Differential Ca^{2+} Influx, K_{Ca} Channel Activity, and Ca^{2+} Clearance Distinguish Th1 and Th2 Lymphocytes[1]

Christopher M. Fanger, Amber L. Neben, and Michael D. Cahalan[2]

In Th1 and Th2 lymphocytes, activation begins with identical stimuli but results in the production of different cytokines. The expression of some cytokine genes is differentially induced according to the amplitude and pattern of Ca^{2+} signaling. Using fura-2 Ca^{2+} imaging of murine Th1 and Th2 clones, we observed that the Ca^{2+} rise elicited following store depletion with thapsigargin is significantly lower in Th2 cells than in Th1 cells. Maximal Ca^{2+} influx rates and whole-cell Ca^{2+} currents showed that both Th1 and Th2 cells express indistinguishable Ca^{2+}-release-activated Ca^{2+} channels. Therefore, we investigated other mechanisms controlling the concentration of intracellular Ca^{2+}, including K^+ channels and Ca^{2+} clearance from the cytosol. Whole-cell recording demonstrated that there is no distinction in the amplitudes of voltage-gated K^+ currents in the two cell types. Ca^{2+}-activated K^+ (K_{Ca}) currents, however, were significantly smaller in Th2 cells than in Th1 cells. Pharmacological equalization of Ca^{2+}-activated K^+ currents in the two cell types reduced but did not completely eliminate the difference between Th1 and Th2 Ca^{2+} responses, suggesting divergence in an additional Ca^{2+} regulatory mechanism. Therefore, we analyzed Ca^{2+} clearance from the cytosol of both cell types and found that Th2 cells extrude Ca^{2+} more quickly than Th1 cells. The combination of a faster Ca^{2+} clearance mechanism and smaller Ca^{2+}-activated K^+ currents in Th2 cells accounts for the lower Ca^{2+} response of Th2 cells compared with Th1 cells. *The Journal of Immunology,* **2000, 164: 1153–1160.**

The activation of mature Th lymphocytes results in the synthesis and secretion of a battery of different cytokines. Effector Th cells fall into two general categories, Th1 and Th2, defined by the type of cytokines they secrete. The particular cytokines produced during an immune response determine the types of other immune cells recruited. Th1 cells secrete IL-2 and IFN-γ and tend to promote cytotoxic responses and macrophage activation, whereas Th2 cells secrete IL-4, IL-5, and IL-10 and promote Ab-dependent responses by activating mast and B cells (1). During their initial stimulation, Th cells irreversibly differentiate to secrete a fixed set of cytokines (2). Genetic predisposition, level of stimulation, and type of costimulation may all play a role in determining whether Th1 or Th2 cells predominate in any given immune response (3–5).

The same signal transduction cascade is launched in both Th1 and Th2 cells by TCR binding to Ag in the context of MHC class II on an APC. The part of the pathway leading to cytokine production can be divided into three different spatiotemporal domains. The proximal domain of membrane-delimited events initiates the cascade and includes TCR ligation, tyrosine kinase activation, and the stimulation of phospholipase C to generate diacylglycerol and inositol-1,4,5-trisphosphate. Inositol-1,4,5-trisphospate and diacylglycerol activate events in the middle domain, which is largely dominated by the Ca^{2+} and K^+ channel-regulated movement of Ca^{2+} ions and which culminates in the activation of calcineurin and protein kinase C by Ca^{2+}/calmodulin and Ca^{2+}/diacylglycerol, respectively (reviewed in Ref. 6). Finally, calcineurin and the serine/threonine kinases activate a set of transcription factors that promote gene expression. All three domains have been implicated in controlling differential cytokine expression in Th1 and Th2 cells.

Distinctions between Th1 and Th2 proximal signal transduction have been detected in the tyrosine kinases Zap-70 and Fyn and in the p38 mitogen-activated kinase and CD28 costimulatory pathways (7–9). However, the importance of these differences is unclear because the method of proximal stimulation does not alter the type of cytokine generated by Th1 or Th2 clones (2, 10–12). At the opposite end of the signal transduction cascade, Th subtype-specific transcription factors have been described, such as GATA-3 and c-maf in Th2 cells and Stat-4 and Ying-Yang 1 in Th1 cells (Refs. 13 and 14; reviewed in Refs. 15 and 16). The involvement of the ubiquitous transcription factor NF-AT in regulation of the IL-4 and IFN-γ genes has also been documented, but a controversy exists as to whether NF-AT is most influential in Th1 or Th2 cytokine production (13, 17). Although both proximal and distal events seem to play a vital role in determining cytokine expression, the mechanisms enforcing transcription factor specificity remain to be elucidated.

The contribution of the central domain of Ca^{2+} flux to the control of cytokine expression remains largely unexplored. A sustained rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$),[3] mediated by Ca^{2+} release-activated Ca^{2+} (CRAC) channels, is necessary for calcineurin-driven translocation to the nucleus of the transcription factor NF-AT (Refs. 18–21; reviewed in Ref. 6). In Th2 cells, TCR stimulation results in a lower amplitude rise in $[Ca^{2+}]_i$ than in Th1 cells (11). The Ca^{2+} response in Th2 cells appears to be lost

Department of Physiology and Biophysics, University of California, Irvine, CA 92697

Received for publication August 30, 1999. Accepted for publication November 10, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants NS14609 and GM41514 (to M.D.C.) and National Cancer Institute Grant 5T32CA09054 (to C.M.F.).

[2] Address correspondence and reprint requests to Dr. Michael D. Cahalan, Room 285, Irvine Hall, University of California, Irvine, CA 92697-4560. E-mail address: mcahalan@uci.edu

[3] Abbreviations used in this paper: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_{ss}$, steady-state Ca^{2+} concentration; CRAC, Ca^{2+} release-activated Ca^{2+}; CTX, charybdotoxin; K_{Ca}, Ca^{2+}-activated K^+; K_V, voltage-gated K^+; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; TEA, tetraethylammonium; Tg, thapsigargin.

0022-1767/00/$02.00

during differentiation from a naive T cell to an effector (22). Two groups have suggested that a distinct pathway for Ca^{2+} influx might participate in Th2 activation (7, 23). However, the mechanism and role of the modified Ca^{2+} response observed in Th2 lymphocytes remains elusive.

The pharmacological agent thapsigargin (Tg), which specifically inhibits the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) responsible for Ca^{2+} uptake into stores (24), bypasses proximal signaling events and permits the direct examination of store release and Ca^{2+} influx. We compared Ca^{2+} signaling and ion channel expression in Th2 and Th1 cells, focusing on the Ca^{2+} channels and on voltage-gated K^+ (K_V) and Ca^{2+}-activated K^+ (K_{Ca}) channels that control the membrane potential. Although we found no differences in the Ca^{2+} channels that mediate Ca^{2+} entry, we found two primary distinctions between the Th subpopulations: 1) increased expression of functional K_{Ca} channels in Th1 cells; and 2) increased rate of Ca^{2+} clearance from the cytosol in Th2 cells. A preliminary report describing this work has appeared in abstract form (50).

Materials and Methods

Cell maintenance

All reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified. Mouse T cell clones D1.6 and CDC35 were obtained from R. Noelle (Dartmouth Medical School) and have been previously described (10, 25). Both are specific for rabbit IgG and are restricted to I-A^d. Cells were maintained in Click's medium supplemented with 10 mM HEPES, 2 mM glutamine, 10% FBS (Summit Biotechnology, Greeley, CO), 3.5×10^{-4} % 2-ME, and 10 ng/ml IL-2 at 37°C with room air plus 5% CO_2. Biweekly, cells were passed by placing 1–2 $\times 10^5$ T cells in each well of a 12- or 24-well culture plate and were activated by the addition of 5×10^6 lethally irradiated BALB/c (The Jackson Laboratory, Bar Harbor, ME) splenocytes plus 0.1 mg/ml rabbit IgG. To remove cells from culture plates for experiments, the medium was removed and cells were incubated for 5 min with 1 ml of PBS plus 0.5 mM EDTA. They were then washed once in medium and used within 4 h. All experiments were performed on cells during days 5–12 following passage and activation, and none of the characteristics described herein varied systematically over this time frame.

FACS

Th1 and Th2 clones were stimulated for 4–8 h with 50 nM PMA plus 2 μM ionomycin (Calbiochem-Novabiochem, San Diego, CA) in the presence of GolgiPlug (PharMingen, San Diego, CA; trade name for brefeldin A). Cells were stained according to the Cytofix/Cytoperm Plus Kit (PharMingen). All staining steps were performed with mAbs directly conjugated to fluorophores, including anti-IL-4 and anti-CD8 conjugated to PE, anti-IFN-γ and anti-CD4 conjugated to FITC, and anti-CD3 conjugated to TRICOLOR. All Abs were obtained from PharMingen or Caltag (South San Francisco, CA). A FACScan (Becton Dickinson, Los Angeles, CA) and CellQuest software were used for data collection and analysis. Data presented are corrected for cross-talk between fluorophores by offline compensation based on singly stained samples.

