in some manner senses the state of filling of internal stores and responds. This necessarily implies the existence of a second messenger that could signal the state of filling of the intracellular stores to the Ca^{2+} entry pathway. In this report, we suggest that Ca^{2+}/calmodulin is the effector molecule between intracellular stores and initiation of Ca^{2+} entry.

According to the modified capacitative hypothesis, depletion of intracellular Ca^{2+} stores induces Ca^{2+} entry. It would be expected from this hypothesis that replete Ca^{2+} stores would, in some manner, signal this fact to the Ca^{2+} entry pathway and cause the cessation of Ca^{2+} entry. However, filled stores do not feed back to shut down this entry pathway. During the sustained portion of elevated $[Ca^{2+}]_i$ after receptor stimulation, $[Ca^{2+}]_i$ could be rapidly and completely reduced to basal levels by blockade of Ca^{2+} influx with Ni^{2+}. This indicates that the sustained portion of the increase in $[Ca^{2+}]_i$ is entirely due to Ca^{2+} influx. Subsequent to blockade of Ca^{2+} entry, $[Ca^{2+}]_i$ could again be elevated by an agent known to induce the release of Ca^{2+} from intracellular stores in the absence of Ca^{2+} entry. These results indicate that refilling of intracellular Ca^{2+} stores is a rapid event after receptor stimulation and that the sustained portion of elevated $[Ca^{2+}]_i$ is independent of the state of filling of intracellular Ca^{2+} stores.

These data, that continued Ca^{2+} entry was independent of the state of filling of the internal stores, coupled with the implied existence of a second messenger pathway between the stores and the entry pathway, led us to speculate that it was the increase in $[Ca^{2+}]_i$ subsequent to release of Ca^{2+} from the internal stores that was responsible for initiating Ca^{2+} entry. To test this hypothesis, $[Ca^{2+}]_i$ was directly increased by photolysis of the Ca^{2+} cage nitr-5. In the absence of Ca^{2+} entry blockade, there were two components to the increase in $[Ca^{2+}]_i$. The first component was due to release of Ca^{2+} from nitr-5 in response to an increase in the K_d for Ca^{2+}. The second component of the rise, which was more sustained and of a greater magnitude, was eliminated by Ni^{2+}. These data indicate that a rise in $[Ca^{2+}]_i$ itself, independent of the status of the intracellular Ca^{2+} stores, can initiate influx of extracellular Ca^{2+}.

It is possible that Ca^{2+} derived from photolysis of nitr-5 caused Ca^{2+} to be released from internal stores (Randriamampita *et al.*, 1991) and that the initiation of Ca^{2+} entry seen in the presence of photolyzed nitr-5 was due to partial depletion of the intracellular Ca^{2+} stores. However, the thapsigargin-induced release of Ca^{2+} from intracellular stores was similar in the presence and absence of nitr-5, with or without photolysis. Thus, it is unlikely that there was release of Ca^{2+} from intracellular stores either in response to the capacity of unphotolyzed nitr-5 to chelate Ca^{2+} or to Ca^{2+}-induced Ca^{2+} release after photolysis of nitr-5. More importantly, chelation of cytosolic Ca^{2+} by BAPTA eliminated initiation of Ca^{2+} entry induced by thapsigargin. Thus, even if there were a component of Ca^{2+}-induced Ca^{2+} release not seen with the methods used in the current study, the final mediator for initiation of Ca^{2+} entry is a change in the concentration of free intracellular Ca^{2+} derived from any source.

Thapsigargin increases $[Ca^{2+}]_i$ by inhibiting the reuptake of Ca^{2+} into the internal storage pool (Thastrup *et al.*, 1990). The capacity of thapsigargin to empty internal Ca^{2+} stores and provoke Ca^{2+} entry has led to the suggestion that these two phenomena are linked (Kwan and Putney, 1990; Menniti *et al.*, 1991). However, chelation of free intracellular Ca^{2+} eliminated the ability of thapsigargin to initiate Ca^{2+} entry. Thus, in the absence of an increased concentration of cytosolic Ca^{2+}, empty internal Ca^{2+} stores do not initiate Ca^{2+} entry. These data are inconsistent with the capacitative model for Ca^{2+} entry. Instead, they indicate that intracellular free Ca^{2+}, derived from any source, initiates Ca^{2+} entry.

The fact that increased $[Ca^{2+}]_i$ induces Ca^{2+} entry suggests that Ca^{2+} entry is regulated, in part, by a feedforward control mechanism. Because Ca^{2+} entry could be delayed by chelation of extracellular Ca^{2+} and initiated with subsequent reintroduction of Ca^{2+}, there is unlikely to be a role for Ca^{2+} itself in the cessation of Ca^{2+} influx. We have shown previously that an increased intracellular cyclic AMP concentration can inhibit receptor-dependent Ca^{2+} entry (Gray *et al.*, 1988), and it is possible that this can account for the return of $[Ca^{2+}]_i$ to basal levels after receptor stimulation. Additionally, we recently have shown that receptor-dependent Ca^{2+} influx is mediated by a voltage-gated Ca^{2+} channel in Jurkat cells (Densmore *et al.*, 1992). Because this channel is under complex regulation by membrane potential (Densmore *et al.*, 1992), it is plausible that membrane potential plays a role in inactivating Ca^{2+} entry as well. However, this is clearly an area requiring further investigation.

It is unlikely that Ca^{2+} itself is the final effector in initiation of Ca^{2+} entry in nonexcitable cells. In electrically excitable cells, there is a large body of recent evidence suggesting a role for calmodulin action on voltage-gated Ca^{2+} entry pathways. This evidence is based primarily on sequencing and homology studies of Ca^{2+} channels and consensus motifs for calmodulin binding (Hardie and Minke, 1992; Jan and Jan, 1992; Phillips *et al.*, 1992). In one case, it was shown that inhibition of calmodulin resulted in a decrease in a Ca^{2+} current (McCarron *et al.*, 1992). Data presented here show that at least a portion of the rise in $[Ca^{2+}]_i$ resulting from release of Ca^{2+} from intracellular stores in a receptor-dependent or -independent manner, or direct elevations of $[Ca^{2+}]_i$ subsequent to release from a Ca^{2+}-cage, can be blocked by any one of three phenothiazines or by W-7. These compounds do not act as direct blockers of the Ca^{2+} entry pathway as has been recently suggested for a different class of calmodulin/calmodulin kinase inhibitors (Li *et al.*, 1992), because the phenothiazines or W-7 did not reduce $[Ca^{2+}]_i$ when added after initiation of Ca^{2+} entry. Rather, as shown by the ability of trifluoperazine to eliminate thapsigargin-stimulated Mn^{2+} entry, the effect of these compounds was to block the initiation of Ca^{2+} entry.

Taken together, these data suggest a model in which an elevation in $[Ca^{2+}]_i$, regardless of the source, activates a Ca^{2+} entry pathway that may involve calmodulin. In the specific case of the T-cell antigen receptor on Jurkat cells, receptor occupancy is followed by $InsP_3$ generation, resulting in release of Ca^{2+} from internal stores and an increase in the intracellular concentration of Ca^{2+}. This initial increase in $[Ca^{2+}]_i$ may then lead to activation of a Ca^{2+}/calmodulin-dependent pathway that culminates in a further increase in $[Ca^{2+}]_i$ due to Ca^{2+} entry.

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