Target-Cell Contact Activates a Highly Selective Capacitative Calcium Entry Pathway in Cytotoxic T Lymphocytes

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Abstract. Calcium influx is critical for T cell activation. Evidence has been presented that T cell receptor–stimulated calcium influx in helper T lymphocytes occurs via channels activated as a consequence of depletion of intracellular calcium stores, a mechanism known as capacitative Ca\(^{2+}\) entry (CCE). However, two key questions have not been addressed. First, the mechanism of calcium influx in cytotoxic T cells has not been examined. While the T cell receptor–mediated early signals in helper and cytotoxic T cells are similar, the physiology of the cells is strikingly different, raising the possibility that the mechanism of calcium influx is also different. Second, contact of T cells with antigen-presenting cells or targets involves a host of intercellular interactions in addition to those between antigen–MHC and the T cell receptor. The possibility that calcium influx pathways in addition to those activated via the T cell receptor may be activated by contact with relevant cells has not been addressed. We have used imaging techniques to show that target-cell–stimulated calcium influx in CTLs occurs primarily through CCE. We investigated the permeability of the CTL influx pathway for divalent cations, and compared it to the permeability of CCE in Jurkat human leukemic T cells. CCE in CTLs shows a similar ability to discriminate between calcium, barium, and strontium as CCE in Jurkat human leukemic T lymphocytes, where CCE is likely to be mediated by Ca\(^{2+}\) release–activated Ca\(^{2+}\) current (CRAC) channels, suggesting that CRAC channels also underlie CCE in CTLs. These results are the first determination of the mechanism of calcium influx in cytotoxic T cells and the first demonstration that cell contact–mediated calcium signals in T cells occur via depletion-activated channels.

Key words: CTL \(\cdot\) Ca\(^{2+}\) release–activated Ca\(^{2+}\) current \(\cdot\) Fura-2 \(\cdot\) granule exocytosis \(\cdot\) perforin

Introduction

Ca\(^{2+}\) influx is critical for T cell activation (Berke, 1994; Weiss and Littman, 1994). In helper (CD\(^{4}\)) T cells, Ca\(^{2+}\) influx leads to the activation of cytokine genes (Weiss and Littman, 1994), and different patterns of calcium signals can regulate which cytokines are produced (Dominquez et al., 1998). In cytotoxic T cells (CTLs), Ca\(^{2+}\) influx participates in the exocytosis of lytic granules that contain perforin and granzymes (Berke, 1994). In both T cell types, interaction of antigen–MHC with the T cell receptor (TCR) leads to production of inositol 1,4,5 trisphosphate (IP\(_{3}\)) and diacylglycerol (Berke, 1994; Weiss and Littman, 1994). IP\(_{3}\) opens IP\(_{3}\) receptor channels in the ER, causing release of sequestered Ca\(^{2+}\), and diacylglycerol activates protein kinase C (PKC). The combination of Ca\(^{2+}\) ionophores such as ionomycin or A 23187 and PKC activators like phorbol myristate acetate (PMA) can substitute for antigen–MHC in promoting proliferation and cytokine production in CD\(^{4}\) T cells and proliferation and lytic granule exocytosis in CD\(^{8}\) cells (Berke, 1994; Weiss and Littman, 1994).

Recent work has provided support for the idea that TCR–dependent Ca\(^{2+}\) influx in helper T cells (or lines with helper phenotype) occurs through a class of channels that are activated as a consequence of depletion of the cell’s intracellular Ca\(^{2+}\) stores (Zweifach and Lewis, 1993; Partiseti et al., 1994; Premack et al., 1994; Fanger et al., 1995), a mechanism known as capacitative Ca\(^{2+}\) entry (CCE).
(Putney, 1990). While other mechanisms have been proposed to participate in Ca\(^{2+}\) influx, including IP\(_3\)-gated channels (Kuno et al., 1986), voltage-gated Ca\(^{2+}\) channels (Dansmore et al., 1996), and reverse Na\(^+\)-Ca\(^{2+}\) exchange (Wacholtz et al., 1992), evidence has been presented that argues against the involvement of these pathways (for review see Lewis and Cahalan, 1995).

In T cells, store depletion is believed to activate influx via a functionally defined subtype of store-operated channels (SOCs) called Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels (Zweifach and Lewis, 1993; Partiseti et al., 1994; Premack et al., 1994; Fanger et al., 1995), first identified in Jurkats (Lewis and Cahalan, 1989) and mast cells (Hoth and Penner, 1992). A monog the properties of CRAC channels are the following: an inwardly rectifying current-voltage relationship, fast inactivation during hyperpolarizing voltage pulses, a lack of current noise when external divalent cations are present, and a high selectivity for Ca\(^{2+}\) over other divalent cations and Na\(^+\) (Parekh and Penner, 1997). Selectivity for Ca\(^{2+}\) is a key property that distinguishes CRAC channels from other SOCs, as CRAC channels are the only SOCs known that exhibit Ca\(^{2+}\) selectivity (Parekh and Penner, 1997). For example, in X enucleated oocytes, store depletion activates an inwardly rectifying current that exhibits fast inactivation, but is permeable to Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\) (Yao and Tsien, 1997). A CCE pathway in A 431 cells was found to be more permeable to Ba\(^{2+}\) than Ca\(^{2+}\) (Luckhoff and Clapham, 1994). In vascular endothelial cells, a depletion-activated current was described that was essentially equally permeable to Ca\(^{2+}\) and Ba\(^{2+}\) (Vaca and Kunze, 1993). In pancreatic acinar cells, a depletion-activated nonselective influx pathway has been described previously (Krause et al., 1996).

The selectivity of CRAC channels for Ca\(^{2+}\) over other ions reflects a blocking effect of internal ions (primarily M\(^{2+}\)) that must be knocked out of the pore to allow current flow (Hoth, 1995; Kerschbaum and Cahalan, 1998). Ca\(^{2+}\), but not Ba\(^{2+}\), can serve as the function of, and, thus, Ca\(^{2+}\) carries sustained currents through CRAC channels whereas Ba\(^{2+}\) does not. The most complete analysis of CRAC channel divalent permeation to date was performed by Hoth (1995). He found that switching from Ca\(^{2+}\) to Ba\(^{2+}\) solutions gave rise to kinetically complex currents and, further, that CRAC channels in Jurkats behaved differently than CRAC channels in mast and rat basophilic leukemia cells. In rat basophilic leukemia cells, Ba\(^{2+}\) can actually pass through the pore better than Ca\(^{2+}\) because Ba\(^{2+}\) currents at some potentials are transiently larger than Ca\(^{2+}\) currents. These results suggest that there may be different subtypes of CRAC channels expressed in different cell types. Full understanding of the distinctions between different CCE pathways will require an analysis of the molecular identity of the protein(s) forming the influx pathway. Although there is evidence that homologues of the Drosophila trp gene product may form SOCs (see for example Warna et al., 1999), the molecular basis of CCE in mammalian cells remains unknown.