Imaging

Cells were loaded for 30 min at 21–24°C in 1 μM fura-2-acetoxymethyl ester (fura-2-AM) (Molecular Probes, Eugene, OR) plus normal growth medium. They were then adhered to poly-L-lysine-coated glass coverslips and placed on the stage of a Zeiss Axiovert 35 microscope (Carl Zeiss, Thornwood, NY) equipped with a Zeiss 63× Neofluar objective (NA 1.25). A complete videoprobe video-microscopic Ca^{2+} imaging system (ETM Systems, Petaluma, CA) was used for $[Ca^{2+}]_i$ measurements. Light from a xenon arc lamp (Hamamatsu Photonics, Bridgewater, NJ) was passed alternately through excitation bandpass filters of 350 ± 5 or 380 ± 5 nm, which were exchanged by a computer controlled Lambda-10 and filter wheel unit (Sutter Instruments, Novato, CA). A 400-nm dichroic mirror and 480-nm long-pass emission filter supplied light to the Hamamatsu SIT camera. All optical filters were from Chroma Optics (Brattleboro, VT). Typically, data acquisition occurred at a rate of one dual-wavelength image every 5 s, although this acquisition rate was increased to one image every 3 s for the Ca^{2+} clearance rate experiments.

$[Ca^{2+}]_i$ was estimated using the formula $[Ca^{2+}]_i = K^* (R - R_{min})/(R_{max} - R)$, where the values of K^*, R_{min}, and R_{max} were determined using Ca^{2+} standards containing 10 mM $CaCl_2$ or 10 mM EGTA and fura 2 pentapotassium salt for in vitro calibrations in a thin, glass chamber.

The values of R_{min} and R_{max} determined in this way were then adjusted to the anticipated in vivo values using correction factors derived from T cells dialyzed with the above solutions or known Ca^{2+} concentration standards from Molecular Probes. In these experiments, simultaneous whole-cell patch-clamp and imaging data were acquired with fura-2 pentapotassium salt inside the pipette. To derive these correction factors, three to four D1.6 cells were examined at each of three Ca^{2+} concentrations: 0 nM, 1 mM, and 250 nM. These measurements yielded a calculated K_d of fura 2 for Ca^{2+} inside T cells of 248 nM.

During imaging experiments, cells were bathed in normal Ringer solution consisting of 155mM NaCl, 4.5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 10 D-glucose, 5mM HEPES, pH 7.4. Ca^{2+}-free Ringer solution is identical except that it contains 1 mM EGTA in place of $CaCl_2$ and a total of 3 mM $MgCl_2$. K^+ Ringer solution consists of 159.5mM KCl, 1mM $MgCl_2$, 22mM $CaCl_2$, 10mM D-glucose, 5mM HEPES, pH 7.4. Ca^{2+}-free K^+ Ringer is identical but contains 1 mM EGTA instead of $CaCl_2$ and a total of 3 mM $MgCl_2$. In all imaging experiments, cells were mounted in a chamber permitting rapid (~1 s) solution exchange by a syringe-driven perfusion system. Tg was obtained from Alexis Chemical (San Diego, CA).

Patch-clamp experiments

All patch-clamp experiments used the whole-cell configuration, a holding potential of −80 mV (except for CRAC current experiments in which the holding potential was −20 mV) and the equipment and techniques previously described (26). All data were corrected for a liquid junction potential of −13 mV for aspartate-based internal solutions. For K_{Ca} experiments, high Ca^{2+} internal solution consisted of 130mM potassium aspartate, 10mM K_2EGTA, 8.55mM $CaCl_2$, 2.08mM $MgCl_2$, 10mM HEPES, pH 7.2, 290 mOsm, with a calculated free $[Ca^{2+}]$ of 1 μM. Low Ca^{2+} internal solution was used for K_V experiments and was identical except that it contained 2.28 mM added $CaCl_2$, yielding a free $[Ca^{2+}]$ of 50 nM. K_V and voltage-gated Ca^{2+}-channel experiments employed a p/4 leak-subtraction routine in which a leak pulse was applied after each voltage pulse was completed. K_V data are shown after leak subtraction. In experiments attempting to detect voltage-gated Ca^{2+} channels, the internal solution consisted of 118mM cesium aspartate, 10mM cesium HEPES, 1.2mM EGTA, 0.23mM $CaCl_2$, 2mM $MgCl_2$, 40mM mannitol, pH 7.2. This solution had a calculated free $[Ca^{2+}]$ of 50 nM. To elicit voltage-gated Ca^{2+} currents, we used a protocol of 10 ms depolarization from the holding potential to 0 mV. For CRAC experiments, the internal solution consisted of 138mM cesium aspartate, 10mM HEPES, 12mM *O,O′*-bis(2-aminophenyl)ethyleneglycol-*N,N,N′,N′*-tetraacetate cesium salt (CsBAPTA; Molecular Probes), 0.3mM $CaCl_2$, 2.46mM $MgCl_2$, pH 7.2, with a calculated free $[Ca^{2+}]$ of 10 nM. CRAC experiments employed a holding potential of −20 mV, during which 200-ms voltage ramps were applied once per second from −140 mV to +60 mV. Free $[Ca^{2+}]$ was calculated using MaxChelator (Chris Patton, Stanford University). All external solutions were as described above under *Imaging*. Additionally, some patch-clamp experiments used tetraethylammonium (TEA) Ringer solution, in which 155 mM TEA was substituted for NaCl in the normal Ringer formula. By mixing TEA Ringer solution with normal Ringer solution we derived the desired concentration of TEA. Charybdotoxin (CTX) was obtained from Bachem Biosciences (King of Prussia, PA).

Data analysis

All initial analyses were performed using Igor Pro software (Wavemetrics, Lake Oswego, OR) with home-written macros and extensions. The analysis of rates of initial Ca^{2+} influx and of Ca^{2+} clearance involved the calculation of maximal increase or decrease rate for each cell by finding the maximal absolute value of the slope between each pair of points. Histograms were normalized for the total number of cells analyzed in the following way: the histogram data is divided by the total number of cells, then multiplied by 100 to permit an axis without numbers <1. For statistical analysis, data were exported to Excel (Microsoft, Redmond, WA) and analyzed using a two-tailed unpaired Student's *t* test assuming unequal variances. Data were considered statistically significant when $p < 0.01$. All data are reported as mean ± SEM (number of experiments) except in the tables, where results are reported as mean ± SD (number of cells).

Results

Differential Ca^{2+} response in Th1 and Th2 lymphocytes

Surface markers and cytokine expression of two mouse T cell clones, D1.6 and CDC35, were analyzed by flow cytometry; >90% of cells in both clones were $CD3^+$ $CD4^+$ $CD8^-$ (data not

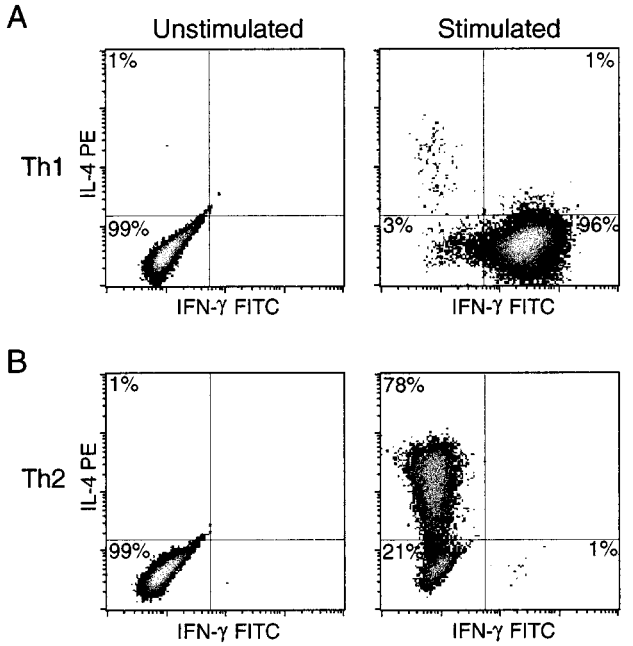

FIGURE 1. Clones D1.6 and CDC35 are a Th1 and a Th2 clone, respectively. Approximately 1 wk after their previous passage and stimulation, the clones D1.6 and CDC35 were incubated for 6 h in the presence of GolgiStop with or without stimulation by PMA and ionomycin. Fixation, permeabilization, and staining for cell-surface markers and cytokines was followed by FACS analysis of 2×10^4 cells per condition. Cells that were positive for the cell-surface marker CD3 (>90% of all samples) are shown. Numbers represent the percentage of cells in each quadrant, and the absence of a number indicates that <1% of cells are found in this quadrant. *A*, Unstimulated (*left*) and stimulated (*right*) clone D1.6, which is a Th1 clone. *B*, Unstimulated (*left*) and stimulated (*right*) clone CDC35, which is a Th2 clone. Data shown are from a single experiment, but these results were confirmed in two separate repetitions. Additional control experiments not shown included staining with isotype control Abs or preincubation of the anti-IL-4 or anti-IFN-γ Ab with a 10-fold excess of its respective target cytokine before staining. In both conditions, positive cytokine staining was eliminated, confirming the specificity of our reagents (data not shown).

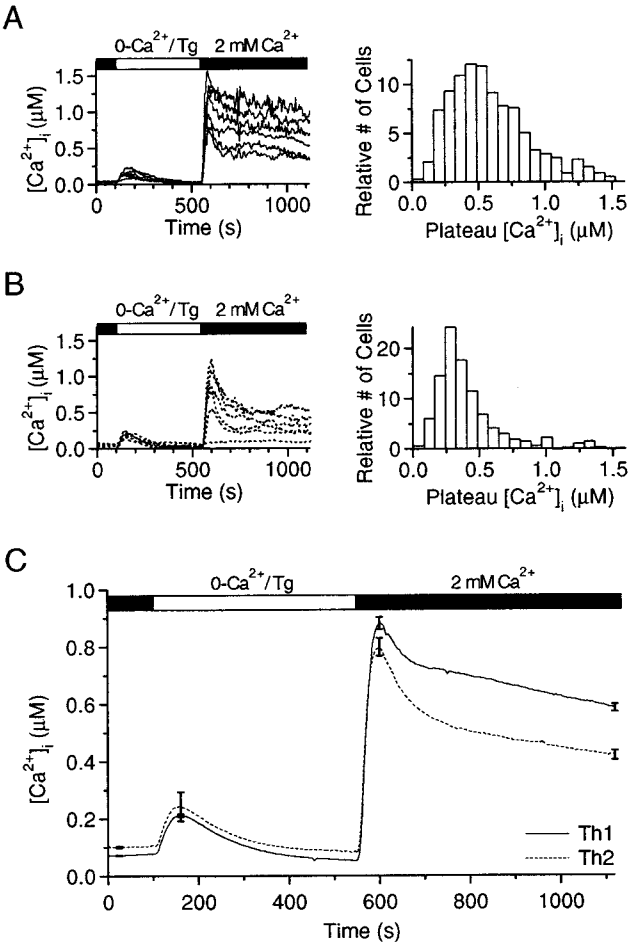

FIGURE 2. Tg-stimulated Ca^{2+} responses are lower in Th2 cells than in Th1 cells. After 100 s baseline in normal Ringer solution, fura-2-loaded Th1 and Th2 cells were stimulated with 1 μM Tg in Ca^{2+}-free Ringer solution, followed after ~8 min by perfusion with normal Ringer solution. (see bars above each graph). *A*, The overlaid responses of six typical Th1 cells from one imaging experiment (*left*) and a histogram showing the plateau $[Ca^{2+}]_i$ levels ~9 min after Ca^{2+} reintroduction for all Th1 cells (*right*, 511 cells from 12 experiments). *B*, The responses of six typical Th2 cells from one imaging experiment (*left*) and a histogram showing the plateau $[Ca^{2+}]_i$ levels for all Th2 cells (*right*, 259 cells from 11 experiments). *C*, The averaged responses of all Th1 cells (solid line) and Th2 cells (dotted line) are shown. Error bars show the SE at baseline, store release peak, influx peak, and plateau and are omitted elsewhere for clarity.

shown). The $CD3^+$ population was 96% IFN-γ^+ IL-4^- in the clone D1.6 and 78% IFN-γ^- IL-4^+ in the clone CDC35 (Fig. 1). In each clone, 1% or fewer cells expressed the other cytokine or both cytokines, and unstimulated cells expressed neither cytokine. Throughout the rest of the study, we refer to the D1.6 and CDC35 clones simply as Th1 and Th2, respectively.

To determine whether the Ca^{2+} signaling differences previously noted in Th1 and Th2 clones resulted from a change in the factors directly regulating Ca^{2+} entry, we used single-cell fura-2 Ca^{2+} imaging techniques. Resting $[Ca^{2+}]_i$ in Th2 cells was significantly higher than in Th1 cells (102 ± 3 nM compared with 74 ± 2 nM, $p < 0.0001$). Treatment of cells with Tg in the absence of extracellular Ca^{2+} resulted in a transient rise in $[Ca^{2+}]_i$, revealing a slow leak in intracellular Ca^{2+} stores. Ca^{2+} store release transients were similar in both Th1 and Th2 cells (Fig. 2). However, upon readdition of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ level achieved by Th2 cells was substantially lower than in Th1 cells and exhibited a more rapid decline (Fig. 2, *A* and *B*, *left panels*). By analyzing plateau $[Ca^{2+}]_i$ values for each cell, we generated histograms (Fig. 2, *A* and *B*, *right panels*) permitting comparison of our data, in which Tg was used as a stimulant, with previously published data employing an APC-mediated stimulus. Despite the different mode of activation and different clones used, the Ca^{2+} plateau histograms in Fig. 2 are very similar to those shown by Sloan-Lancaster et al. (22). At the end of the experiment, ~9 min after Ca^{2+} readdition, mean $[Ca^{2+}]_i$ for Th1 cells was 585 ± 13 nM (511 cells from 12 runs) as compared with 407 ± 16 nM for Th2 cells (259 cells from 11 runs; $p < 0.0001$). The averaged traces (Fig. 2*C*) also demonstrate a significantly lower Ca^{2+} influx component in Th2 cells compared with Th1 cells, but disguise the rapid decline from peak $[Ca^{2+}]_i$ seen in single Th2 cells. We conclude that mechanisms governing the Ca^{2+} response of lymphocytes must differ in Th1 and Th2 cells.

Channels contributing to the lower Ca^{2+} response of Th2 cells

Ca^{2+} channels. Upon Ca^{2+} readdition following store depletion, the maximal rate of the $[Ca^{2+}]_i$ rise constitutes a rough measure of influx rate through Ca^{2+} channels (21). Using the protocol illustrated in Fig. 3*A*, we generated histograms of the maximal rates of Ca^{2+} rise in Th1 and Th2 cells (Fig. 3*B*), in which mean influx rates were 42.8 ± 1.4 nM/s and 39.2 ± 2.2 nM/s, respectively

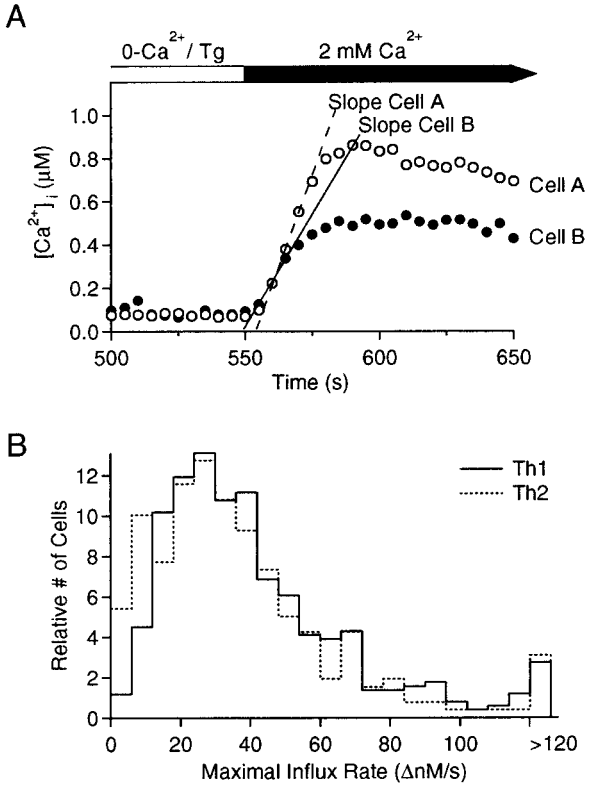

FIGURE 3. Maximal Ca^{2+} influx rates are indistinguishable in Th1 and Th2 cells. Data from the imaging experiments shown in Fig. 2 were reanalyzed for maximal Ca^{2+} influx rate in each cell. *A,* Individual data points are shown on a magnified time scale for two Th1 cells at the time of Ca^{2+} readdition following store depletion. The maximal rate of Ca^{2+} increase in nM/s was calculated for each cell, as illustrated by the slopes of the lines. *B,* Histogram of Ca^{2+} influx rates for all 511 Th1 (solid line) and 259 Th2 (dotted line) cells.