Despite the progress in understanding the mechanism of TCR-stimulated Ca\(^{2+}\) influx, two key issues regarding Ca\(^{2+}\) influx in T cells have not been addressed. First, it has not been determined whether CCE is responsible for the Ca\(^{2+}\) influx that accompanies granule exocytosis in CTLs.

The similarity of upstream signaling events in helper T cells and CTLs might suggest that the Ca\(^{2+}\) influx pathway is similar. However, the physiology of the two cell types is sufficiently different that different Ca\(^{2+}\) influx pathways might be required. Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\text{i}) levels must be elevated for hours to activate cytokine genes in CD 4\(^{+}\) cells (Crabtree, 1989). By contrast, granule-dependent killing by CTLs is an extremely rapid process, occurring in minutes (Poenie et al., 1987; this report), that does not require new gene expression. While several studies have demonstrated that contact of CTLs with appropriate targets leads to an increase in [Ca\(^{2+}\)]\text{i}, and the contribution of influx to the response has been demonstrated (Gray et al., 1987, 1988; Poenie et al., 1987; Haverstick et al., 1991; Hesser et al., 1993), the mechanism of Ca\(^{2+}\) influx in CTLs has not been determined.

The second issue that has not been addressed is whether influx pathways in addition to CCE contribute significantly to influx stimulated by contact of a T cell with an antigen-presenting cell (APC) or target. Interactions of T cells with APCs or targets involves a host of intercellular interactions in addition to the binding of antigen–MHC to the TCR. A cersory molecules such as CD 3, CD 4, and CD 8 also interact with antigen–MHC, and adhesion molecules such as LFA-1 and CD 2/LFA-2 are known to be critical for APC or target cell binding (Berke, 1994). The involvement of adhesion molecules in T cell interactions with APCs and targets is particularly intriguing, as it has been demonstrated that integrin binding to RGD-containing peptides can trigger a non-CCE pathway in MDCK cells (Sjaastad et al., 1996), and integrin binding has been shown to stimulate [Ca\(^{2+}\)]\text{i}, increases in Jurkats (Weismann et al., 1997). The adhesion molecule LFA-1 has been shown to activate signaling pathways in CTLs distinct from those activated by the TCR (Ni et al., 1999). Findings such as these raise the possibility that a non-CCE Ca\(^{2+}\) influx pathway might be activated in parallel to CCE in T cells stimulated by contact with an APC or target. A ll studies to date in which the mechanism of calcium influx in T cells has been addressed have relied on mAbs, mitogenic lectins, or store-depleting drugs (Lewis and Cahalan, 1989; Hess et al., 1993; Zweifach and Lewis, 1993; Partiseti et al., 1994; Premack et al., 1994; Fanger et al., 1995). Studies that have demonstrated cell contact–stimulated [Ca\(^{2+}\)]\text{i}, signals in T cells have not addressed the mechanism(s) of influx (Poenie et al., 1987; Gray et al., 1987, 1988; Donnadieu et al., 1992; Arawal and Linderman, 1995; Negulescu et al., 1996; DeIon et al., 1998).

In the present study we have used imaging techniques to provide evidence that target-cell–stimulated Ca\(^{2+}\) influx accompanying CTL lytic granule exocytosis occurs primarily via CCE. Imaging techniques offer advantages over patch clamp techniques for such an investigation, as the conditions required to record CCE, which include high levels of intracellular Ca\(^{2+}\) buffers, pipette solutions designed to block contaminating currents and voltage protocols designed to maximize CCE, might obscure the presence of other Ca\(^{2+}\) entry pathways. Our results are the first demonstration that CCE can account for [Ca\(^{2+}\)]\text{i}, signals stimulated by contact of a T cell with a relevant cellular partner. We show that CCE in CTLs displays a similar ability to discriminate between Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\) as...
CCE in Jurkat cells, suggesting that CRAC channels may also underlie CCE in CTLs. Significantly, the fact that CCE is responsible for target cell–stimulated Ca\(^{2+}\) influx in CTLs indicates that CCE can serve Ca\(^{2+}\)-dependent T cell functions as diverse as gene expression and lytic granule exocytosis.

**Materials and Methods**

**Chemicals and Reagents**

Salts for physiological solutions. N-\(\beta\) -benzoxycarbonyl-\(\gamma\)-lysine thio-\(\beta\)-benzyl ester (BLT), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), and poly-\(\gamma\)-lysine were purchased from Sigma-Aldrich. Fura-2 AM and calcine AM were from Molecular Probes. Thapsigargin was purchased from A lexis Biochemicals. A nile-CD3 beads (M-450 Pan T) were purchased from Dynal A.S. RPMI 1640 and tissue culture plastic was obtained from Fisher Scientific. FCS, glutamine, and antibiotics were purchased from Gemini Bioproducts. T-stim IL-2 containing conditioned medium was purchased from Bioproducts. T-stim IL-2 containing conditioned medium was purchased from Biozol. T-stim IL-2 containing conditioned medium was purchased from Biozol.

**Cells**

JY (HLA-A2, -B7, and -D14,6), and Jurkat E 6-1 cells were maintained in RPMI supplemented with 2 mM glutamine, 10% heat-inactivated FCS, and antibiotics (complete cell culture medium). The JY CTL line (HLA-A3, B7) was maintained in complete medium supplemented with 15% T-stim. JYs were given irradiated JYs (10,000 R) once a week (ratio of JYs to JYS ~ 10–20:1), and used for experiments 4–6 d after stimulation. JYs and JYS were the gift of Dr. Carol Claybergher (Stanford University, Stanford, CA); Jurkat E6-1s were provided by Dr. M. Michael Calahan (University of California, Irvine, Irvine, CA).  

**Solutions**

Ringer’s solution contained the following (in mM): 145 NaCl, 4.5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 5 Hapes, and 10 glucose (pH 7.4 with NaOH). Zero Ca\(^{2+}\)-Ringer’s was identical, except CaCl\(_2\) was replaced with MgCl\(_2\). Zero Ca\(^{2+}\)-Ringer’s plus EGTA was identical to zero Ca\(^{2+}\)-Ringer’s, but was supplemented with 1 mM EGTA. K\(^{+}\)-Ringer’s contained (in mM): 160 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 5 Hapes, and 10 glucose, pH 7.4 with K Hapes, free K\(^{+}\)-Ringer’s contained (in mM): 160 KCl, 1 MgCl\(_2\), 5 Hapes, and 10 glucose, pH 7.4 with K Hapes. In experiments with different cations (Ba\(^{2+}\), Sr\(^{2+}\), and Mn\(^{2+}\)), 0.1 M or 1 M stock solution of the chloride salt was prepared in distilled water. For Mn\(^{2+}\) quench experiments, Mn\(^{2+}\) was added to normal Ringer’s. To investigate the permeability of Ca\(^{2+}\) entry pathways, Ba\(^{2+}\) or Sr\(^{2+}\) was added to Ca\(^{2+}\)-free K\(^{+}\)-Ringer’s.

**Loading of Cells with Dyes**

JYs were loaded with calcine by incubating them with 2.5 \(\mu\)M calcine-A M in cell culture medium for 30 min at room temperature. JYs were loaded with Fura-2 by incubating them with 1 \(\mu\)M Fura-2 AM in cell culture medium for 30 min at room temperature. Cells were washed twice with fresh medium before use.

**Imaging**

Experiments were performed with an imaging system built around a Nikon Diaphot 300 inverted microscope (Nikon Inc.) equipped for epifluorescence. A Nikon PlanFluor 40X oil objective (NA 1.4) was used for all experiments. Excitation light was provided by a 150-W Xeon arc lamp (Optipquin), and was attenuated by 12% for Fura-2 experiments or 1% for calcine experiments using neutral density filters. Excitation wavelength was selected using appropriate excitation filters in a Sutter Lambda-10 filter wheel (Sutter Instruments). A Fura-BCECF dichroic mirror was used in the filter cube, and emission light was filtered with a second Sutter filter wheel. Filters and the dichroic mirror were obtained from Chroma. Images were acquired with a Cooke Sensicam peltier-cooled interline CCD camera (PCO). For Fura-2 experiments, ratios were collected every 6 or 8 s. Emission exposure times were 150 or 200 ms for F340 and F360, and 75 or 100 ms for F380. Typically, the CCD array was binned 8 x 8 to increase sensitivity and decrease data density. Hardware was controlled and images were acquired and analyzed with SlideBook software from Intelligent Imaging Innovations, Inc., running on a Dell Dimensions Pentium II computer with 500 MB of RAM.

Imaging experiments were conducted at room temperature. The experimental chambers consisted of lids of 35-mm petri dishes in which a 5-mm diam hole was milled and a coverslip was attached to the bottom with Sylgard. The volume of chambers was ~30 \(\mu\)l. This small volume was kept from evaporating by covering the chamber with a lid to which a sponge, wetted with Ringer’s, was attached. Solutions were changed using a handheld suction line and a 1-ml pipetter in experiments in which targets were applied, or using fixed-position syringe-driven perfusion inlet and vacuum-driven outlet lines. In either case, because of the extremely small volume of the chamber, solution exchange was complete within 2 s. Coverslips were coated with poly-\(\gamma\)-lysine to promote cell adherence.

For analysis of Fura-2 ratio data, background-subtracted images were thresholded on F340, and the ratios were computed pixel-by-pixel for all pixels above threshold. Ratiograms were converted to estimates of (Ca\(^{2+}\)), using the method of Gryniewicz et al. (1985). Calibration values were measured in vitro, assuming a dissociation constant of 300 nM for the binding of Ca\(^{2+}\) to Fura-2 (Nagel, 1996). For Mn\(^{2+}\) quench experiments, (Ca\(^{2+}\)), was determined as above, whereas F360 was measured with no thresholding. A fitter initial processing, all fluorescence data were exported to Igor Pro for subsequent analysis.

To align (Ca\(^{2+}\)), responses (see Fig. 4), an automatic routine was written in Igor Pro. For each cell, resting (Ca\(^{2+}\)), was computed for the 7–2 s before addition of JYs. A threshold was set at 50 nM above this value, and the routine automatically detected the first threshold crossing. All points >60 s before the threshold crossing were deleted from the trace, resulting in temporally aligned traces. A nother routine written in Igor Pro was used to inspect traces and add them to a running average. A fitter the average was calculated, standard deviations for the data set were computed. Statistical significance of the data in Fig. 5 was computed using an unpaired t test.

**BLT-esterase Assays**

BLT-esterase activity released from JYs (Takayama et al., 1987) was measured by resuspending JYs at a density of 1.6 \(\times\) 10\(^6\) cells/ml of cell culture medium. The cell suspension was pipetted into wells of a v-bottom 96-well microtiter plate (5 \(\mu\)l well), and 100 \(\mu\)l of appropriate experimental solution was added to each well (see above for composition). The cells were incubated for 2 h at 37°C in 5% CO\(_2\). After the incubation period, the cells were resuspended by careful pipetting, and centrifuged at 200 g for 0.5 min. 50 \(\mu\)l of supernatant was transferred to another 96-well plate for analysis of BLT-esterase activity. 75 \(\mu\)l of BLT solution (0.2 mM BLT and 0.22 mM DTNB) in PBS, pH 7.2, was added to the supernatant of each condition. The 96-well plate was covered with parafilm and incubated in the dark at room temperature for 40 min. BLT activity was determined from absorbance measurements made on a Dynatech Mini-reader II, read at 410 nm after subtraction of an appropriate blank. The percentage of BLT-esterase release in each experimental condition was calculated as follows:

Release (%) = \(\frac{E - S}{T - S}\) \times 100,

where E is the BLT-esterase activity in the supernatant of experimental wells, S is BLT-esterase activity in the supernatant of a well containing normal Ringer’s with no stimuli, and T is the total amount of BLT activity determined by adding 0.1% Triton X-100 to cells in normal Ringer’s. A1 determinations were made in triplicate.