($p = 0.16$). There is no significant difference between the rates of rise in Th1 and Th2 cells. Thus, both clones have similar numbers of Ca^{2+} channels activated by Ca^{2+} store depletion.

Both CRAC channels and voltage-gated Ca^{2+} channels have been suggested to mediate Ca^{2+} influx in T lymphocytes (20, 21, 23, 27, 28). To assess Ca^{2+} channel activity directly, we used whole-cell patch-clamp experiments with an internal solution containing cesium aspartate to block K^+ channels and a high concentration of the Ca^{2+} chelator BAPTA (12 mM) to deplete Ca^{2+} stores. Over ~2 min, both Th1 and Th2 cells developed a small, inwardly rectifying current with a reversal potential more positive than +40 mV and no voltage-dependence (data not shown). This current was present at similar levels in both Th1 and Th2 cells and developed without any obvious increase in noise, indicating a tiny unitary conductance. We identified the current as CRAC current on the basis of its similarity to CRAC currents previously observed in murine and Jurkat T cells (20, 28). No voltage-gated Ca^{2+} influx was ever observed in either Th1 (0 of 16) or Th2 (0 of 5) cells (data not shown). Thus, both Th1 and Th2 cells express CRAC channels, but lack voltage-gated Ca^{2+} channels.

K_v and K_{Ca} channels. Ca^{2+} influx is regulated by the number of open Ca^{2+} channels and by the electrochemical gradient driving Ca^{2+} ions into the cell. Under conditions of normal extracellular Ca^{2+} concentration, the driving force for Ca^{2+} entry in T cells is governed by the membrane potential, which is in turn controlled predominantly by K^+ channels (Refs. 29 and 30; reviewed in Ref. 31). Both K_V and K_{Ca} channels have been shown to exist and alter membrane potential in T lymphocytes (32).

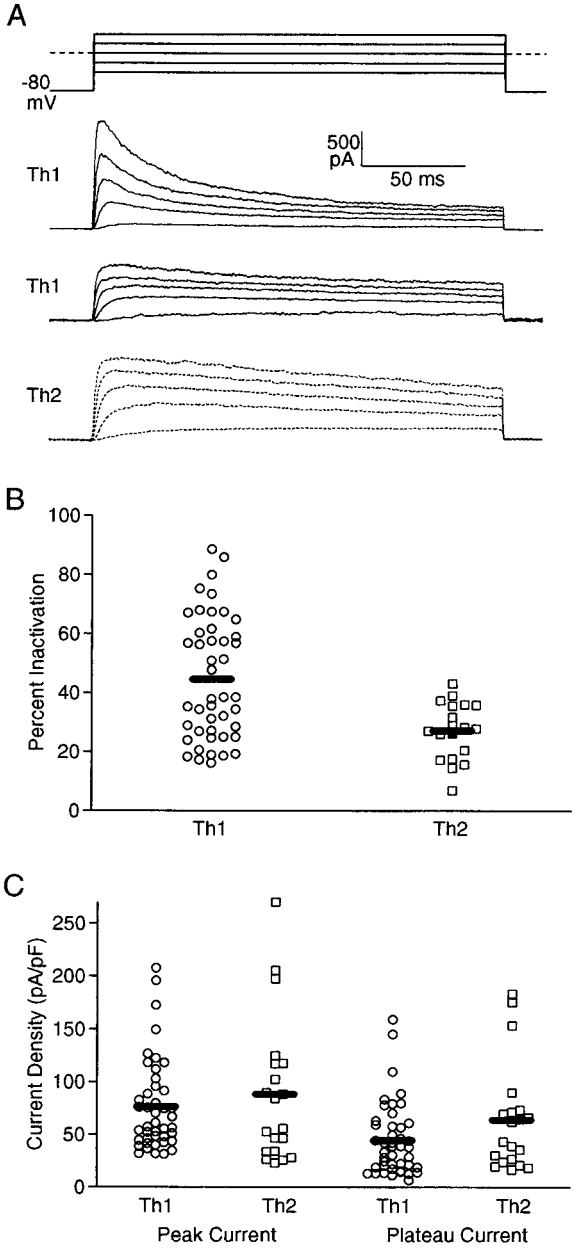

FIGURE 4. K_V currents in Th1 and Th2 cells have similar amplitudes but different rates of inactivation. Whole-cell patch-clamp experiments with low-Ca^{2+} internal solution (50 nM free Ca^{2+}) prevent the activation of K_{Ca} channels (32). *A,* K_V currents were evoked by 200-ms pulses to +40 mV through −40 mV in −20 mV decrements, as illustrated (*top*). Between each pulse, 35 s were allowed for the channels to recover from inactivation. Families of leak-subtracted currents elicited in two example Th1 cells (*middle traces*) and one Th2 cell (*bottom trace*) are shown. *B,* The degree of inactivation within a single 200-ms pulse to +40 mV is plotted for each of 44 Th1 and 20 Th2 cells at a time shortly after break-in. Percent inactivation is calculated as 1 − (plateau current/peak current), where the plateau current is the current in the last 10 ms of the pulse. Mean values are shown by the solid bars. *C,* Mean peak and plateau currents for each cell shown in *B* during pulses to +40 mV shortly after break-in. The displayed current density equals current divided by cell capacitance to correct for cell size. Bars represent mean values.

Whole-cell patch-clamp experiments with low Ca^{2+} internal solution revealed voltage-dependent outward currents that activated near −40 mV, were maximal at the most depolarized potentials, and partially inactivated during the course of each pulse (Fig. 4*A*).

Table I. *Pharmacology of K_V channels in Th1 and Th2 cells*[a]

Cell Type	TEA (1 mM)	CTX (100 nM)	Clotrimazole (50 nM)
Th1	11 ± 6 (7)	86 ± 11 (9)	3 ± 5 (3)
Th2	7 ± 5 (5)	96 ± 1 (3)	ND

[a] Numbers represent mean percent block ± SD (number of cells measured). Block equals 1 − (post-block current/pre-block current). Whole-cell patch-clamp measurements were made using 200-ms pulses to +40 mV from a holding potential of −80 mV. Leak-subtracted peak current amplitudes were noted before and after application of TEA, CTX, or clotrimazole at the concentration shown, and block was calculated after 2 min exposure to drug, except in the case of clotrimazole, where it was calculated at 15 min after cell treatment. Differences in percent block between Th1 and Th2 cells are not statistically significant.

Repeated pulses to +40 mV at 1-s intervals caused a rapid, use-dependent inactivation of the current (data not shown). The predominant current in both cell types corresponds to the previously described lymphocyte *n*-type K_V current encoded by the KV1.3 gene, as demonstrated by the voltage dependence, inactivation, and pharmacology of TEA and CTX block (Table I; Refs. 33 and 34). The degree of K_V current inactivation during each voltage pulse varied from cell to cell. For many Th1 and Th2 cells, ~30% of the current inactivated within each pulse (Fig. 4*A*, *middle* and *bottom traces*), but a number of Th1 cells showed more rapid inactivation (Fig. 4*A*, *top trace*). Inactivation for all cells is displayed in Fig. 4*B*. On average, K_V currents inactivated more completely in Th1 cells than in Th2 cells (45 ± 3% in 44 Th1 cells compared with 27 ± 2% in 20 Th2 cells; $p < 0.0001$). The Th1 cells with more rapid current inactivation were also somewhat less sensitive to CTX treatment, suggesting that additional K_V channels with different pharmacology are expressed in Th1 cells. However, the overall amplitude of K_V currents was indistinguishable in Th1 and Th2 cells. Mean peak current densities measured at +40 mV and corrected for cell size were 76 ± 7 pA/pF (44 cells) in Th1 cells and 88 ± 15 pA/pF (20 cells) in Th2 cells (Fig. 4*C*); values that are not significantly different ($p = 0.48$). Plateau current densities at the end of a 200-ms pulse were also not significantly different (44 ± 5 pA/pF for Th1 cells and 64 ± 11 pA/pF for Th2 cells; $p = 0.13$). We conclude that similar levels of K_V current are present in both Th1 and Th2 cells; thus, K_V channels probably do not contribute to differences in Ca^{2+} signaling in the Th subtypes.