**Results and Discussion**

**A J Y S K i ll J Y S U sing G ranule E xocytosis**

This study was designed to investigate the mechanism of target-cell–stimulated Ca\(^{2+}\) influx accompanying lytic granule exocytosis by CTLs. We chose A J Y CTLs, a long-term human HLA-A2 allospecific CTL line, for these studies. Previous work has shown that these cells are CD8\(^+\), and that mAbbs against CD8, LFA-1, LFA-2, and LFA-3 inhibit lysis of JYs (Koller et al., 1987). We developed a novel imaging technique derived from the calcine-
release assay (CARE-LASS) (Lichtenfels et al., 1994) to confirm that AJYs use granule exocytosis rather than the FAS pathway (Berke, 1994) to kill JYs, and to explore the kinetics of granule exocytosis, as the relevant \([Ca^{2+}]\) rise must occur before hitting. Calcein is a membrane-impermeant dye that is well-retained in cells with intact plasma membranes. CARE-LASS measures the amount of calcein released into the extracellular solution from loaded targets to monitor hitting. We reasoned that, as perforin pore formation following granule exocytosis is accompanied by an increased membrane permeability to calcein, we should be able to monitor hitting as a decrease in target cell calcein fluorescence in imaging experiments. JY targets were loaded with calcein-AM, and allowed to adhere to coverslips coated with poly-L-lysine. An excess of AJYs was added to the chamber, and allowed to settle into contact with the JYs. Addition of AJYs in this manner established contact with JYs in \(\sim 30–60\) s. Fig. 1 shows results from a typical experiment. After a delay, target cells exhibited sudden decreases in calcein fluorescence, consistent with perforin-induced increases in membrane permeability to calcein. The shortest latency observed was \(\sim 400\) s and the mean latency was 1,031 s. By 2,000 s, \(\sim 65\%\) of JYs had lost their fluorescence. Jurkats, which are not targets for JYs, did not demonstrate similar fluorescence decreases when exposed to AJYs (data not shown).

To confirm that the decreases in fluorescence we observed correspond to perforin-induced membrane damage, we repeated the experiment in Ca\(^{2+}\)-free external solution (Fig. 1 C) as cell killing because of granule exocytosis but not FAS has been shown to depend absolutely on the presence of external Ca\(^{2+}\) (Berke, 1994; Griffiths, 1995). In the absence of external Ca\(^{2+}\) (Ca\(^{2+}\)_0), sudden decreases in calcein fluorescence were not observed over a period of \(\sim 2,000\) s. When the external solution was replaced with Ca\(^{2+}\)-containing Ringer’s, decreases in calcein fluorescence occurred within 60 s. We conclude based on the kinetics and Ca\(^{2+}\)_0 dependence of CTL-induced calcein fluorescence changes that AJYs use granule exocytosis to kill JYs.

An alternative imaging technique for monitoring target cell killing based on Fura-2 fluorescence has been reported (Poenie et al., 1987). In preliminary experiments, we used Fura-2 to measure target cell killing, and found that AJYs were capable of causing robust \([Ca^{2+}]\) increases in JYs. However, there are three problems associated with the use of Fura-2 to measure target cell killing. First, the earliest signal is an increase in \([Ca^{2+}]\), because of Ca\(^{2+}\) in-
Influx Contributes to [Ca\textsuperscript{2+}], Signals in AJYs

To examine the [Ca\textsuperscript{2+}] signals elicited in CTLs by target-cell contact, we loaded AJYs with Fura-2, let them adhere to coverslips coated with poly-L-lysine, and then allowed an excess of JY targets to settle onto them (Fig. 2). Experiments of this sort, originally performed by Poenie et al. (1987), are essentially the converse of the calcein experiments shown in Fig. 1. In these experiments, [Ca\textsuperscript{2+}], responses were observed in 62% of cells (172 out of 280 cells from 7 experiments). AJYs exposed to Jurkats in the identical fashion did not exhibit [Ca\textsuperscript{2+}] increases (data not shown).

The most striking feature of the [Ca\textsuperscript{2+}] responses we observed was their heterogeneity (Fig. 2 A). Both the latency until the onset of a [Ca\textsuperscript{2+}] rise and the magnitude and shape of the [Ca\textsuperscript{2+}] response were extremely variable (Fig. 2 A). In most cases, the earliest [Ca\textsuperscript{2+}] signal consisted of a rapid rise from baseline to \(\sim 200-400\) nM. The mean latency to the onset of [Ca\textsuperscript{2+}], signals was 674 \pm 472 s (mean \pm SD, \(n = 172\)). After this initial response, individual cells displayed very variable responses ranging from a series of small transients to large, sustained oscillations. The mean peak [Ca\textsuperscript{2+}] increase was 319 \pm 282 nM (mean \pm SD, \(n = 172\)). Comparison of the mean latency to the initiation of the [Ca\textsuperscript{2+}] signal to the mean latency of killing suggests that JY-induced [Ca\textsuperscript{2+}], signals precede perforin-induced target cell membrane permeability changes by 300-400 s on average. When tested, \(\sim 1,200\) s after the addition of JYs, [Ca\textsuperscript{2+}] increases in AJYs were reduced when Ca\textsuperscript{2+} was removed, when extracellular Na\textsuperscript{+} was replaced by K\textsuperscript{+}, and by application of 5 mM NiCl\textsubscript{2} (data not shown). Reduction of [Ca\textsuperscript{2+}], after replacement of Na\textsuperscript{+} with K\textsuperscript{+} provides evidence that influx is not mediated by Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange operating in the reverse mode, as reverse Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange should be enhanced by Na\textsuperscript{+} removal, resulting in a decrease in [Ca\textsuperscript{2+}]. Furthermore, Donnadieu and Trautmann (1993) have presented evidence that macrophages, but not lymphocytes have active Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange only functions in the forward mode.

Averaging the single-cell [Ca\textsuperscript{2+}], responses (\(n = 280\) cells) results in a trace that is not a good representation of the single-cell behavior (Fig. 2 B), as has been reported previously for Jurkats (Lewis and Cahalan, 1989). Averaging [Ca\textsuperscript{2+}], rises from a resting level of 66 to 236 nM, a difference of 171 nM. Thus, the average behavior, similar to what would be measured in a population assay, underestimates the magnitude of the peak change in [Ca\textsuperscript{2+}], by a factor of approximately two.
We confirmed that influx contributes to the \([\mathrm{Ca}^{2+}]_i\), elevation by measuring target-cell-induced \([\mathrm{Ca}^{2+}]_i\), responses in \(\mathrm{Ca}^{2+}\)-free external solution (Fig. 2 C). Under these conditions, the only source of \(\mathrm{Ca}^{2+}\) is intracellular stores; differences between \([\mathrm{Ca}^{2+}]_i\), responses measured in the presence and absence of \(\mathrm{Ca}^{2+}\) reflect the contribution of influx. As in the experiments performed in the presence of \(\mathrm{Ca}^{2+}\), \(~65\%\) of cells responded (117 out of 180 cells from 6 experiments). In the absence of \(\mathrm{Ca}^{2+}\), \([\mathrm{Ca}^{2+}]_i\) responses were of a smaller amplitude and shorter duration than in the presence of \(\mathrm{Ca}^{2+}\) (Fig. 2 C), which is consistent with previous reports that \(\mathrm{Ca}^{2+}\) influx plays a role in CTL [\(\mathrm{Ca}^{2+}\)] signals (Poenie et al., 1987; Gray et al., 1987, 1988; Haverstick et al., 1991; Hess et al., 1993). In fact, the \([\mathrm{Ca}^{2+}]_i\) signals measured in the absence of \(\mathrm{Ca}^{2+}\) were strikingly similar to the earliest responses observed in the presence of \(\mathrm{Ca}^{2+}\). The mean peak \([\mathrm{Ca}^{2+}]_i\) increase was 153.8 \pm 76 nM (mean \pm SD, \(n = 117\)), approximately half as large as the rise seen in the presence of \(\mathrm{Ca}^{2+}\). The latency until responses were initiated was 741 \pm 383 s (mean \pm SD, \(n = 117\)), similar to the results obtained in the presence of \(\mathrm{Ca}^{2+}\). This result indicates that early signaling events proximal to release of \([\mathrm{Ca}^{2+}]_i\) from stores occur at the same rate in the presence and absence of \(\mathrm{Ca}^{2+}\). Averaging the single-cell \([\mathrm{Ca}^{2+}]_i\) responses (Fig. 2 D) underestimates the single-cell responses much more dramatically than in the presence of \(\mathrm{Ca}^{2+}\). The average rises from a resting value of 72 to 92 nM, a change of only 20 nM, whereas the single-cell responses show that \([\mathrm{Ca}^{2+}]_i\), can actually be sustained at levels of hundreds of nanomolar above baseline for several hundred seconds. The discrepancy between the single-cell behavior and the population behavior in the absence of \(\mathrm{Ca}^{2+}\) highlights the importance of single-cell measurements.

**Figure 3. Release of \(\mathrm{Ca}^{2+}\) from intracellular stores precedes influx.** (A) Alignment of \([\mathrm{Ca}^{2+}]_i\), responses like those shown in Figs. 2 and 3. The earliest portion of the responses are identical. Differences become apparent \(~80\) s after the initial response. Error bars are \(2\) SEM, and differences between them thus represent 95% confidence intervals. (B) Computed time course of \(\mathrm{Ca}^{2+}\) influx obtained by subtracting the aligned \(0\) \(\mathrm{Ca}^{2+}\) response from the aligned response in 2 mM \(\mathrm{Ca}^{2+}\). (C) \(\mathrm{Mn}^{2+}\) quench allows determination of the relationship between store release and influx in single cells. The top trace is \([\mathrm{Ca}^{2+}]_i\), recorded in a single cell after contact with JYs. The bottom trace is F360. \(144\) s after the initial \([\mathrm{Ca}^{2+}]_i\), rise, F360 begins to decrease, demonstrating that influx was activated with a delay in this cell.
The alignment procedure described above allows determination of the average behavior of CTLs. To examine the temporal relationship between release of Ca\textsuperscript{2+} from stores and initiation of influx in single CTLs, we exploited the fact that Mn\textsuperscript{2+} can permeate many Ca\textsuperscript{2+} influx pathways and quench the fluorescence of intracellular Fura-2 (Gryniewicz et al., 1985; Hallam et al., 1988). In the presence of extracellular Mn\textsuperscript{2+}, measuring the fluorescence of Fura-2 at its Ca\textsuperscript{2+}-independent isosbestic wavelength (360 nm) in addition to the F340/F380 ratio can, therefore, be used to monitor changes in [Ca\textsuperscript{2+}], and influx across the plasma membrane simultaneously (Fig. 3 C).

For these experiments, the extracellular solution was supplemented with 0.5 mM MnCl\textsubscript{2}, and the fluorescence of Fura-2 excited at 360 nm (F360) was measured in addition to the F340/F380 ratio. Control in vitro experiments demonstrated that F360 changed <10% when [Ca\textsuperscript{2+}] was increased from 0 (10 mM EGTA) to 10 mM. Because AJYs are highly motile cells, it was necessary to conduct these experiments on sparse fields so that the regions of interest used for analysis could entirely contain a given cell at all times. Fig. 3 C shows results of a Mn\textsuperscript{2+} quench experiment. Initially, there was a small slow decline in F360 because of photobleaching. Target cell contact initiated a [Ca\textsuperscript{2+}], rise with no significant change in F360. In the cell shown in Fig. 3 C, the rate of decline of F360 increased over its basal level 144 s after the initial increase in [Ca\textsuperscript{2+}]. These data indicate that the plasma membrane permeability to Mn\textsuperscript{2+} increased detectably at this time, confirming that the release of Ca\textsuperscript{2+} from stores preceded influx. Results compiled from 17 cells indicate that the average delay between release of Ca\textsuperscript{2+} from stores and opening of plasma membrane Ca\textsuperscript{2+} channels was 189 ± 40 s (mean ± SEM). The shortest delay observed was 16 s, whereas the longest was 552 s.

The results of the alignment procedure and of the Mn\textsuperscript{2+} quench experiments both indicate that release of Ca\textsuperscript{2+} from intracellular stores precedes initiation of influx by ~80–200 s. Taken together with the observation that [Ca\textsuperscript{2+}]\textsuperscript{r} rises precede hitting by 300–400 s, these results suggest that, on average, influx begins ~100–300 s before target-cell hitting. That store release precedes influx is consistent with the idea that target cell-stimulated influx in AJYs occurs via a CCE mechanism.

**Thapsigargin Stimulates [Ca\textsuperscript{2+}], Increases and Granule Exocytosis in CTLs**

We investigated whether AJY CTLs possess CCE by measuring [Ca\textsuperscript{2+}], responses to thapsigargin (TG), a drug that activates CCE without generating IP\textsubscript{3} in a wide variety of cell types (Fig. 4). Fig. 4 A demonstrates that treatment of cells with 1 \mu M TG in Ca\textsuperscript{2+}-free extracellular solution caused a small transient increase in [Ca\textsuperscript{2+}]. A fter the [Ca\textsuperscript{2+}], transient has returned to baseline, addition of Ca\textsuperscript{2+} to the external solution results in a large increase in [Ca\textsuperscript{2+}]. Experiments like the one shown in Fig. 4 A are considered diagnostic of CCE (Putney, 1990), and argue against the participation of a Ca\textsuperscript{2+}-activated Ca\textsuperscript{2+} conductance. Maximal [Ca\textsuperscript{2+}], was typically higher in cells treated with TG as compared with cells stimulated by contact with targets, likely reflecting the greater degree of store depletion that occurs in TG-treated cells. A s was the case with target-cell stimulation, and as has been reported for Jurkat human leukemic T cells (Lewis and Cahalan, 1989), TG-stimulated [Ca\textsuperscript{2+}], responses were dependent on the presence of Ca\textsuperscript{2+}\textsubscript{i}. Inhibited when extracellular Na\textsuperscript{+} was replaced by K\textsuperscript{+}, and blocked by 5 mM NiCl\textsubscript{2} (data not shown). A s stated above, reduction of [Ca\textsuperscript{2+}], after replacement of Na\textsuperscript{+} with K\textsuperscript{+} provides evidence that influx is not mediated by Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange operating in the reverse mode.