K_{Ca} channels also help to establish lymphocyte membrane potential and thereby alter the driving force for Ca^{2+} entry (29, 30). Calmodulin prebound to the intermediate-conductance K_{Ca} channel found in T lymphocytes mediates channel activation upon Ca^{2+} binding (26, 35). High-Ca^{2+} internal solution elicited a combination of K_{Ca} and K_V current. We analyzed the K_{Ca} current in isolation by measuring the slope of the ramp at potentials more negative than the activation threshold of K_V currents (Fig. 5*A*). The slope conductance corrected for cell size (specific conductance) obtained for each cell is displayed in Fig. 5*C*. The mean K_{Ca} conductance was significantly different in the two Th subtypes (0.42 ± 0.04 nS/pF in 53 Th1 cells compared with 0.24 ± 0.02 nS/pF in 44 Th2 cells; $p = 0.0001$). On average, Th2 cells have only 57% of the K_{Ca} current found in Th1 cells. Fig. 5, *A* and *B* and Table II demonstrate that both Th1 and Th2 cells express K_{Ca}

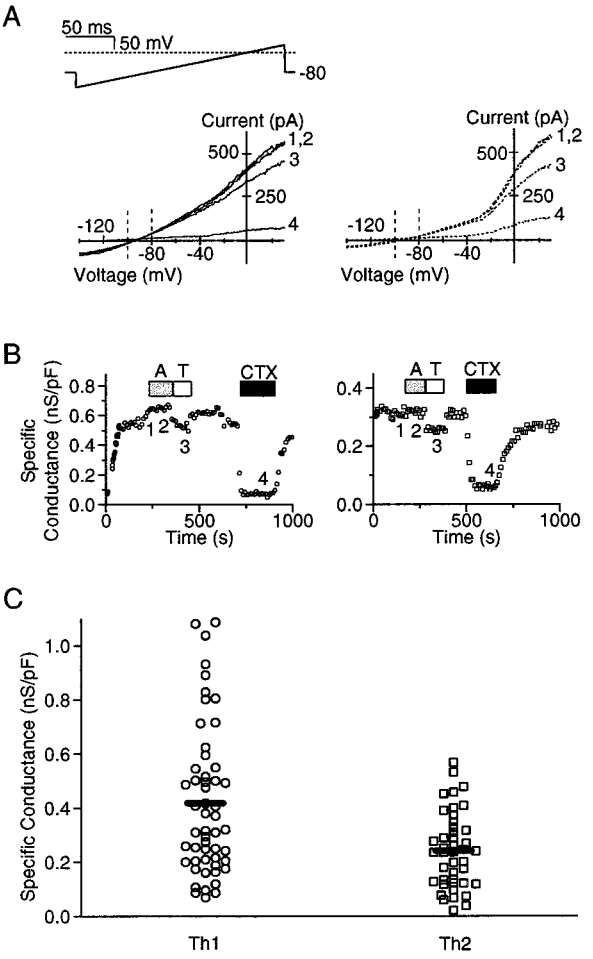

FIGURE 5. Th2 K_{Ca} conductance is significantly lower than Th1 K_{Ca} conductance. In whole-cell patch-clamp experiments with high-Ca^{2+} internal solution (1 μM free Ca^{2+}), we applied 200-ms ramps every 10 s from −140 mV to +30 mV. *A*, Currents shown were observed in a single Th1 cell (*left*) and a single Th2 cell (*right*) using the voltage ramp protocol illustrated at the top. Ramps shown are from the times in *B* denoted by the numbers. The dashed lines define the region in which we measured slope conductance to isolate pure K_{Ca} conductance. *B*, Time course of K_{Ca} conductance in a single Th1 cell (*left*) and Th2 cell (*right*) exposed to 3 nM apamin (A), 10 mM TEA (T), and 10 nM CTX (see bars above each graph). Drugs were added by rapid perfusion and exchange of the bath solution, and the end of each bar represents wash with normal Ringer solution except in the case of the switch from apamin directly to TEA. Slope conductances of K_{Ca} current were measured near −90 mV, as described in *A*, and corrected for cell size by dividing by cell capacitance to yield specific conductance. *C*, The specific conductance measurement for each of 53 Th1 and 44 Th2 cells, with mean values denoted by solid bars.

Table II. *The pharmacology of K_{Ca} channels in Th1 and Th2 cells is identical*[a]

Cell Type	TEA (10 mM)	CTX (10 nM)	Apamin (3 nM)	Clotrimazole (50 nM)
Th1	16 ± 6 (8)	84 ± 2 (3)	0 ± 1 (4)	44 ± 18 (3)
Th2	13 ± 7 (10)	81 ± 8 (5)	13 ± 15 (5)	ND

[a] Numbers represent the mean percent block of K_{Ca} conductance ± SD (number of cells). Measurements were made during whole-cell recordings in which slope conductance was measured from voltage ramps as described (Fig. 5). Block was determined after 2 min exposure to drug, except in the case of clotrimazole, where block was calculated at 15 min after cell treatment. Block was calculated as 1 − (post-block conductance/pre-block conductance). For both cell types, the pharmacological profile is consistent only with the intermediate-conductance K_{Ca} channel (32), and none of the differences between percent block in Th1 and Th2 cells are significant.

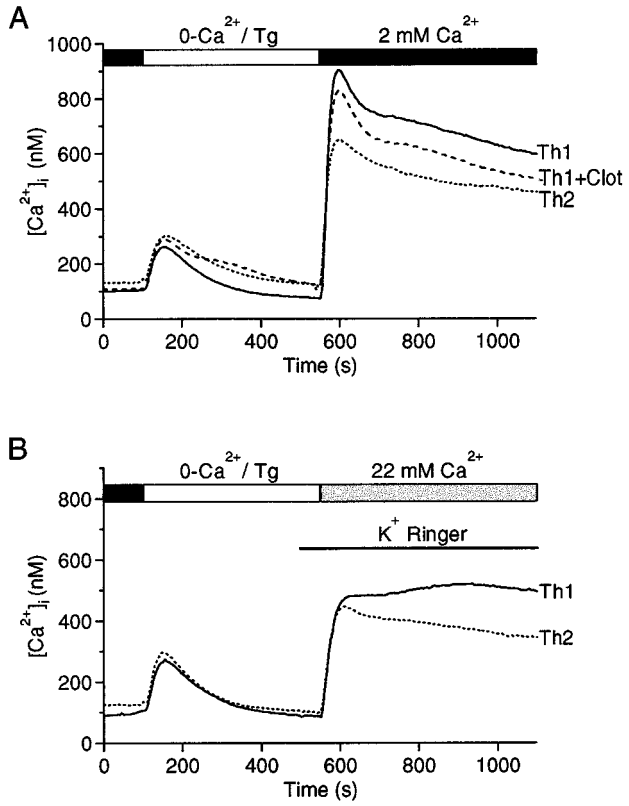

FIGURE 6. K_{Ca} channels are partially responsible for the lower Ca^{2+} response of Th2 cells. Fura-2 imaging experiments used the protocol from Fig. 2, in which depletion of Ca^{2+} stores with 1 μM Tg in Ca^{2+}-free Ringer solution is followed by readdition of extracellular Ca^{2+} (see bars above graphs). *A*, Treatment of Th1 cells with 50 nM clotrimazole resulted in a slowly developing block of K_{Ca} conductance plateauing at 44% block after ~15 min treatment (Table II). This level of inhibition corresponds well with the 43% lower K_{Ca} conductance observed in Th2 cells. K_V currents were unaffected by clotrimazole (Table I). Averaged Ca^{2+} responses of all Th2 (dotted line, 90 cells from five experiments) and clotrimazole-treated (dashed line, 286 cells from seven experiments) or untreated (solid line, 233 cells from seven experiments) Th1 cells are shown. Where used, treatment with 50 nM clotrimazole began ~5 min before time zero, so that cells had been exposed to it for 15 min at the time of Ca^{2+} readdition. Plateau $[Ca^{2+}]_i$ was measured at the end of the experiment, ~9 min after Ca^{2+} reintroduction. *B*, Averaged Ca^{2+} responses of Th1 cells (solid line, 108 cells from four experiments) and Th2 cells (dotted line, 106 cells from four experiments) in the presence of K^+ Ringer solution. One minute before Ca^{2+} reintroduction, the extracellular solution was changed to Ca^{2+}-free K^+ Ringer solution by perfusion. The extracellular solution was then again exchanged by perfusion with K^+ Ringer solution containing 22 mM Ca^{2+}.

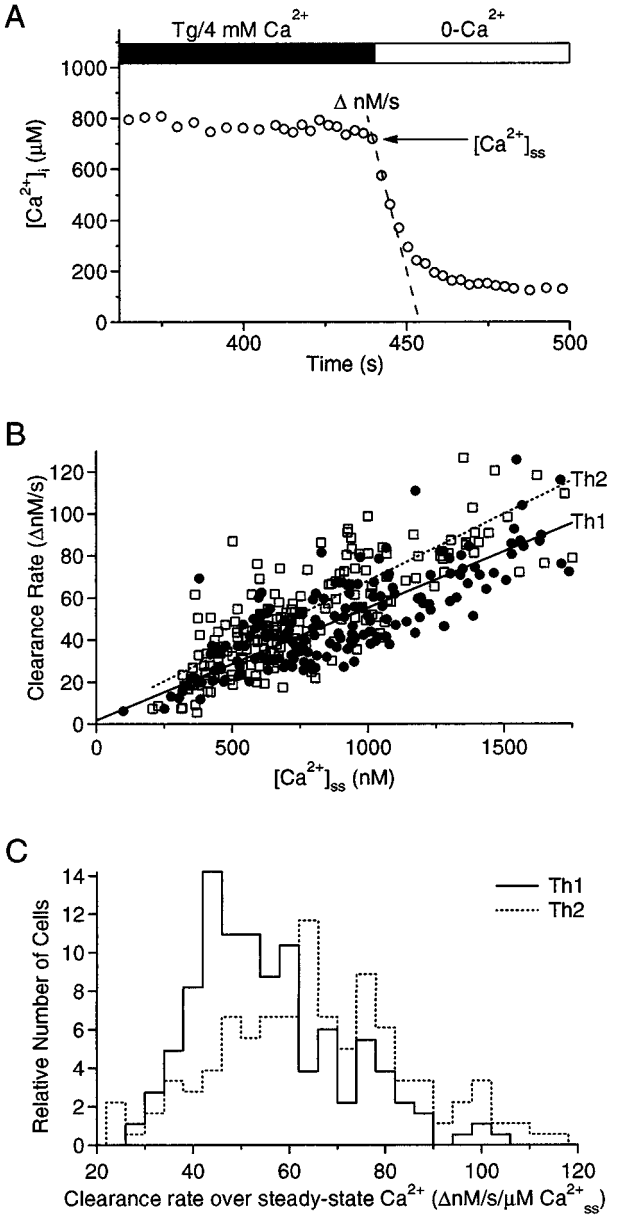

FIGURE 7. Th2 cells clear cytosolic Ca^{2+} more rapidly than Th1 cells. $[Ca^{2+}]_i$ was measured in Th1 and Th2 cells following stimulation with 1 μM Tg in the presence of normal Ringer solution with 4 mM extracellular Ca^{2+}. After $[Ca^{2+}]_i$ had stabilized (~6 min), Ca^{2+} was rapidly removed from the extracellular solution (~1 s) by perfusion with Ca^{2+}-free Ringer solution. *A*, A single Th1 cell illustrates the protocol for data analysis. The rate of Ca^{2+} clearance was measured as the maximal rate of decline following Ca^{2+} removal (dashed line) and was compared in each individual cell with the $[Ca^{2+}]_{ss}$ before Ca^{2+} removal (arrow). *B*, Plot showing clearance rates of all cells. Th2 cells (□, dotted line shows linear fit) appear to exhibit a more rapid Ca^{2+} clearance than Th1 cells (●, solid line shows linear fit) at a given $[Ca^{2+}]_{ss}$. *C*, The Ca^{2+} clearance rate at a given $[Ca^{2+}]_{ss}$ in all Th1 cells (solid line, 183 cells from three experiments) and Th2 cells (dotted line, 180 cells from five experiments). The histogram shows the significantly faster Ca^{2+} clearance rate in Th2 lymphocytes. For each cell, the calculated Ca^{2+} clearance rate is equal to the maximal rate of decrease in $[Ca^{2+}]_i$ following Ca^{2+} removal ($\Delta[Ca^{2+}]_i/s$) divided by $[Ca^{2+}]_{ss}$ before Ca^{2+} removal.

current with pharmacological sensitivities corresponding to the intermediate-conductance CTX-sensitive K_{Ca} current normally seen in human T lymphocytes, rather than to apamin-sensitive small conductance K_{Ca} current found in the Jurkat T cell line (32). We conclude that the number of K_{Ca} channels rather than their subtype is likely to be responsible for smaller K_{Ca} currents in Th2 cells.

Is decreased K_{Ca} current sufficient to account for the lower Th2 Ca^{2+} response?

We pharmacologically equalized the K_{Ca} current in both cell types using clotrimazole, an imidazole derivative that specifically blocks the intermediate-conductance K_{Ca} channel (36, 37). Fig. 6*A* compares the Ca^{2+} responses of Th2 cells with those of Th1 cells either untreated or blocked with sufficient clotrimazole to reduce K_{Ca} current to the level found in Th2 cells. The mean plateau $[Ca^{2+}]_i$ level of clotrimazole-treated Th1 cells was reduced to such a degree that it was no longer significantly different from that of Th2 cells (508 ± 16 nM in 286 clotrimazole-treated Th1 cells

compared with 450 ± 30 nM in 90 Th2 cells; $p = 0.08$). Furthermore, plateau $[Ca^{2+}]_i$ levels of both Th2 and clotrimazole-treated Th1 cells were significantly different from plateau $[Ca^{2+}]_i$ levels of untreated Th1 cells (mean plateau at 595 ± 21 nM in 233 cells; $p = 0.0001$ and 0.001, respectively). Thus, the decreased K_{Ca} current in Th2 cells accounts for much of the difference between Th1 and Th2 Ca^{2+} responses.

To evaluate whether additional mechanisms of $[Ca^{2+}]_i$ regulation differ in Th1 and Th2 cells, we neutralized the contribution of K^+ channels to the membrane potential and repeated the experiment of Fig. 2. Replacing extracellular Na^+ with K^+ results in membrane depolarization to between 0 mV and −20 mV (30), eliminating the effects of differential K^+ channel activity. Such a treatment should equalize Th1 and Th2 Ca^{2+} responses if K^+ channels are the sole cause of altered $[Ca^{2+}]_i$ responses in the Th clones. The result is shown in Fig. 6*B*. The peak $[Ca^{2+}]_i$ values attained following Ca^{2+} readdition were not significantly different (548 ± 23 nM in 108 Th1 cells compared with 502 ± 23 nM in 106 Th2 cells; $p = 0.17$). However, the Th1 and Th2 responses diverge with time, and by ~9 min after Ca^{2+} reintroduction, the mean plateau $[Ca^{2+}]_i$ in the two clones was significantly different (500 ± 24 nM compared with 343 ± 17 nM, $p < 0.0001$). Because differences between Th1 and Th2 Ca^{2+} responses persist despite neutralization of the effects of K^+ channels on the membrane potential, additional features must distinguish $[Ca^{2+}]_i$ regulation in the Th subtypes.

Ca^{2+} clearance

In addition to the influx mechanisms already discussed, $[Ca^{2+}]_i$ is governed by a number of mechanisms of Ca^{2+} efflux that could contribute to the difference in Th1 and Th2 Ca^{2+} response amplitude. Because the rate of Ca^{2+} extrusion in T cells depends on the $[Ca^{2+}]_i$ (38), we first elevated $[Ca^{2+}]_i$ to a steady plateau and then rapidly perfused Ca^{2+}-free Ringer solution and observed the rate of decrease in $[Ca^{2+}]_i$, as illustrated on a magnified time scale in Fig. 7*A*. The relationship between the rate of $[Ca^{2+}]_i$ decrease and steady-state Ca^{2+} ($[Ca^{2+}]_{ss}$) before extracellular Ca^{2+} removal is shown for both Th1 and Th2 cells (Fig. 7*B*). A histogram of the clearance rates for all cells illustrates significantly faster Ca^{2+} clearance in Th2 cells (Fig. 7*C*). Mean general rates of Ca^{2+} clearance were 56 ± 1 nM/s/μM Ca^{2+} for Th1 cells (183 cells) vs 70 ± 2 nM/s/μM Ca^{2+} for Th2 cells (180 cells; $p < 0.0001$). More rapid Ca^{2+} clearance in Th2 cells represents a second difference in Th1 and Th2 Ca^{2+} regulation.