If target-cell contact stimulates Ca\textsuperscript{2+} influx through CCE, then treatment of cells with TG + PMA should be able to stimulate granule exocytosis. We confirmed that treating AJYs with TG + PMA can stimulate granule exocytosis using BLT-esterase assays (Takayama et al., 1987) (Fig. 4 B). These assays measure the release of granzyme A from CTLs and, thus, allow determination of exocytosis in CTL populations. TG in combination with PMA was as effective as anti-CD3-coated beads. TG + PMA stimulated granule exocytosis requires Ca\textsuperscript{2+}. Results are mean ± SD from two identical experiments performed in triplicate.
duced in the absence of extracellular Ca$^{2+}$, Ba$^{2+}$ could not substitute for Ca$^{2+}$ in promoting granule release.

**CCE Is the Major Influx Pathway Activated by Target-Cell Contact**

The results presented above indicate that CTLs possess a CCE pathway. We would expect target cell contact to activate CCE, as the data presented in Fig. 2 B demonstrate that target cell contact causes release of Ca$^{2+}$ from intracellular stores. We performed two experiments to explore whether target cell contact activates non-CCE pathways as well as CCE. First, we compared the magnitude of Ca$^{2+}$ influx in cells that were stimulated with JYs followed by TG versus cells that were stimulated with TG alone (Fig. 5). We reasoned that, if contact with JYs activates CCE and non-CCE pathways, then there would be more influx in cells stimulated with JYs followed by TG versus cells that were stimulated with TG alone (Fig. 5). We reasoned that, if contact with JYs activates CCE and non-CCE pathways, then there would be more influx in cells stimulated with JYs + TG than cells stimulated with TG alone. Second, we compared the relative permeabilities of Ca$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$ to cause Fura-2 ratio changes in cells stimulated with TG, JYs, or JYs + TG (Fig. 6). We reasoned that, if contact with JYs activates CCE and non-CCE pathways, there should be a difference in divalent cation permeabilities in cells stimulated with TG as compared with cells stimulated with JYs or JYs + TG.

To assess Ca$^{2+}$ influx after JY + TG stimulation, JY targets were dropped onto Fura-2–loaded CTLs in the absence of Ca$^{2+}$, and the initial slope of the averaged [Ca$^{2+}$]$_i$ rise and the peak [Ca$^{2+}$]$_i$ were used as measures of the amount of influx activated. In these experiments, as in the ones shown in Fig. 2, A and C, ~65% of AJYs exhibited [Ca$^{2+}$] responses to JY targets. Only cells that exhibited JY-stimulated [Ca$^{2+}$] rises were included in the average. The [Ca$^{2+}$] responses to TG stimulation alone were identical to those described in Fig. 2 D. To assess responses to TG stimulation alone, the identical protocol was used except that JY targets were never exposed to JY targets.

Comparison of the average [Ca$^{2+}$] responses of AJYs that responded to targets with those that were never exposed to targets revealed two key results. First, cells that exhibited [Ca$^{2+}$] responses to JY targets released less Ca$^{2+}$ when subsequently stimulated with TG than cells that were not exposed to JYs (Fig. 5 B). The peak [Ca$^{2+}$], increase because of TG-stimulated release in cells that responded to JYs was 20 nM, as compared with 107 nM for cells that were never exposed to JYs (P < 0.0005). Cells that responded to JYs also released less Ca$^{2+}$ when treated with TG than cells in the same experiment that did not respond to targets (data not shown). These results con-

### Table I. Relative Permeability of Influx in AJY CTLs and Jurkat Leukemic T Cells

| Cell type | AJY | AJY | AJY | Jurkat | Jurkat |
|-----------|-----|-----|-----|--------|--------|
| Stimulation | Thapsigargin | Target cells | Target cells + thapsigargin | Thapsigargin | Digtomin |
| Divalent: | | | | | |
| Ca$^{2+}$ | 1 | 1 | 1 | 1 | 1 |
| Sr$^{2+}$ | 0.083 | 0.11 | 0.094 | 0.06 | 0.42 |
| (0.076–0.091) | (0.10–0.12) | (0.06–0.13) | (0.048–0.072) |
| Ba$^{2+}$ | 0.031 | 0.043 | 0.023 | 0.022 | 0.37 |
| (0.028–0.032) | (0.037–0.049) | (0.020–0.027) | (0.020–0.023) |

Values are relative slopes determined from the data of Figs. 6 and 7. Numbers in parentheses below are minimum and maximum relative slopes calculated from the standard deviation of the fitted slopes.
firm that contact with JY targets releases Ca\(_{2+}\) from the TG-sensitive Ca\(_{2+}\) stores that control CCE, which is consistent with the idea that CCE participates in the response to target cells. Critically, the slope of the [Ca\(_{2+}\)]\(_i\) rise after Ca\(_{2+}\)\(_0\) addition was not higher (in fact, it was slightly lower) in cells that responded to JY’s than in cells that were not exposed to JY’s, although the difference was not significant. Additionally, peak [Ca\(_{2+}\)]\(_i\) levels were lower in cells that responded to JY’s versus 1,201 nM for cells that were not exposed to JY’s, although the difference was not significant. If target-cell contact activated a second influx pathway, we would expect that the slope of the [Ca\(_{2+}\)]\(_i\) increase and the peak [Ca\(_{2+}\)]\(_i\) would be higher in cells that responded to targets as compared with cells that were not exposed to targets. Thus, the data presented in Fig. 5 suggest that target-cell contact does not open any channels other than those activated by store depletion. The lower slope and peak [Ca\(_{2+}\)]\(_i\) in cells stimulated with JY’s + TG may reflect PKC inhibition of the channels (Parekh and Penner, 1995), caused by TCR engagement.