Discussion

Th1 and Th2 lymphocytes play a critical role in determining the outcome of the immune response by producing discrete subsets of cytokines that govern the recruitment of other immune cells. The distinctions giving rise to differential cytokine production are not yet fully understood. It has been suggested that Th2 cells use an alternate signal transduction pathway that either generates no Ca^{2+} influx or that employs a voltage-gated Ca^{2+} channel (11, 22, 23). Using Tg to bypass TCR-mediated proximal signaling events, we found that both Th subtypes have a Ca^{2+} influx pathway activated by Ca^{2+} store depletion, but that Ca^{2+} signals were larger in Th1 than Th2 cells (Fig. 2). In patch-clamp experiments, we investigated the biophysical properties of ion channels in T cell clones and found a similar complement of ion channels in both Th1 and Th2 cells, including K_V channels (Table I), intermediate-conductance K_{Ca} channels (Table II), and CRAC channels rather than voltage-dependent Ca^{2+} channels (not shown). A subpopulation of Th1 cells expressed a biophysically distinct K_V current with more rapid inactivation and decreased CTX sensitivity, but the overall amplitude of whole-cell K_V currents was indistinguishable between the Th subtypes (Fig. 4). Furthermore, the initial rate of Ca^{2+} influx (Fig. 3) did not differ in the Th subtypes, indicating that a similar number of CRAC channels is present in Th1 and Th2 cells. The only major difference detected by patch-clamp studies was the expression of a greater number of functional K_{Ca} channels in Th1 cells than in Th2 cells (Fig. 5). The importance of this difference was confirmed by the pharmacological equalization of Th1 and Th2 $[Ca^{2+}]_i$ plateaus using the K_{Ca} channel blocker clotrimazole (Fig. 6*A*). Our results are consistent with investigations showing that K_V and K_{Ca} channel blockers depolarize the plasma membrane, reduce Ca^{2+} influx, and inhibit lymphocyte activation (Refs. 29, 35, and 39–41; reviewed in Ref. 31). We conclude that the decreased Th2 Ca^{2+} influx is caused primarily by expression of a lower number of K_{Ca} channels, rather than a discrete type of Ca^{2+} channel.

Ca^{2+} efflux also plays a role in the difference between Th1 and Th2 Ca^{2+} responses. Examination of the kinetics of single-cell traces revealed a more rapid rate of $[Ca^{2+}]_i$ decline in Th2 cells (Fig. 7). Mechanisms that contribute to Ca^{2+} clearance in T lymphocytes are the plasma membrane Ca^{2+} ATPase, the SERCA pump, plasma membrane exchangers, and mitochondrial Ca^{2+} uptake. Our experiments in the presence of Tg rule out the involvement of the SERCA pump (Fig. 7). A Na^+/Ca^{2+} exchanger could not contribute to the differences we observed, because Th2 cells still clear Ca^{2+} more rapidly than Th1 cells in the absence of extracellular Na^+ (Fig. 6*B*). Distinct levels of mitochondrial Ca^{2+} uptake in Th1 and Th2 cells are also unlikely to exist, because these would alter the residual $[Ca^{2+}]_i$ levels following Ca^{2+} removal in Ca^{2+} clearance experiments (42), which we did not observe (data not shown). Previous studies have demonstrated that the plasma membrane Ca^{2+} ATPase is responsible for removal of most of the Ca^{2+} from the cytosol in lymphocytes (38, 43). Differential activity of the plasma membrane Ca^{2+} ATPase is the most likely cause of the more rapid Ca^{2+} clearance by Th2 cells.

It is unlikely that additional differences exist between the Ca^{2+} regulatory mechanisms of Th1 and Th2 cells. Although a distinction in Th1 and Th2 inositol phosphate generation has been documented (11), our results argue that the importance of such differences is limited because the comparative distribution of Th1 and Th2 $[Ca^{2+}]$, plateaus is preserved even using stimuli that bypass inositol phosphate generation (Fig. 2 and Ref. 22). A simple calculation based on the combined contribution of decreased K_{Ca} conductance and increased rates of Ca^{2+} clearance suggests that these two mechanisms alone are sufficient to account for the lower Th2 Ca^{2+} response. In Fig. 6*B*, if we assume that the rates of Ca^{2+} influx in the two clones are equalized by treatment with K^+ Ringer, the faster Ca^{2+} efflux of Th2 cells is adequate to produce the decreasing Ca^{2+} plateau observed in these cells. Thus, no additional distinctions between the Th1 and Th2 Ca^{2+} regulatory processes are required to explain our observations.

Differential Ca^{2+} regulation could optimize cytokine production in Th1 and Th2 cells if the distinct $[Ca^{2+}]_i$ in each cell type were to preferentially activate a particular set of transcription factors. Precedent for this possibility exists in the regulation of NF-AT and NF-κB in Jurkat T cells. Rapid Ca^{2+} oscillations activate both NF-AT and NF-κB, whereas slower Ca^{2+} oscillations activate NF-κB alone, leading to differential production of reporter genes driven by the IL-8 and IL-2 promoters (44). It has been suggested that discrete $[Ca^{2+}]_i$ levels induce each isoform of NF-AT (45). Such a mechanism could be particularly relevant to IL-4 production. The IL-4 promoter contains two NF-AT-binding sites and is induced by NF-AT-stimulatory treatments (46). Furthermore, whereas IL-4 production requires the NF-AT isoform NF-ATc1 (47), NF-ATc2 inhibits IL-4 production (48). If activation of the

IL-4 inhibitory isoform NF-ATc2 requires higher $[Ca^{2+}]_i$ than does NF-ATc1, a "window" of ideal $[Ca^{2+}]_i$ would result. Within the window, stimulatory transcription factor NF-ATc1 would maximally promote IL-4 production. However, when $[Ca^{2+}]_i$ exceeds this window, the IL-4-inhibitory isoform NF-ATc2 would be activated, leading to suboptimal IL-4 production. According to this hypothesis, IL-4 generation would be optimal at a lower $[Ca^{2+}]_i$. Differential NF-AT activity has already been noted in Th1 and Th2 cells (17, 49). Further research should help to clarify the relevance of Ca^{2+} signaling differences to Th1 and Th2 function.

Acknowledgments

We thank Luette Forrest for expert cell culture, Randolph J. Noelle for providing the D1.6 and CDC35 clones, and Heiko Rauer, Christopher Hughes, and Richard Lewis for helpful comments on the manuscript.

References

1. Paul, W. E., and R. A. Seder. 1994. Lymphocyte responses and cytokines. *Cell 76:241.*
2. Swain, S. L. 1994. Generation and in vivo persistence of polarized Th1 and Th2 memory cells. *Immunity 1:543.*
3. Hsieh, C. S., S. E. Macatonia, A. O'Garra, and K. M. Murphy. 1995. T cell genetic background determines default T helper phenotype development in vitro. *J. Exp. Med. 181:713.*
4. Constant, S., C. Pfieffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med. 182:1591.*
5. Kuchroo, V. K., M. Prabhu Das, J. A. Brown, A. M. Ranger, S. S. Zamvil, R. A. Sobel, H. L. Weiner, N. Nabavi, and L. H. Glimcher. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell 80:707.*
6. Crabtree, G. R., and N. A. Clipstone. 1994. Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Biochem. 63:1045.*
7. Tamura, T., H. Nakano, H. Nagase, T. Morokata, O. Igarashi, Y. Oshimi, S. Miyazaki, and H. Nariuchi. 1995. Early activation signal transduction pathways of Th1 and Th2 cell clones stimulated with anti-CD3: roles of protein tyrosine kinases in the signal for IL-2 and IL-4 production. *J. Immunol. 155:4692.*
8. Rincón, M. H. Enslen, J. Raingeaud, M. Recht, T. Zapton, M. S-S. Su, L. A. Penix, R. J. Davis, and R. A. Flavell. 1998. Interferon-γ expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. *EMBO J. 17:2817.*
9. Li-Weber, M., M. Giasi, and P. H. Krammer. 1998. Involvement of Jun and Rel proteins in up-regulation of interleukin-4 gene activity by the T cell accessory molecule CD28. *J. Biol. Chem. 273:32460.*
10. Kurt-Jones, E. A., S. Hamberg, J. Ohara, W.E. Paul, and A. K. Abbas. 1987. Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness. *J. Exp. Med. 166:1774.*
11. Gajewski, T. F., S. R. Schell, and F. W. Fitch. 1990. Evidence implicating utilization of different T cell receptor-associated signaling pathways by Th1 and Th2 clones. *J. Immunol. 144:4110.*
12. Mingari, M.C., E. Maggi, A. Cambiaggi, F. Annunziato, F. Schiavetti, R. Manetti, L. Moretta, and S. Romagnani. 1996. Development in vitro of human $CD4^+$ thymocytes into functionally mature Th2 cells: exogenous interleukin-12 is required for priming thymocytes to produce both Th1 cytokines and interleukin-10. *Eur. J. Immunol. 26:1083.*
13. Sweetser, M. T., T. Hoey, Y.-L. Sun, W. M. Weaver, G. A. Price, and C. B. Wilson. 1998. The roles of nuclear factor of activated T cells and Ying-Yang 1 in activation-induced expression of the interferon-γ promoter in T cells. *J. Biol. Chem. 273:34775.*
14. Zheng, W.-P., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell 89:587.*
15. Rincón, M., and R. A. Flavell. 1997. T-cell subsets: transcriptional control in the Th1/Th2 decision. *Curr. Biol. 7:R729.*
16. Agarwal., S., and A. Rao. 1998. Long-range transcriptional regulation of cytokine gene expression. *Curr. Opin. Immunol. 10:345.*
17. Rincón, M., and R. A. Flavell. 1997. Transcription mediated by NFAT is highly inducible in effector $CD4^+$ T helper 2 (Th2) cells but not in Th1 cells. *Mol. Cell. Biol. 17:1522.*
18. Goldsmith, M. A., and A. Weiss. 1988. Early signal transduction by the antigen receptor without commitment to T cell activation. *Science 240:1029.*
19. Lewis, R. S., and M. D. Cahalan. 1989. Mitogen-induced oscillations of cytosolic Ca^{2+} and transmembrane Ca^{2+} current in human leukemic T cells. *Cell Reg. 1:99.*
20. Zweifach, A., and R. S. Lewis. 1993. Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proc. Natl. Acad. Sci. USA 90:6295.*
21. Fanger, C. M., M. Hoth, G. R. Crabtree, and R. S. Lewis. 1995. Characterization of T cell mutants with defects in capacitative calcium entry: genetic evidence for the physiological roles of CRAC channels. *J. Cell Biol. 131:655.*
22. Sloan-Lancaster, J., T. H. Steinberg, and P. M. Allen. 1997. Selective loss of the calcium ion signaling pathway in T cells maturing toward a T helper 2 phenotype. *J. Immunol. 159:1160.*
23. Badou, A., M. Savignac, M. Moreau, C. Leclerc, R. Pasquier, P. Druet, and L. Pelletier. 1997. $HgCl_2$-induced interleukin-4 gene expression in T cells involves a protein kinase C-dependent calcium influx through L-type calcium channels. *J. Biol. Chem. 272:32411.*
24. Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA 87:2466.*
25. Tony, H.-P., N. E. Phillips, and D. C. Parker. 1985. Role of membrane immunoglobulin (Ig) crosslinking in membrane Ig-mediated major histocompatibility-restricted T cell-B cell cooperation. *J. Exp. Med. 162:1695.*
26. Fanger, C. M., S. Ghanshani, N. J. Logsdon, H. Rauer, K. Kalman, J. Zhou, K. Beckingham, K. G. Chandy, M. D. Cahalan, and J. Aiyar. 1999. Calmodulin mediates calcium-dependent activation of the intermediate conductance K_{Ca} channel, *IKCa1. J. Biol. Chem. 274:5746.*
27. Densmore, J J., G. Szabo, and L. S. Gray. 1992. A voltage-gated calcium channel is linked to the antigen receptor in Jurkat T lymphocytes. *FEBS Lett. 312:161.*
28. Kerschbaum, H. H., P. A. Negulescu, and M. D. Cahalan. 1997. Ion channels, Ca^{2+} signaling, and reporter gene expression in antigen-specific mouse T cells. *J. Immunol. 159:1628.*
29. Lin, C. S., R. C. Boltz, J. T. Blake, M. Nguyen, A. Talento, P. A. Fischer, M. S. Springer, N. H. Sigal, R. S. Slaughter, M. L. Garcia, et al. 1993. Voltage-gated potassium channels regulate calcium-dependent pathways involved in human T lymphocyte activation. *J. Exp. Med. 177:637.*
30. Verheugen, J. A. H., and H. P. M. Vijverberg. 1995. Intracellular Ca^{2+} oscillations and membrane potential fluctuations in intact human T lymphocytes: role of K^+ channels in Ca^{2+} signaling. *Cell Calcium 17:287.*
31. Lewis, R. S., and M. D. Cahalan. 1995. Potassium and calcium channels in lymphocytes. *Annu. Rev. Immunol. 13:623.*
32. Grissmer, S., A. N. Nguyen, and M. D. Cahalan. 1993. Calcium-activated potassium channels in resting and activated human T lymphocytes. *J. Gen. Physiol. 102:601.*
33. Grissmer, S., B. Dethlefs, J. J. Wasmuth, A. L. Goldin, G. A. Gutman, M. D. Cahalan, and K. G. Chandy. 1990. Expression and chromosomal localization of a lymphocyte K^+ channel gene. *Proc. Natl. Acad. Sci. USA 87:9411.*
34. Grissmer, S., A. N. Nguyen, J. Aiyar, D. C. Hanson, R. J. Mather, G. A. Gutman, M. J. Karmilowicz, D. D. Auperin, and K. G. Chandy. 1994. Pharmacological characterization of five cloned voltage-gated K^+ channels, types KV1. 1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol. Pharmacol. 45:1227.*
35. Khanna, R., M. C. Chang, W. J. Joiner, L. K. Kaczmarek, and L. C. Schlichter. 1999. HSK4/hIK1, a calmodulin-binding K_{Ca} channel, in human T lymphocytes. *J. Biol. Chem. 274:14838.*
36. Ishii, T. M., C. Silvia, B. Hirschberg, C. T. Bond, J. P. Adelman, and J. Maylie. 1997. A human intermediate conductance calcium-activated potassium channel. *Proc. Natl. Acad. Sci. USA 94:11651.*
37. Logsdon, N. J., J. Kang, J. A. Togo, E. P. Christian, and J. Aiyar. 1997. A novel gene, *hKCa4*, encodes the calcium-activated potassium channel in human T lymphocytes. *J. Biol. Chem. 272:32723.*
38. Donnadieu, E., G. Bismuth, and A. Trautmann. 1992. Calcium fluxes in T lymphocytes. *J. Biol. Chem. 267:25864.*
39. Verheugen, J. A., F. Le Deist, V. Devignot, and H. Korn. 1997. Enhancement of calcium signaling and proliferation responses in activated human T lymphocytes: inhibitory effects of K^+ channel block by charybdotoxin depend on the T cell activation state. *Cell Calcium 21:1.*
40. Jensen, B. S., N. Ødum, N. K. Jørgensen, P. Christophersen, and S.-P. Olesen. 1999. Inhibition of T cell proliferation by selective block of Ca^{2+}-activated K^+ channels. *Proc. Natl. Acad. Sci. USA 96:10917.*
41. Ehring, G. R., H. H. Kerschbaum, C. Eder, A. L. Neben, C. M. Fanger, R. M. Khoury, P. A. Negulescu, and M. D. Cahalan. 1998. A nongenomic mechanism for progesterone-mediated immunosuppression: inhibition of K^+ channels, Ca^{2+} signaling, and gene expression in T lymphocytes. *J. Exp. Med. 188:1593.*
42. Hoth, M., C. M. Fanger, and R. S. Lewis. 1997. Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. *J. Cell Biol. 137:633.*
43. Mason, M. J., and S. Grinstein. 1993. Ionomycin activates electrogenic Ca^{2+} influx in rat thymic lymphocytes. *Biochem. J. 296:33.*
44. Dolmetsch, R. E., K. Xu, and R. S. Lewis. 1998. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature 392:933.*
45. Abbott, K. L., B. B. Friday, D. Thaloor, T. J. Murphy and G. K. Pavlath. 1998. Activation and cellular localization of the cyclosporine A-sensitive transcription factor NF-AT in skeletal muscle cells. *Mol. Biol. Cell 9:2905.*
46. Wenner, C. A., S. J. Szabo, and K. M. Murphy. 1997. Identification of IL-4 promoter elements conferring Th2-restricted expression during T helper cell subset development. *J. Immunol. 158:765.*
47. Yoshida, H., H. Nishina, H. Takimoto, L. E. M. Marengère, A. C. Wakeham, D. Bouchard, Y. Y. Kong, T. Ohteki, A. Shahinian, M. Bachmann, et al. 1998. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity 8:115.*
48. Kiani, A., J. P. B. Viola, A. H. Lichtman, and A. Rao. 1997. Down-regulation of IL-4 gene transcription and control of Th2 cell differentiation by a mechanism involving NFAT1. *Immunity 7:849.*
49. Barve, S. S., D. A. Cohen, A. De Benedetti, R. E. Rhoads, and A.M. Kaplan. 1994. Mechanism of differential regulation of IL-2 in murine Th1 and Th2 T cell subsets. *J. Immunol. 152:1171.*
50. Fanger, C. M., and M. D. Cahalan. 1999. Depletion-activated calcium influx in Th1 and Th2 T cells. 1999.