We exploited the fact that the fluorescence of Fura-2 is sensitive to the binding of cations other than Ca\(_{2+}\) to compare the permeability properties of the influx pathway(s) stimulated by TG, JY’s, or JY’s + TG. These methods are expected to activate CCE alone (TG) or CCE and non-CCE pathways (JY’s and JY’s + TG), as Fig. 5 demonstrates that contact with JY’s in Ca\(_{2+}\)-free external solution depletes the TG-sensitive stores. A cationization of a non-CCE pathway should change the relative abilities of Ca\(_{2+}\), Ba\(_{2+}\), and Sr\(_{2+}\) to enter cells, unless the CCE and non-CCE pathways have identical divalent cation permeabilities. We initially tested the ability of a panel of cations to cause ratio changes after TG stimulation. We found that Ca\(_{2+}\), Cd\(_{2+}\), Sr\(_{2+}\), and Ba\(_{2+}\) caused ratio increases. However, Cd\(_{2+}\) caused ratio increases in unstimulated cells, an effect that may be related to the known toxic effects of Cd\(_{2+}\) on lymphocytes (Steffenson et al., 1994). Therefore, we restricted further experiments to Ca\(_{2+}\), Ba\(_{2+}\), and Sr\(_{2+}\).

To determine the divalent cation permeability of CCE in AJY’s, cells were treated with 1 μM TG in Ca\(_{2+}\)-free K\(^+\)-Ringer’s solution to deplete stores maximally and activate influx (Fig. 6A). After 1,368 s, the chamber was perfused with K\(^+\)-Ringer’s containing 10 mM Ca\(_{2+}\), Ba\(_{2+}\), or Sr\(_{2+}\). K\(^+\)-Ringer’s was used in all divalent cation experiments to clamp the membrane potential, a critical component of the driving force for cation entry, to near 0 mV. We analyzed the initial slope of the averaged ratio increase and found that the sequence of divalent cation-induced ratio changes in AJY’s stimulated with TG alone was Ca\(_{2+}\)(K\(^+\)-Ringer’s) > Sr\(_{2+}\) > Ba\(_{2+}\) (Table I). As will be discussed below, these results are consistent with an influx pathway that is highly selective for Ca\(_{2+}\).

To assess the divalent cation permeability of target-cell-stimulated influx (Fig. 6B), JY’s were dropped onto Fura-loaded AJY’s in Ca\(_{2+}\)-free K\(^+\)-Ringer’s. After 1,168 s, the chamber was perfused with K\(^+\)-Ringer’s containing 10 mM Ca\(_{2+}\), Ba\(_{2+}\), or Sr\(_{2+}\). Note that, based on the results of Fig. 5 indicating that target-cell contact depletes TG-sensitive stores, we expect target-cell contact to activate CCE in addition to any non-CCE pathways. The initial slope of cation-induced ratio changes followed the sequence Ca\(_{2+}\)(K\(^+\)-Ringer’s) > Sr\(_{2+}\) > Ba\(_{2+}\), similar to the results obtained with TG stimulation. Note that ratio changes...
produced by Ca$^{2+}$ addition were smaller and more transient in CTLs stimulated with J Y's than cells stimulated with TG (Fig. 6 B), indicating that both the peak and steady-state [Ca$^{2+}$]$_i$ were lower. This result is consistent with the idea that target-cell contact activates influx via a CCE pathway, as an increase in [Ca$^{2+}$] would be expected to promote store refilling, which has been shown to inactivate CCE in several cell types including lymphocytes (Jacob, 1990; Zweifach and Lewis, 1995). The relatively lower Ca$^{2+}$ slope observed in target-cell-stimulated A J Y S might cause a systematic error, leading us to overestimate the relative Ba$^{2+}$/Ca$^{2+}$ and Sr$^{2+}$/Ca$^{2+}$ ratios for target-cell stimulation.

We also measured the permeability of influx stimulated by J Y's + TG (Fig. 6 C), which like stimulation with J Y's, should activate CCE and non-CCE pathways. J Y's were dropped onto Fura-loaded A J Y S as above. A fter 1,264 s, the chamber was perfused with Ca$^{2+}$-free K$^+$-Ringer's + 1 μM TG. Finally, after an additional 1,232 s, the chamber was perfused with K$^+$-Ringer's containing 10 mM Ca$^{2+}$, Ba$^{2+}$, or Sr$^{2+}$. The initial slope of cation-induced ratio changes followed the sequence Ca$^{2+}(1) >$ Sr$^{2+}(0.094) >$ Ba$^{2+}(0.028)$, which is similar to the results obtained with targets or TG alone. The similarity of the sequence of divalent cation-induced ratio changes in cells stimulated with TG, J Y's, or J Y's + TG, taken together with observation that cells stimulated with J Y's + TG do not exhibit a higher influx rate than cells stimulated with TG alone, indicates that contact with targets does not activate a significant influx pathway in parallel to CCE.

**Divalent Cation Permeability of CCE in Jurkats Is Similar to CCE in AJYs**

On the basis of the results presented thus far, we conclude that CCE is the only significant Ca$^{2+}$ influx pathway stimulated by target-cell contact in CTLs. Whereas several different channel types mediating CCE have been reported (Parekh and Penner, 1997), a significant body of evidence suggests that CRAC channels mediate CCE in helper (CD4$^+$) T lymphocytes or lines with helper phenotype such as J urkat human leukemic T cells (Lewis and Cahalan, 1995). A s described in the Introduction, a key property distinguishing CRAC channels from other CCE channels is that they are highly selective for Ca$^{2+}$ over other divalent cations (Clapham, 1995; Parekh and Penner, 1997). CRAC channels are unique in their Ca$^{2+}$ selectivity among all CCE pathways described to date. Therefore, we investigated the pattern of divalent cation permeability in J urkats (Fig. 7), and compared it to the pattern of divalent cation permeability that we determined for CTLs. We stimulated Jurkats with 1 μM TG in Ca$^{2+}$-free K$^+$-Ringer's. A fter 1,368 s, we perfused the experimental chamber with K$^+$-Ringer's containing 10 mM Ca$^{2+}$, Ba$^{2+}$, or Sr$^{2+}$ (Fig. 7 A). The ability of the test cations to cause ratio changes was Ca$^{2+}(1) >$ Sr$^{2+}(0.06) >$ Ba$^{2+}(0.023)$, within a factor of two of the results obtained with A J Y S.

We confirmed that Fura-2 can detect differences in selectivity of different influx pathways by inducing nonselective influx using the detergent digitonin. We treated Jurkats with 4 μM digitonin in Ca$^{2+}$-free Ringer's solution (Fig. 7 B). Under these conditions, the membrane is first made permeable to divalent cations before it is made permeable to Fura-2, as determined by simultaneously measuring the F340/F380 ratio and F360 (data not shown). A fter 1,152 s, we perfused the chamber with Ca$^{2+}$-free Ringer's containing 10 mM Ca$^{2+}$, Ba$^{2+}$, or Sr$^{2+}$. We found that the slope of ratio changes in digitonin-treated Jurkats followed the sequence Ca$^{2+}(1) >$ Sr$^{2+}(0.42) >$ Ba$^{2+}(0.37)$, which is very different than the results with TG. These results confirm that Fura-2 can be used to discriminate selective from nonselective divalent cation influx, and indicate that CCE in CTLs is 7 times as selective for Ca$^{2+}$ over Sr$^{2+}$ and 17 times as selective for Ca$^{2+}$ over Ba$^{2+}$ as digitonin-induced nonselective influx.

Divalent cation-induced fluorescence changes can be affected by a number of factors, including the dissociation constant and maximum ratio of Fura-2, the properties of intracellular buffers, and whether the cation is extruded by plasma membrane ATPases. However, if these factors are similar in A J Y S and J urkats, then the similarity of CCE-stimulated divalent cation-induced fluorescence changes in the two cell types would provide evidence that CCE in the two cells has similar abilities to discriminate between divalent cations. To determine whether divalent cations can induce similar fluorescence changes in A J Y S and J urkats, we induced nonselective influx by treating cells with 4 μM digitonin in the presence of 10 mM Ca$^{2+}$, Ba$^{2+}$, or...
Sr$^{2+}$ (data not shown). We measured the F 340/F 380 ratio, as well as F 360 to monitor the progress of permeabilization. Within ~200 s of digitonin treatment, cells began to display ratio increases in the presence of all of the cations. The maximum ratios observed were as follows: Ca$^{2+}$(1.0) > Ba$^{2+}$(0.76) > Sr$^{2+}$(0.54) in Jurkats and Ca$^{2+}$(0.77) > Ba$^{2+}$(0.75) > Sr$^{2+}$(0.54) in A)Y)s. These results suggest that Fura-2 behaves similarly, but not identically, in the two cell types, as Ca$^{2+}$ gives a smaller maximum ratio in A)Y)s than in Jurkats. This suggests that our results may underestimate the Ba$^{2+}$/Ca$^{2+}$ and Sr$^{2+}$/Ca$^{2+}$ ratios for Jurkats by ~30% compared with A)Y)s. Correcting for this effect would make the results from Jurkats and A)Y)s more similar. We conclude that CCE in Jurkats and CTLs is similarly highly selective for Ca$^{2+}$ over Ba$^{2+}$ and Sr$^{2+}$.

**Significance**

We have demonstrated that target cells activate Ca$^{2+}$ influx in CTLs primarily via CCE. The evidence for these conclusions can be summarized as follows. [Ca$^{2+}$], responses in A)Y)s consist of both release from intracellular stores and influx, and release of Ca$^{2+}$ from intracellular stores precedes influx, as required if influx occurs through depletion-activated channels. A)Y) CTLs possess capacitative Ca$^{2+}$ entry as demonstrated by the ability of TG to stimulate robust [Ca$^{2+}$], increases, and contact with target cells depletes the TG-sensitive Ca$^{2+}$ store, as required if target-cell-stimulated Ca$^{2+}$ entry occurs via depletion-activated channels. Two tests for the existence of non-CCE pathways gave negative results. The amount of influx was not higher in cells stimulated with both JYs and TG than in cells stimulated by TG alone, and there was no difference in the divalent cation permeability of influx stimulated by target cells, TG, or the combination of TG and target-cell contact. Note that we cannot entirely rule out minor participation by an additional Ca$^{2+}$ influx pathway.

We have demonstrated that CCE in CTLs has a similar degree of selectivity for Ca$^{2+}$ over Sr$^{2+}$ and Ba$^{2+}$ as CCE in Jurkats, which is believed to occur via CRAC channels (Lewis and Cahalan, 1995). We have as yet been unable to detect the CCE pathway in CTLs in patch clamp experiments, largely because of the presence of a contaminating inwardly rectifying current carried by monovalent cations (Zweifach, A., unpublished observations). Electrophysiological recordings of the CCE current in CTLs would allow determination of such properties as the I-V relationship, amount of current noise, and inactivation properties of the influx pathway, thus providing further points of comparison with I$_{\text{CRAC}}$. However, as described in the Introduction, these properties are not unique to CRAC channels, and may, therefore, not be good discriminators between CCE pathways. A)S I$_{\text{CRAC}}$ is the only CCE pathway described to date that is selective for Ca$^{2+}$ over Ba$^{2+}$ and Sr$^{2+}$, our results suggest that CCE in CTLs is mediated by CRAC channels, although it is possible that there are Ca$^{2+}$-selective CCE pathways that are not CRAC that have not yet been discovered.

Our results are significant for three reasons. First, we have shown that target-cell-stimulated Ca$^{2+}$ influx in CTLs occurs primarily via CCE. Previous work has demonstrated Ca$^{2+}$ influx in CTLs in response to target-cell contact, but has not addressed its mechanism. Second, this report is the first time it has been demonstrated that contact of a T cell with a relevant cellular partner activates influx primarily via CCE. Previous studies in which the mechanism of influx in T cells has been addressed used mAbs (Hess et al., 1993; Partiseti et al., 1994; Premack et al., 1994), mitogenic lectins (Lewis and Cahalan, 1989; Zweifach and Lewis, 1993), or pharmacological agents like TG (Zweifach and Lewis, 1993; Premack et al., 1994) to stimulate cells. In studies in which contact-mediated [Ca$^{2+}$], signals have been reported, the mechanism of influx was not determined (Gray et al., 1987, 1988; Poonie et al., 1987; Donnadieu et al., 1992; A grawal and Linderman, 1995; Negulescu et al., 1996; Delon et al., 1998). Our results rule out the possibility that a significant non-CCE influx pathway is activated by one of the many different cell surface molecules that participate in interactions between T cells and APCs or targets (Berke, 1994; Ni et al., 1999). The third significant finding of this study is that CCE is able to play very diverse roles in T cell physiology. In helper T cells, CRAC channel activity over a period of hours plays a key role in activation of cytokine genes.

Our results demonstrate that CCE, likely also mediated by CRAC channels, can contribute to a very different and much more rapid T cell behavior, i.e., exocytosis of lytic granules. Work on excitable cells has shown that voltage-gated Ca$^{2+}$ channels play roles in processes as diverse as vesicle exocytosis and gene transcription (Ghosh and Greenberg, 1995). The present work demonstrates that CCE plays similarly diverse roles in T cells.

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